UNIVERSITY OF SOUTHAMPTON

SCHOOL OF MEDICINE

DEPARTMENT OF MICROBIOLOGY AND PATHOLOGY,

DIVISION OF CANCER SCIENCES

"Functional characterisation of bone marrow stromal cells and their responses to leukaemia therapy"

by

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ABSTRACT

FACULTY OF MEDICINE, HEALTH & BIOLOGICAL SCIENCES
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INTRO. The bone marrow (BM) micro-environment contains a variety of stromal cells (SC) and extracellular matrix components essential for support and regulation of haemopoiesis. Isolation and culture of BMSC is hindered by the rarity of these cells in aspirated BM and lack of *in vitro* models in which to study individual stromal components. **AIMS.** (1) To develop methods to isolate and characterise BMSC and investigate their functional characteristics. (2) To use this information to study stromal changes that occur in the pathogenesis of idiopathic myelofibrosis (IMF) and pre/post chemotherapy for acute leukaemias. **METHODS.** Magnetic activated cell sorting (MACS) was adapted to optimise multi-step sequential MACS separations to isolate specific SC populations. These were cultured on porous polyethylene terephthalate (PET) cell culture inserts, which more closely resemble the *in vivo* micro-environment than do cultures on glass or plastic surfaces as they permit access of nutrients to both apical and basolateral cells surfaces. Single and double immunocytochemistry and Northern blot analysis were used to characterise these SC to provide insight into possible functions and origins. Immunohistochemical studies using BM trephine biopsies were carried out to investigate stromal changes that occur in BM from patients with idiopathic myelofibrosis (IMF) and from patients before and after chemotherapy for acute leukaemias.

RESULTS. Stromal components of BM were successfully isolated, cultured and characterised. A number of fundamental changes were identified in BM stroma of patients with IMF compared with normal individuals, in particular a reduction in the proportion of marrow occupied by adipocytes and the increased proportion of marrow occupied by α-smooth muscle actin (αSMA) positive stromal fibroblasts. Unexplained variations in haemopoietic re-growth occur in patients after chemotherapy for acute leukaemias. Analysis of BM stroma in patients before and after consecutive courses of chemotherapy identified that, after each course of chemotherapy, there was a progressive decline in low-affinity nerve growth factor receptor (L-NGFR) expression by stromal fibroblasts. This was paralleled by a progressive increase in expression of αSMA by these cells. **CONCLUSIONS.** The results obtained provide insight into possible functions/origins of BMSC. Immunohistochemical analyses have identified a potential mechanism for reduced ability of BM stroma to support haemopoiesis after chemotherapy for acute leukaemias.

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LIST OF PUBLICATIONS/PRESENTATIONS ARISING FROM THIS STUDY

1. **Kovacs, I.E.L.**, Wilkins, B.S. and Jones, D.B. (1999) Isolation and culture of human bone marrow stromal cells. *Br. J. Haematol.* **105s1,** p33. Abstract submitted and presented as a poster at the British Society for Haematology meeting in April 1999 at the Brighton Conference Centre, UK. The abstract was one of 11 out of 294 short-listed for a Young Investigators Award.

2. **Kovacs, I.E.L.**, Cheung, M., Myint, H., Wilkins, B.S. and Jones, D.B. (2000) Are Stromal Cell Precursors Present in Peripheral Blood Mobilised for Transplantation? *J. Pathol.* **190**. Abstract. No.131. Abstract submitted and presented as an oral presentation at the Pathological Society of Great Britain and Ireland Winter Meeting in January 2000 at the Queen Elizabeth II Conference Centre, Westminster, UK.

3. Wilkins, B.S., **Kovacs, I.E.L.**, Taylor, P., Jones, D.B. (2000) Characterisation of Human Bone Marrow Stromal Cells Expressing Low-Affinity Nerve Growth Factor Receptor (L-NGFR; p75). Abstract submitted and presented by Dr. B.S. Wilkins at the European Meeting for Haematopathology in May 2000.

4. **Kovacs, I.E.L.**, Cheung, M., Myint, H., Wilkins, B.S. and Jones, D.B. Peripheral blood stem cells do not generate stromal cells *in vitro*. Manuscript in preparation for publication.

5. **Kovacs, I.E.L.**, Wilkins, B.S. and Jones, D.B. Immunomagnetic Isolation and Characterisation of Cellular Components of Bone Marrow Stroma. Manuscript in preparation for publication.

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LIST OF ABBREVIATIONS

ADE ..... Cytarabine, daunorubicin and etoposide
AML ..... Acute myeloid leukaemia
ALL ..... Acute lymphoblastic leukaemia
ANLL ..... Acute non-lymphoblastic leukaemia
APES ..... 3`aminopropyl-triethoxysilane
APML ..... Acute promyelocytic leukaemia
α-SMA ..... α-smooth muscle actin

B-ALL ..... B-cell ALL
BCNU ..... 1,3-bis-(2-chloroethyl)-1-nitrosurea
BDNF ..... Brain-derived neurotrophic factor
BMT ..... Bone marrow trephine
BSA ..... Bovine serum albumin

c-ALL ..... Common-ALL
CD ..... *Cluster designation*
CFU-F ..... Fibroblast colony-forming unit
CFU-GM ..... Granulocyte-macrophage colony-forming unit
CFU-RF ..... Reticulo-fibroblast colony-forming unit
CMPD ..... Chronic myeloproliferative disorders
CNS ..... Central nervous system

DAB ..... 3-3` diaminobenzidine tetrahydrochloride
DEPC ..... Diethyl pyrocarbonate
DIC ..... Double immunocytochemistry
DMSO ..... Dimethyl sulphoxide
DNA ..... Deoxyribonucleic acid

EDTA ..... Ethylenediaminetetra-acetic acid
ECGF ..... Endothelial cell growth factor
EGF ..... Epidermal growth factor
EMA ..... Epithelial membrane antigen

FAB ..... French, American and British
(Classification of Acute leukaemias)

FACS ..... Fluorescence-activated cell sorting

FBS ..... Fetal bovine serum

G-CSF ..... Granulocyte-colony stimulating factor

GITC ..... Guanidium isothiocyanate

GM-CSF ..... Granulocyte macrophage-colony stimulating factor

HBMEC ..... Human bone marrow endothelial cells

HBSS ..... Hanks buffered salt solution

hLTBMC ..... Human long-term bone marrow culture

HPLC ..... High performance liquid chromatography

HRP ..... Horseradish peroxidase

ICAM-1 ..... Intercellular adhesion molecule-1

IC ..... Immunocytochemistry

ICE ..... Idarubicin, cytosine arabinoside and etoposide

IFN ..... Interferon

IL ..... Interleukin

IM ..... Iscove`s Modified Dulbecco`s Medium

ISH ..... *in situ* hybridisation

L-NGFR ..... Low-affinity nerve growth factor receptor

MACE ..... M-amsacrine, cytarabine and etoposide

MACS ..... Magnetic-activated cell sorting

MAE ..... Mitozantrone, cytarabine and etoposide

M-CSF ..... Macrophage-colony stimulating factor

MDS ..... Myelodysplastic syndromes

MIC-M ..... Morphologic-immunologic-cytogenetic and molecular genetic (Classification of acute leukaemias)

MIDAC ..... Mitozantrone and ara-C

MOPS ..... 3-(N-Morpholino) propanesulphonic acid

MRC ..... Medical Research Council

mRNA ..... Messenger RNA

msMS ..... Multi-step sequential MACS separations

NCBI ..... National Centre for Biotechnology Information

NCI ..... National Cancer Institute

NGF ..... Nerve growth factor

OD ..... Optical density

PBS ..... Phosphate buffered saline
PBSC ..... Peripheral blood stem cell
PDGF ..... Platelet-derived growth factor
PECAM-1 (CD31) ..... Platelet-endothelial cell adhesion molecule-1
PET ..... Polyethylene Terephthalate
RNA ..... Ribonucleic acid
RNAse ..... Ribonuclease
RO ..... Reverse osmosis
RRNA ..... ribosomal RNA
RT-PCR ..... Reverse-transcriptase polymerase chain reaction

SDS ..... Sodium dodecyl sulphate
SSC ..... Standard saline citrate
St-AB-HRP ..... Streptavidin-biotinylated horseradish peroxidase
SUHT ..... Southampton University Hospital NHS Trust

T-ALL ..... T-cell ALL
TBS ..... Tris buffered saline
TdT ..... Terminal deoxynucleotidyl transferase
TGF ..... Transforming growth factor
TNF ..... Tumour necrosis factor
Trk ..... Tyrosine kinase

u-ALL ..... Unclassified ALL

VCAM-1 ..... Vascular cell adhesion molecule-1
VWF ..... von Willebrand factor

1. INTRODUCTION

1.1 HAEMOPOIESIS AND BONE MARROW STROMA

1.1.1 Introduction

Haemopoiesis is the term used to describe the formation and development of blood cells. It is derived from the Greek word "haima," meaning blood and "poiesis" meaning to create. Sites of haemopoiesis change during embryonic, fetal and post-natal life although the majority of haemopoietic activity in adult mammals occurs within the marrow cavities of flat bones, especially the sternum, vertebra, ribs and iliac crest. It is believed that approximately 95% of all haemopoiesis that takes place in adult mammals occurs in the bone marrow (Mayani *et al.*, 1992).

Bone marrow is a highly ordered tissue consisting of a network of bony trabeculae and a complex marrow vasculature providing nutrients to and removing waste products from, the haemopoietic tissue. Haemopoietic cells are situated within the inter-trabecular regions of the marrow (Figure 1.1), supported by a network of interconnecting stromal cells and extracellular matrix components. These stromal cells provide both physical and regulatory influences that act, via complex interactions, on developing haemopoietic cells (Liesveld *et al.*, 1989; Eaves *et al.*, 1991b).

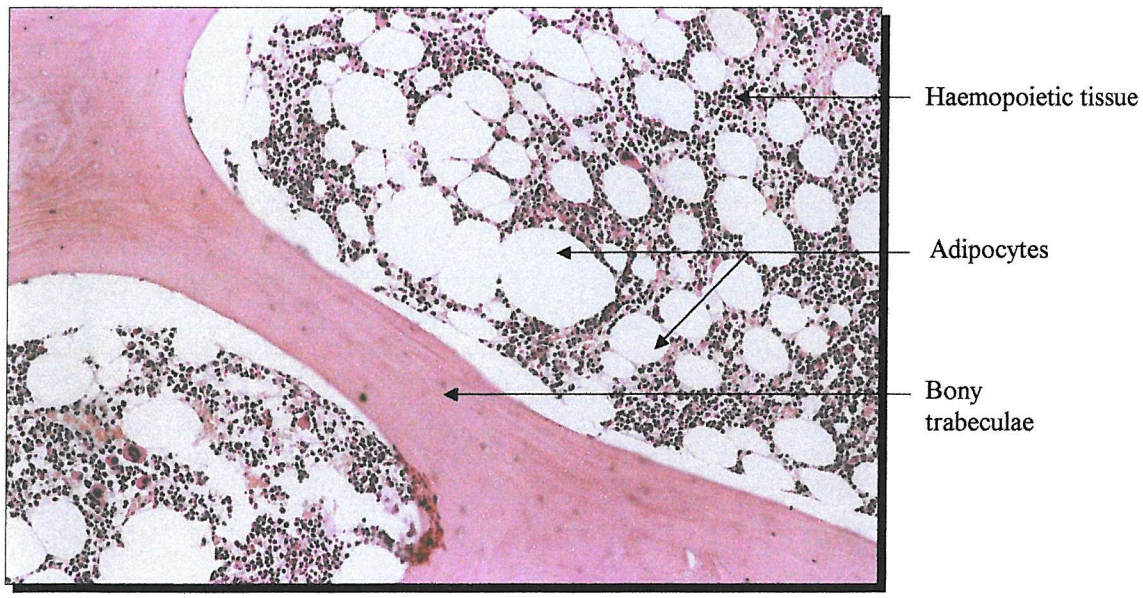


Figure 1.1. Normal bone marrow trephine biopsy section (x100).

1.1.2 Sites of haemopoiesis

The principal sites of haemopoiesis vary throughout mammalian development (Figure 1.2). Initially, all blood cells are derived from primitive mesenchymal stem cells present in the blood islands of the yolk sac (Haen, 1995). As these blood islands develop, they interconnect and begin to infiltrate the developing tissues and organs, forming a simple vascular network. During the second and third months of gestation, primitive haemopoietic stem cells migrate out of the bloodstream into developing organs where they continue their development (Haen, 1995).

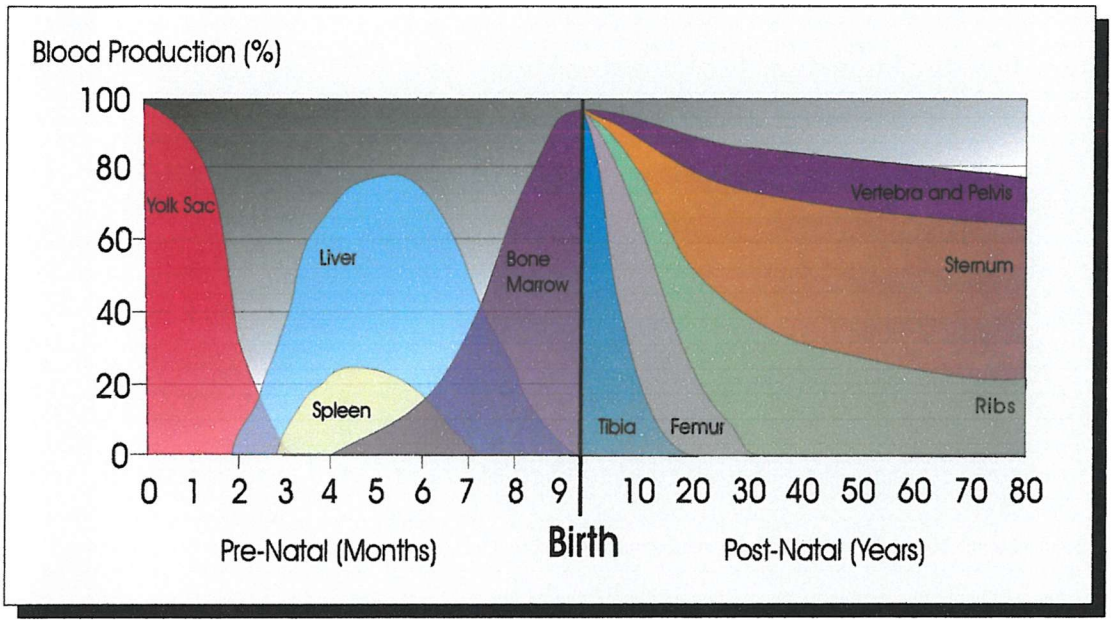


Figure 1.2. Blood production in haemopoietic organs during pre- and postnatal life. Adapted from Haematology, an illustrated colour text, 1997 (Howard & Hamilton, 1997).

The first organ to become a site of haemopoiesis is the liver. In humans, haemopoietic activity in the liver begins at week six of gestation. This becomes and remains, the main site for haemopoiesis after the yolk sac disappears, until towards the end of gestation. In addition, during this time the spleen becomes an organ for haemopoiesis (Tavassoli, 1991), at least in some mammalian species. The bone marrow becomes the main site for haemopoiesis from birth and throughout post-natal life (Zanjani *et al.*, 1993). It is believed that stromal components of bone marrow, prior to birth, may be insufficiently developed to support the differentiation and maturation of haemopoietic stem cells (Zanjani *et al.*, 1993). The liver and spleen are not haemopoietically active

post-natally, although they retain their capacity to support haemopoiesis throughout adult life. These organs may subsequently become haemopoietically active under certain pathological conditions that render the bone marrow incapable of supplying an adequate source of blood cells.

It is well documented that the micro-environment of the bone marrow plays a vital role in the support of haemopoiesis (Eaves *et al.*, 1991a). During embryonic development, a similar micro-environment must also exist to support developing stem cells in the yolk sac, liver and spleen. The changes in the sites of haemopoiesis that occur throughout mammalian development may therefore be explained by changes in the micro-environment that supports the development of haemopoietic stem cells in these sites. This idea is supported by experiments carried out by Van den Heuvel *et al.*, 1987. In these experiments, clonal assay techniques were used to measure colony formation from haemopoietic stem cells and fibroblast colony-forming stem cells (CFU-F) in yolk sac, liver, spleen and bone marrow of mice during gestation and the post-natal period. In these organs, an increase in CFU-F was found to precede an increase in the number of haemopoietic stem cells. In addition, as the site of haemopoiesis changed from the liver to the spleen, the number of CFU-F decreased in the liver and increased in the spleen. This observation remained true as haemopoiesis changed from the spleen to the bone marrow suggesting that stromal stem cells migrate in accordance with changes in sites of haemopoiesis. (Van Den Heuvel *et al.*, 1987; Wolf *et al.*, 1995).

1.1.3 Haemopoietic variations with age

Haemopoietically active marrow is red in appearance due to the abundance of haemopoietic cells in comparison to adipocytes. This contrasts with haemopoietically inactive marrow, which is yellow in appearance due to the increased number of adipocytes relative to haemopoietic cells. A balance exists between red and yellow bone marrow, with progressive change from predominantly red marrow during early life to predominantly yellow marrow during late adult life (Hartsock *et al.*, 1965).

It has been suggested that, throughout adult life, the changes that occur in the percentage of haemopoietic tissue found in bone marrow may be divided into three

phases. A study of trephine biopsy samples obtained from the anterior iliac crest suggests there is an initial phase during which the percentage of haemopoietic tissue in the bone marrow decreases steadily (Hartsock *et al.*, 1965). This occurs until the fourth decade. Phase two, in which the percentage of haemopoietic cells in the marrow stabilises, lasts until approximately the eighth decade, after which a further decrease in the amount of haemopoietic tissue occurs (phase three). The increased adipogenesis that occurs with ageing, in association with the decline in haemopoiesis, can alter the production of certain extracellular matrix components and proteins involved in the adhesion of haemopoietic cells to marrow stroma (Gimble *et al.*, 1996). Expression of tenascin and collagen decreases with increased adipogenesis while expression of the CD44 ligand, hyaluronate, increases. These changes may, as a consequence of age, adversely affect the ability of stromal cells to support and regulate specific haemopoietic cell lineages.

1.1.4 Bone marrow stroma

The bone marrow stroma, or bone marrow micro-environment, is composed of a complex array of cells and extracellular matrix components that span the mesenchymal compartment of bone marrow. This stroma forms both a physical and chemical support upon which haemopoietic cells are arranged (Singer *et al.*, 1985). Interactions between maturing haemopoietic cells and the stroma are fundamental for the maintenance and regulation of normal blood cell development (Eaves *et al.*, 1991a). The details of these interactions are poorly understood. It is widely believed, however, that stromal abnormalities may play a pivotal role in the development and/or support of many haematological malignancies, including those that can result in fibrosis within the marrow (Dührsen & Hossfeld, 1996; Thiele *et al.*, 1997).

Marrow stroma comprises at least four main cellular constituents. These include reticular cells or marrow fibroblasts, macrophages, adipocytes and endothelial cells. The latter are restricted to the bone marrow vasculature. In addition, cells of the osteoblastic and chondrocytic cell lineages are also found within the bone marrow and are considered by bone researchers to represent a component of bone marrow stroma (Bianco & Riminucci, 1998). Differences between what are considered to be bone marrow stromal components result in differences in concepts of the functions of

stromal precursor cells between groups of researchers. Bone researchers concentrate on the osteogenic potential of stromal tissue in contrast to haemopoietic researchers, who predominantly study the role that marrow stroma plays in the physical and functional support of haemopoiesis.

In normal bone marrow conditions, adipocytes are the most prominent marrow stromal cell type. Their relationships to fibroblasts and whether or not the latter are heterogeneous, are unknown issues at present. Numerous extracellular matrix components have been identified in the stroma of the bone marrow, mostly studied using a variety of *in vitro* culture models. These include the interstitial (I and III) and basement membrane (IV, V and VI) collagen types, reticulin, fibronectin, laminin, tenascin and proteoglycans (Campbell & Wicha, 1988; Zuckerman *et al.*, 1989; Wilkins & Jones, 1995a). These macromolecules are produced and secreted by stromal cells and mediate adhesion of haemopoietic components of bone marrow to the stroma, as well as regulating the maturation and differentiation of blood cell lineages (Zuckerman *et al.*, 1989).

1.2 STROMAL FIBROBLASTS

1.2.1 Description of stromal fibroblasts

Stromal fibroblasts represent a sub-population of stromal cells that are poorly characterised due to a current lack of specific phenotypic or functional markers. It is unclear from the published literature whether this component of bone marrow stroma represents a single or heterogeneous population of cells. It has been described that stromal fibroblasts include stromal progenitor cells (fibroblast colony forming units; CFU-F) capable of generating adherent fibroblasts, smooth muscle cells and adipocytes (Simmons & Torok Storb, 1991). In addition, stromal cells have been described which, when cultured, give rise to colonies with reticulofibroblastic appearances, termed CFU-RF (Lim *et al.*, 1986). These reticular fibroblasts are believed to synthesise reticulin into the extracellular matrix and represent adipocyte precursors (Bianco & Riminucci, 1998). However, it is unclear from the reported literature whether CFU-F and CFU-RF represent different cell populations or reflect different culture conditions, as many stromal cells arising from CFU-F seem to be

equivalent of CFU-RF (Allen *et al.*, 1990). Lim *et al.* (1986) distinguish these cell types by the ability of CFU-RF to synthesise laminin and collagen type IV.

It has recently been demonstrated that a population of fibroblastic stromal cells expresses the low-affinity nerve growth factor receptor (L-NGFR) (Cattoretti *et al.*, 1993; Wilkins & Jones, 1995a; Caneva *et al.*, 1997). Previous characterisation of these cells has identified two morphological variants of marrow fibroblast (Wilkins & Jones, 1995a; Caneva *et al.*, 1997). There are those that appear star-shaped with long, cytoplasmic processes. Others have a more elongated morphology. Characterisation of both morphological types has demonstrated expression of a number of extracellular matrix components including collagen types (II, IV and VI) and laminin (Wilkins & Jones, 1995a). According to Lim and colleagues (1986), these features would be characteristic of CFU-RF and not CFU-F.

1.2.2 Studying stromal fibroblasts

Until recently, phenotypic markers that specifically identified stromal fibroblasts within the bone marrow were lacking. In 1991, a murine IgM monoclonal antibody, STRO-1 was produced which was shown to identify bone marrow stromal progenitor cells (CFU-F) and lacked reactivity with haemopoietic multipotential progenitor cells (Simmons & Torok Storb, 1991). However, the STRO-1 antibody was shown also to be highly reactive with nucleated erythroid precursors. Cell sorting using the STRO-1 antibody, showed that CFU-F were exclusive to the STRO-1+ population and that a subset of these STRO-1-positive CFU-F expressed L-NGFR (Gronthos & Simmons, 1995).

When isolated and cultured, STRO-1 positive cells gave rise to heterogeneous stromal cells containing adipocytes, fibroblasts, smooth muscle cells (Simmons & Torok Storb, 1991) and osteoblasts (Gronthos *et al.*, 1994). Upon reaching confluence, these adherent layers were shown to be able to support the haemopoietic development of highly enriched CD34+ long-term culture-initiating cells. However, the ability of adherent stromal layers generated from STRO-1-positive cells to support haemopoiesis was not significantly different to adherent stromal layers generated in cultures formed from unseparated bone marrow (Simmons & Torok Storb, 1991).

In addition to STRO-1, other antibodies reactive with marrow stromal fibroblasts include HOP26 and SB10. These antibodies are reactive with, and have been used to isolate, populations of marrow fibroblasts with osteogenic potential (Joyner *et al.*, 1997; Bruder *et al.*, 1997). Potential expression of L-NGFR, αSMA or the STRO-1 reactive antigen by cells expressing the antigens recognised by HOP26 or SB10 is unknown.

Demonstration of L-NGFR expression by bone marrow stromal fibroblastic cells has led to further studies investigating the phenotype and function of these cells (Cattoretti *et al.*, 1993; Caneva *et al.*, 1997; Wilkins & Jones, 1998). More recently, expression of the p140, high-affinity NGF receptor (trkA) and other tyrosine kinase receptors (trkB and trkC) has also been demonstrated in fibroblastic bone marrow stromal cells (Labouyrie *et al.*, 1999), albeit at a lower level than that of L-NGFR. Further studies will enhance our understanding of this cell type in the marrow micro-environment and determine the functions of NGF receptors in these cells. The findings may have a variety of clinical and therapeutic implications. For example, bone marrow stroma has been previously suggested to play a key role in the pathogenesis of some haematological malignancies including multiple myeloma, in which malignant plasma cells home selectively to the bone marrow stroma (Cook *et al.*, 1997). In terms of therapeutic possibilities, it may be possible to modify stromal fibrosis in chronic myeloproliferative diseases, such as idiopathic myelofibrosis, and to protect stromal cells from bystander toxic injury during chemotherapy for leukaemias.

1.2.3 Functions of stromal fibroblasts

The functions of stromal fibroblasts in bone marrow remain largely unknown. Histological examination of trephine bone marrow biopsy cores, identifying the location of these cells within bone marrow, have contributed to most of our current understanding of the functions of this cell type. The long cytoplasmic processes of L-NGFR positive stromal fibroblasts have been shown to envelop haemopoietic cells, acting as a physical support for developing blood cells (Cattoretti *et al.*, 1993; Caneva *et al.*, 1997). These cells have been identified in close contact with myeloid, erythroid and lymphoid cells as well as immature cells suggesting that interactions between stromal and haemopoietic cells may have important regulatory functions (Caneva *et*

al., 1997). In addition to forming a network of dendritic processes throughout the bone marrow, L-NGFR positive stromal fibroblasts have been identified surrounding venous sinusoids in a number of tissues including liver, pancreas, stomach, kidney (Garin Chesa *et al.*, 1988) and bone marrow (Cattoretti *et al.*, 1993). This may suggest a role, in addition to haemopoietic support, in regulating the migration of mature blood cells from bone marrow into peripheral blood.

1.2.4 L-NGFR structure

The nucleotide sequence of the human L-NGFR has been determined by cloning of cDNA containing the entire coding sequence of the receptor (Johnson *et al.*, 1986). The mature 427 amino acid protein is a type I, integral trans-membrane protein encoded by a 3.8kb receptor mRNA. The protein comprises a 28 amino acid (aa) signal sequence, a 222aa amino-terminal extracellular domain, a 21aa membrane-spanning region and a carboxy-terminal cytoplasmic domain (Figure 1.3).

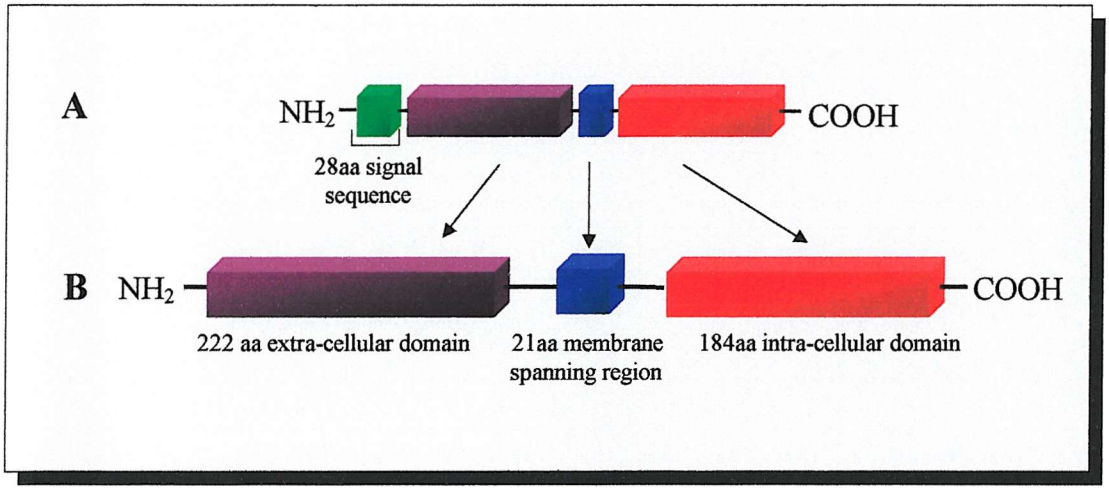


Figure 1.3. Simplified representation of the L-NGFR protein structure. Illustrating (A), the pro-protein preceded by a 28 amino-acid signal sequence and (B), the mature, 427 amino-acid receptor

The structural mechanisms underlying the presence of high- (p140, TrkA) and low-affinity (p75, L-NGFR) forms of the NGF receptor remain unclear. The biologically active form of NGF exists as a non-covalent dimer of two identical 13,000 dalton chains. It has been suggested that the four extracellular domains of NGFR may form either 2 or 4 NGF binding sites to allow bivalent binding of NGF. Alternatively, NGFR is known to be capable of dimerisation through disulphide bond formation

(Grob *et al.*, 1983). The NGF dimer may then bind simultaneously to two, linked receptor molecules achieving higher-affinity binding. Similarly, interaction of the receptor with other proteins, or post-translational modifications of the receptor may also account for high and low-affinity binding.

1.2.5 L-NGFR expression in normal tissue

The L-NGFR is expressed by a wide variety of cell types of both neuronal and non-neuronal origins. An immunohistochemical study of approximately 40 human adult tissue types (Garin Chesa *et al.*, 1988) demonstrated expression of L-NGFR in several epithelial cell types from the epidermis, tongue, oesophagus, cervix uteri, urothelium, breast and prostate gland. The results of this study are summarised in Table 1.1. Strong expression of L-NGFR was observed in follicular dendritic cells from the spleen and lymph nodes. Cells of the peripheral nervous system were also positive for L-NGFR. These included peripheral nerve fibres, autonomic ganglia from the intestine and bladder and nerve fibres from the spinal cord.

Expression of L-NGFR in liver, pancreas, stomach, colon and kidney was found in the study by Garin Chesa and colleagues (1988), to be restricted to nerve fibres and perivascular connective tissue. Muscle tissues, endocrine organs and the thymus were negative for L-NGFR. Tissues of the human central nervous system investigated in the study by Garin Chesa and co-workers (1988) included the cerebral cortex, basal ganglia, hypothalamus, substantia nigra and cerebellum, all of which were negative for L-NGFR. This is in contrast with other studies involving rat central nervous system, demonstrating L-NGFR mRNA production (Ojeda *et al.*, 1990) and receptor expression (Berg-von der Emde *et al.*, 1995) by some dopaminergic and some peptidergic neurons of the hypothalamus. Garin Chesa and colleagues (1988) also investigated expression of L-NGFR in fetal tissue. The results demonstrated that L-NGFR expression in the fetus almost parallels that of adult tissues except that strong expression was found in all fetal connective tissues including skeletal muscle.

Table 1.1. Expression of L-NGFR in normal tissue investigated using the avidin-biotin immunoperoxidase technique in frozen sections

Tissue	NGFR-expressing cell types
Nervous System	
Peripheral Nerves	Nerve fibres, perineurium
Autonomic ganglia	Ganglion, satellite
(Intestine, bladder)	Schwann cells
Spinal Cord	Nerve fibres (posterior horn)
Cerebral Cortex	No NGFR expression
(Frontal, temporal, occipital lobes)	
Basal Ganglia (gl. Pallidus, putamen)	----
Internal capsule	----
Thalamus	----
Hypothalamus	----
Substantia Nigra	----
Crus cerebri	----
Pons	----
Cerebellum	----
Cranial nerves (N.I., N.II)	----
Skin	Basal keratinocytes, sweat glands Myoepithelial cells
Mammary gland	Duct epithelium, myoepithelial cells
Gastro-intestinal system	
Tongue	Epithelium (basal layer)
Salivary gland	Myoepithelial cells
Oesophagus	Epithelium (basal layer)
Stomach	----
Colon	----
Liver	----
Pancreas	----
Respiratory system	
Bronchus	Epithelium (basal layer)
Lung	----
Gastro-urinary system	
Kidney	Glomerulus
Urinary bladder	Urothelium (basal layer)
Prostate	Epithelium, myoepithelial cells
Uterus	----
Cervix uteri	Exocervical epithelium (basal layer) Endocervical glands
Ovary	----
Testis	----
Lymphoid system	
Lymph node	Lymphocyte subpopulation
Spleen	Lymphocyte subpopulation
Thymus	----
Endocrine system	
Thyroid gland	----
Pancreas	----
Muscle tissue	Only in fetal skeletal muscle
Connective tissue	Perivascular stroma (widespread in fetus)

Reproduced, with permission, from Garin Chesa *et al.*, 1988

Over the last few years it has emerged that L-NGFR is expressed by a subset of stromal cells within bone marrow (Cattoretti *et al.*, 1993; Wilkins & Jones, 1995a; Caneva *et al.*, 1997). Antibodies reactive with L-NGFR have been shown by immunohistochemistry to label cells in bone marrow described as having an oval nucleus and dendritic projections (Cattoretti *et al.*, 1993). These dendritic processes have been shown by electron microscopy to be closely associated with bone marrow adipocytes, to surround mature plasma cells and bone marrow sinusoids and to form a complex network upon which haemopoietic cells lie (Caneva *et al.*, 1997). In normal adult bone marrow, L-NGFR positive cells are present in greatest concentration at trabecular margins and around larger stromal blood vessels (Wilkins & Jones, 1995a). Aspirated bone marrow samples have also been shown to contain a small population of cells that express L-NGFR (Wilkins & Jones, 1998).

1.2.6 L-NGFR expression in malignant tissue

The study by Garin Chesa and co-workers (1988) also investigated L-NGFR expression in over 200 tumour biopsy samples, including both frozen and fixed, paraffin-embedded tissue. Expression of L-NGFR was found most commonly in various subtypes of sarcoma; namely, embryonal rhabdomyosarcoma, Ewing`s sarcoma and synovial sarcoma. A number of neuro-ectodermal tumours were studied, of which expression of L-NGFR was demonstrated in approximately half the schwannomas and a smaller proportion of melanomas but was absent from all neuroblastomas studied. Approximately 25% of lymphomas studied showed expression of L-NGFR. These consisted of malignant B-cell lymphoma (1 case), plasmacytoma (1 case) and Hodgkin's disease (2 cases). Other tumours that have been shown to express L-NGFR include Wilm`s tumour (Donovan *et al.*, 1994) and adenocarcinoma of the prostate gland (Pflug *et al.*, 1992).

1.2.7 L-NGFR functions

Due to the relatively recent identification of L-NGFR expression by bone marrow stromal fibroblasts, there have been no studies to date investigating the functions of this receptor in the bone marrow micro-environment. In a recent publication (Miller, 1998), antagonistic functions of L-NGFR and the tyrosine kinase receptor, TrkA, are

described in neuronal development. This model may have implications for L-NGFR function in bone marrow development and/or regeneration following cytotoxic or radiation damage.

During the period of target organ innervation, *in vivo* survival of sympathetic neurons is dependent upon NGF-mediated TrkA activation to prevent these neurons from apoptotic cell death. By contrast, cell culture experiments demonstrate that activation of L-NGFR results in apoptosis of neural tumour cells. The L-NGFR contains a death domain motif believed to bind death effector molecules.

Miller (1998) postulates that relative levels of TrkA and L-NGFR activation determine survival of developing sympathetic neurons. If a neuron innervates at the correct time and location, it can sequester optimal levels of NGF, resulting in TrkA-mediated activation. This overrides NGFR activation, prevents L-NGFR-mediated cell death and allows neuron survival (Figure 1.4, part A). However, if the neuron innervates at either an inappropriate time or location, only low levels of NGF will be present. This will result in low activation of TrkA. Consequently, L-NGFR which, unlike TrkA, can be activated by other neurotrophins (for example, brain-derived neurotrophic factor; BDNF), will be sufficiently activated to override TrkA-mediated activation signals. This will result in neuronal apoptosis (Figure 1.4, part B).

The TrkA/L-NGFR system may thus provide a model for the elimination of inappropriate neurons during neuronal development. Similarly, this mechanism could operate in the bone marrow micro-environment to prevent inappropriate fibroblast growth. Clearly, functional studies will be required before any conclusions can be made regarding this possible function of L-NGFR expression in the bone marrow stroma.

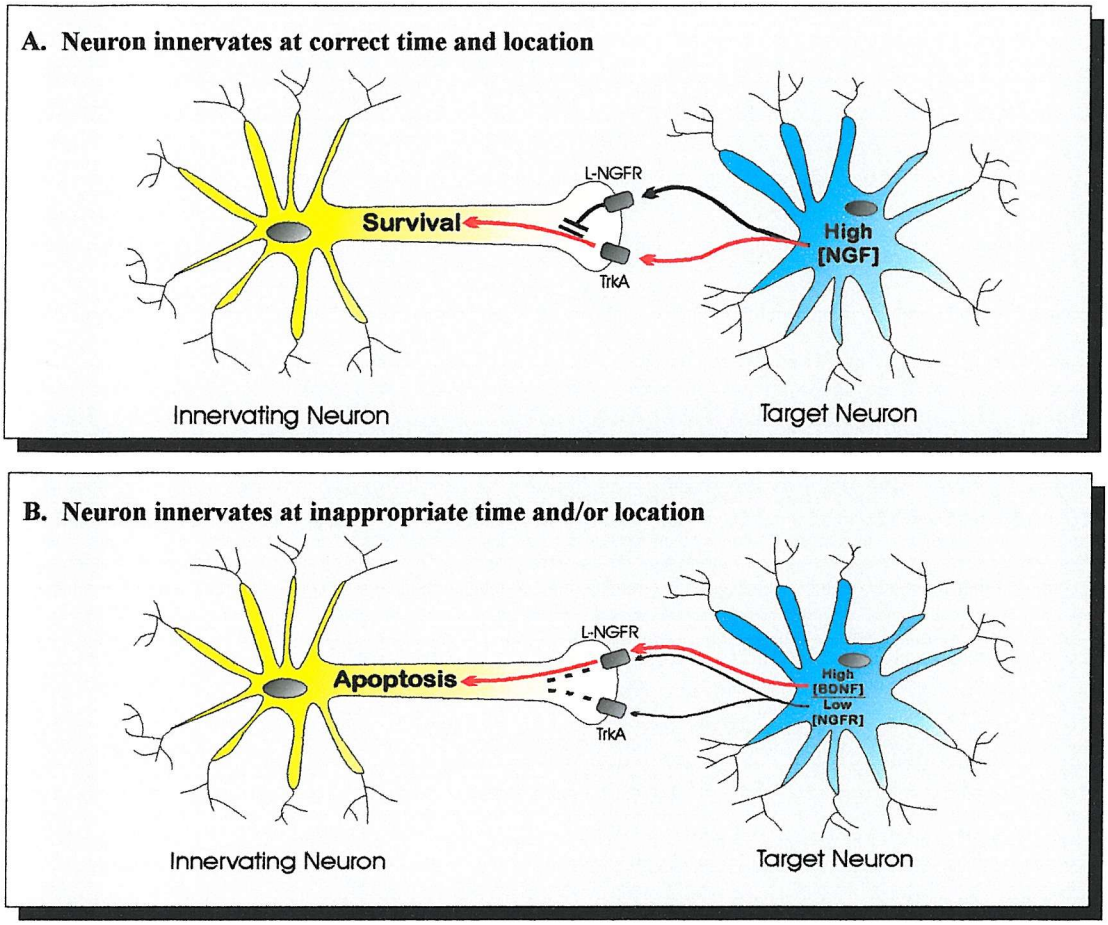


Figure 1.4. Model proposed by Miller (1998) for the regulation of neuronal innervation. See 1.2.7 for explanatory text.

1.3 STROMAL ENDOTHELIUM

1.3.1 Description of stromal endothelium

The bone marrow vasculature comprises a centrally located nutrient artery that runs longitudinally through the marrow of long bones, with arterial vessels branching radially to the marrow periphery (Weiss & Chen, 1965). A thin layer of endothelium, a thicker layer of smooth muscle cells and an outer, perivascular layer of stromal fibroblasts surround these arterioles. Thin-walled venous sinuses are present that run from the periphery of the marrow towards the centre where they connect to a central vein that runs alongside the major arteries (Weiss & Chen, 1965). These venous sinusoids are composed of a thin layer of flattened endothelium with individual endothelial cells connecting to each other via zonula adherens-type junctions.

Human bone marrow endothelial cells (HBMEC) have been isolated and cultured previously by immunomagnetic cell separation techniques based on the selective binding of *Ulex europaeus* agglutin-1 lectin (Masek & Sweetenham, 1994). Analyses of these cells, cultured *in vitro,* describe two morphological variants of HBMEC. There are those that grow rapidly, which are polygonal and spindle-shaped and others that have a more rounded morphology and a slower growth rate (Masek & Sweetenham, 1994). It has been suggested that endothelial cells from human bone marrow represent a distinct type of endothelium in terms of their functions and expression of many adhesion molecules (Rafii *et al.*, 1994). However, morphologically they are thought to be similar to human umbilical vein, bovine aortic and brain micro-vascular endothelial cells (Rafii *et al.*, 1994).

1.3.2 Antigen expression by stromal endothelium

Immunohistochemical analysis of cultured HBMEC has demonstrated expression of von Willebrand factor (vWF) and CD31 (platelet-endothelial cell adhesion molecule 1 or PECAM-1), with stronger expression of CD31 on endothelial cells with round morphology. Subpopulations of HBMEC also expressed intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin (Masek & Sweetenham, 1994). Cultured endothelial cells have also been shown to express CD34 (Rafii *et al.*, 1994).

Vascular structures within hLTBMC have also been studied by immunohistochemistry and shown to express CD31, CD34 and CD105 (endoglin). Equal expression of vWF and VCAM-1 was demonstrated by vascular endothelium and stromal fibroblasts in hLTBMC (Wilkins & Jones, 1995b). Many of the antigens expressed by stromal endothelium are involved in haemopoietic cell adhesion and may be involved in the regulation of migration of blood cells into and out of the bone marrow.

1.3.3 Functions of stromal endothelium

Stromal endothelial cells in culture have been shown to bind selectively CD34 positive haemopoietic stem cells and megakaryocytes (Rafii *et al.*, 1994) and are known to play a fundamental a role in the homing of stem cells to the bone marrow following

peripheral blood or bone marrow stem cell transplantation. The mechanisms involved in this homing are largely unknown but entail complex interactions between stromal endothelium and haemopoietic stem cells to ensure specificity of stem cell adhesion to bone marrow endothelium. Such mechanisms may involve regulation by CD31, which has been shown to be highly expressed on a subset of CD34 positive haemopoietic stem cells with early myeloid and B-lymphoid phenotypes (Watt *et al.*, 1993). Binding of CD31 can occur in a homophilic manner; i.e., CD31 molecules bind to each other on adjacent cells (Newman & Albelda, 1992). This could provide an adhesive mechanism by which haemopoietic stem cells might recognise and subsequently enter the bone marrow following transplantation.

However, more recent evidence suggests that other adhesion molecules, namely the integrins and their receptors (Imai *et al.*, 1999; Vermeulen *et al.*, 1998) and the selectins (Mazo *et al.*, 1998; Frenette *et al.*, 1998) expressed by stromal endothelial cells, may be more important in the homing of progenitor cells to the bone marrow. Studies carried out using lethally irradiated mice deficient in both P- and E-selectin have shown that the selectins may function in conjunction with the $\alpha 4\beta 1$ integrin receptor, vascular cell adhesion molecule-1 (VCAM-1) (Frenette *et al.*, 1998). In this study, mice deficient in P- and E-selectin, did not have abnormal numbers of progenitor cells in their bone marrow. Similarly, wild-type mice treated with functional blocking antibodies against VCAM-1 did not demonstrate greatly reduced homing of progenitor cells to the bone marrow. Interestingly, when mice deficient in P- and E-selectin were treated with VCAM-1 blocking antibodies, the numbers of circulating stem cells in the peripheral blood was greatly increased, demonstrating that these cells were not homing to the bone marrow. These data imply potentially important roles for P-and E-selectin in conjunction with VCAM-1 in the homing of progenitor cells to bone marrow endothelium

Stromal endothelium forms a physical barrier to circulating blood cells and, as such, is likely also to be involved the control of migration of mature blood cells out of the marrow. Many cytokines with roles in haemopoietic lineage maturation are produced by stromal endothelium, including granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), macrophage-colony

stimulating factor (M-CSF), kit-ligand and interleukin 6 (IL-6) (Rafii *et al.*, 1997). Stromal endothelium may therefore also be an important, if not vital, component of stroma for haemopoiesis.

1.4 STROMAL MACROPHAGES

1.4.1 Description of stromal macrophages

Stromal macrophages, although functionally considered part of the bone marrow stroma, differ from other stromal cells by virtue of their haemopoietic rather than stromal origin (Allen & Dexter, 1984). Two morphologically distinct types of bone marrow macrophage have been described in the adherent stromal layers of hLTBMC (Wilkins & Jones, 1995a; Wilkins & Jones, 1996). There are those with round morphology containing intracytoplasmic, phagocytosed granules and those that appear flattened and elongated with no evidence of cytoplasmic debris. The elongated macrophage variant, upon establishment of hLTBMC, generally took longer (by up to 4 weeks) to become as numerous as the rounded type. Equivalent macrophage populations have also been demonstrated in trephine biopsy sections (Wilkins & Jones, 1996).

1.4.2 Antigen expression by stromal macrophages

Studies investigating antigen expression by stromal macrophages in hLTBMC have shown that both types of stromal macrophages described above strongly express CD68, BerMac3 antigen (CD168), CD31, CD14, MHC class II, the antigens recognised by RFD 1 and 7 and factor XIIIA (Wilkins & Jones, 1995a; Wilkins & Jones, 1996). In addition, weak expression of endoglin, CD35, GM-CSF, CD31, CD44, VCAM-1, vimentin and vWF was demonstrated. It was found that in general, expression of these antigens was greater in the more round macrophages than in the flattened, elongated type.

1.4.3 Functions of stromal macrophages

The role of stromal macrophages in the support and maintenance of haemopoiesis remains unclear. They may be involved in the autocrine regulation of monocyte/macrophage differentiation, as they have been shown to produce mRNA encoding macrophage-colony stimulating factor (M-CSF) (Wilkins & Jones, 1996). Interactions between stromal macrophages and undifferentiated haemopoietic cells have been described (Lambertsen, 1984), which suggest that stromal macrophages may provide both physical and regulatory influences on haemopoietic stem cells.

1.5 STROMAL ADIPOCYTES

1.5.1 Description of stromal adipocytes

Stromal adipocytes represent one of the least studied stromal cell types in human bone marrow yet are the largest and most prominent stromal cell type, occupying a considerable area of the marrow. Mature adipocytes are large, with a thin band of cytoplasm, containing the nucleus, surrounding a single central lipid vacuole. In more immature adipocytes, lipid vacuoles are smaller and more numerous (Gimble *et al.*, 1996). In hLTBMC, the first signs of adipogenesis occur after approximately 2 to 3 week's growth, following formation of a confluent fibroblastic layer (unpublished observations). The adipocytes that develop, contain multiple lipid vacuoles, resembling small bunches of grapes. As hLTBMC develop further, these lipid vacuoles increase in size and become less numerous but the cells rarely, if ever become truly unilocular.

1.5.2 Functions of stromal adipocytes

The functions of stromal adipocytes and their contribution to haemopoiesis remain uncertain. Several hypotheses have been proposed for their function (Gimble *et al.*, 1996). They may act to fill space within the marrow cavity not required for haemopoiesis or may be involved in lipid metabolism, acting as a local energy source for emergency situations. Alternatively, they may serve similar supportive functions to other stromal cell types, providing physical support for haemopoietic cells and supplying contact-mediated or chemical signals.

1.5.3 Origin of stromal adipocytes

It is unclear whether stromal adipocytes develop as a separate lineage from other stromal cells or whether they originate from a common precursor stromal cell capable of divergent differentiation. It has been suggested that stromal adipocytes may originate from fibroblastic stromal cells, following observation that fat cells in the bone marrow of patients being treated with chemotherapy for leukaemia are positive for alkaline phosphatase, a marker for fibroblastic stromal cells (Bianco *et al.*, 1988). Thus, after chemotherapy when bone marrow haemopoietic cellularity decreases, fibroblastic stromal cells may convert to adipocytes to occupy the remaining space. Further evidence for a shared origin of bone marrow fibroblasts and adipocytes is provided by the observation that fibroblastic colonies grown in collagen gel developed into adipocytes (Mori *et al.*, 1987). There is also evidence that stromal adipocytes have osteogenic potential (Nuttall *et al.*, 1998). Cultured cells from human trabecular bone have been shown to be capable of generating both osteocytes and adipocytes. The authors of this study suggested that their results support the existence of a link between decreased bone density and increased stromal adipocyte formation observed with advancing age.

1.6 MYELOFIBROSIS

1.6.1 Description and causes of myelofibrosis

Marrow fibrosis is rarely, if ever, a primary disorder but usually occurs as a secondary consequence of haemopoietic or non-haemopoietic malignancies, or as a repair phenomenon following mechanical or toxic injury to bone marrow. Such injury includes chemotherapy and radiation therapy, which can cause marrow fibrosis during the course of treatment for neoplastic diseases (Fliedner *et al.*, 1986). Haemopoietic neoplasms that show a tendency to progress to myelofibrosis include the chronic myeloproliferative disorders (CMPD) and myelodysplastic syndromes (MDS). Many patients with CMPD, particularly those with primary thrombocythaemia (previously called essential thrombocythaemia), develop myelofibrosis progressively as their disease advances. Myelofibrosis has also been linked to abnormalities associated with megakaryocytes, macrophages, mast cells, lymphocytes, plasma cells and tumour cells (McCarthy, 1985).

Myelofibrosis arising as a primary manifestation of CMPD is usually associated with extramedullary haemopoiesis (so-called "myeloid metaplasia") and is currently designated in the U.K. as idiopathic myelofibrosis (IMF). It is characterised by an increase in fibroblasts and endothelial cells in the marrow, with excess deposition of their extracellular matrix products (Dilly & Jagger, 1990). Clinical features characteristic of this disorder include massive splenomegaly and leuko-erythroblastic anaemia (Lisse *et al.*, 1991; Weinstein, 1991). As the disease progresses, excessive pathological extramedullary haemopoiesis may also cause gastric bleeding and peritoneal ascites (Weinstein, 1991).

It is widely believed that myelofibrosis in the bone marrow in IMF is caused by abnormalities of the megakaryocytic cell lineage (Lisse *et al.*, 1991; Thiele *et al.*, 1997; Weinstein, 1991). Under normal circumstances, megakaryocytes interact closely with marrow fibroblasts and secrete a number of regulatory cytokines essential to their growth and development (Mayani *et al.*, 1992). These include interleukins (IL) 1, 6 and 7, granulocyte-colony stimulating factor (G-CSF), interferon-γ (IFN-γ), platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β) and tumour necrosis factor-α (TNF-α) (McCarthy, 1985; Bernabei *et al.*, 1986). Interestingly, the development of fibrosis in CMPD has been shown to correlate positively with the number of malignant megakaryocytes in the bone marrow (McCarthy, 1985).

1.6.2 Stromal changes in idiopathic myelofibrosis

Malignant megakaryocytes in CMPD overproduce and show premature release of these regulatory cytokines, especially PDGF and TGF-β (Bernabei *et al.*, 1986). This activates marrow fibroblast proliferation (Figure 1.5) and stimulates the synthesis and release of extracellular matrix components such as reticulin fibres. The latter can be shown by silver staining of hLTBMC (Chen *et al.*, 1991). In early stages of fibrosis, these reticulin fibres are associated with the predominance of interstitial collagen type III and fibronectin. In advanced stages of the disease, collagen type I predominates over type III and proliferating endothelial cells produce basement membrane collagens.

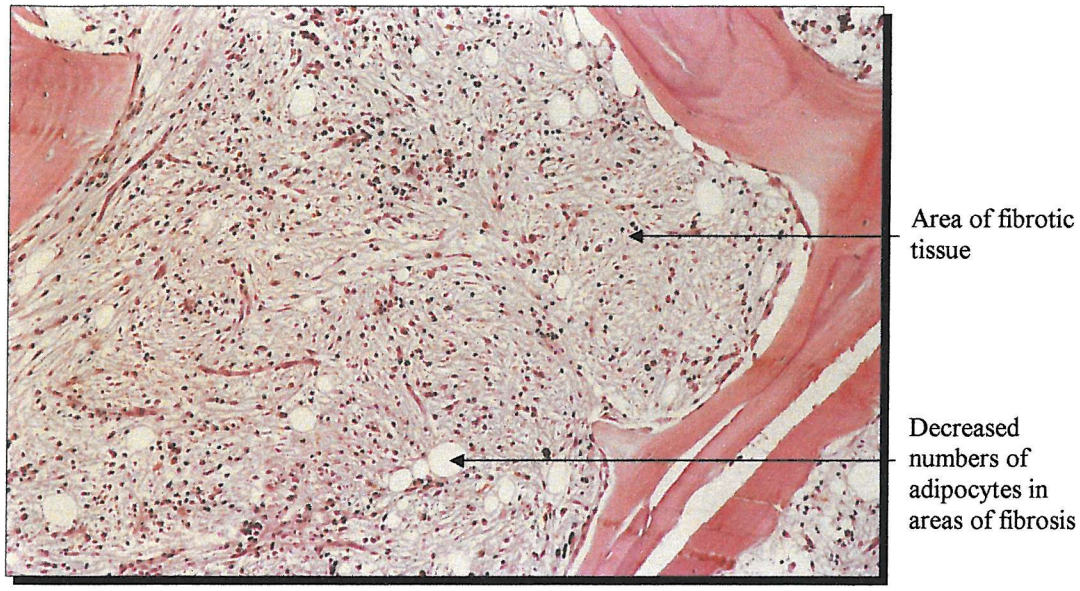


Figure 1.5. Myelofibrotic bone marrow trephine biopsy section (x100)

A study of 35 biopsy samples from patients with IMF showed that in all cases, intravascular haemopoiesis in these patients was associated with distended marrow sinusoids (Wolf & Neiman, 1985). In patients with chronic myeloid leukaemia, it has been shown that marrow fibroblasts do not cover sinus endothelial cells to the same extent as in normal marrow (Kuto *et al.*, 1984). In addition, trans-cellular endothelial pores, through which mature blood cells leave the marrow, are more numerous and larger in size. This may be a common feature of marrow fibrosis arising from various causes and would explain the intravascular and extramedullary haemopoiesis seen in IMF.

Other studies have also demonstrated dilation of marrow sinusoids in IMF. This has been associated with neo-angiogenesis and enhanced blood flow (Hasselbach, 1990). Increased proliferation of endothelial cells in association with marrow fibrosis, leading to increased production of basement membrane collagen and other extracellular matrix proteins may, in part, explain these observations.

In summary, marrow fibrosis is a complicated abnormality arising as either a manifestation of haemopoietic neoplasia or in the course of some other malignancy and/or treatment for malignant disease. It occurs as a result of complex interactions between haemopoietic and stromal cells leading to stimulation of the latter and subsequent overproduction of extracellular matrix proteins, in particular reticulin

fibres. Histological examination of bone marrow trephine biopsy sections in IM reveals an excess of stromal fibroblasts and endothelial cells, accompanied by increased size and dilation of marrow sinusoids. These factors contribute to the intravascular and extramedullary haemopoiesis seen in IMF. It is attractive to speculate that anti-fibrogenic intervention might ameliorate patients` symptoms in this disease and in secondary myelofibrosis occurring in other disorders.

1.7 ACUTE MYELOID LEUKAEMIA

1.7.1 Diagnosis and classification of acute myeloid leukaemia

Acute myeloid leukaemia (AML) is a neoplastic disease of leukocytes characterised by excessive proliferation of immature myeloid cells within the bone marrow (Figure 1.6). It is often referred to as acute non-lymphoblastic leukaemia (ANLL), to emphasise its distinction from acute leukaemias of lymphoid derivation. The incidence of AML is greatest in adults and older adolescents, with peaks of incidence occurring at 15 to 20 years of age and above 50 years of age (Haen, 1995). By current criteria, AML exists when more than 30% of all non-erythroid cells in the bone marrow are myeloid blast cells (Bennett *et al.*, 1985), although recent proposals have been made to lower this threshold to 20% (Harris *et al.*, 2000).

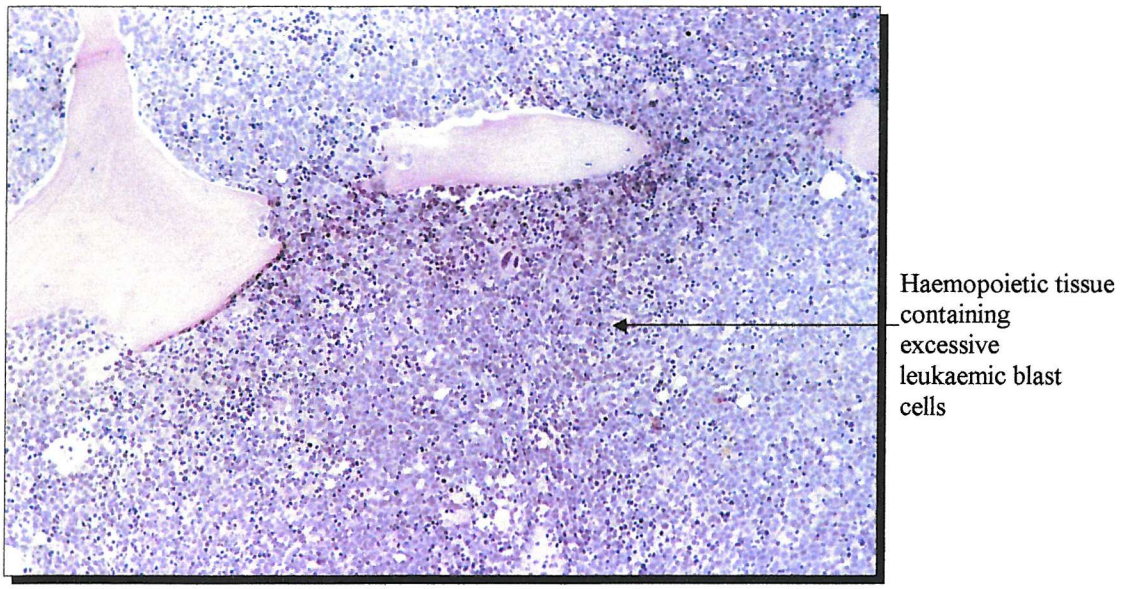


Figure 1.6. Bone marrow trephine biopsy section from a patient with AML (x100).

Acute myeloid leukaemia is commonly divided into 8 subtypes, based upon morphological and cytological characteristics (Bennett *et al.*, 1976; Bennett *et al.*, 1980; Bennett *et al.*, 1985; Bennett *et al.*, 1991). The different subtypes are classified according to the degree of differentiation along the various myeloid cell lineages and the stage of maturation within that lineage.

This classification for the acute leukaemias was proposed in 1975 by a group of 7 French, American and British haematologists after it was observed that the first cytotoxic drugs used gave better responses in patients with acute leukaemia affecting the lymphoid cell lineages (Bennett *et al.*, 1976). It was intended that, as subtypes within the acute leukaemias were understood better, cytotoxic therapy could be tailored to suit individual subtypes and hence improve responses to chemotherapy. The resultant French, American and British classification (FAB) for acute leukaemias initially proposed 6 subtypes of acute myeloid leukaemia; M1 to M6. In this system, AML-M1 to M3 demonstrate predominantly granulocyte differentiation, M4 both granulocyte and monocyte differentiation, M5 mainly monocyte differentiation and M6 predominantly erythroblastic differentiation.

A more recent classification, the MIC-M (morphologic-immunologic-cytogenetic and molecular genetic) classification has been devised due to the advances made in recent years with immunocytochemical, cytogenetic and molecular biological techniques and the association of genetic abnormalities with various acute leukaemia subtypes. For the purposes of this study, however, FAB terminology will be used because full data for MIC-M classification were not available for many of the archival specimens studied. The MIC-M classification is based on leukaemic cell morphology, expression of lineage specific markers, such as CD13, CD33, CD65 and CD117, specific for myeloid cell lineages and molecular genetic analysis of leukaemic cell DNA. Seventy percent of AML cases show genetic abnormalities, the most common being trisomy 8 and abnormalities of chromosome 7 (Bain, 1999). The most common chromosomal translocations in patients with AML is t(8;21)(q22;q22) representing a translocation of band 22 on the long arm of chromosome 8 with band 22 on the long arm of chromosome 21. The molecular events arising from this translocation result in the fusion of part of the AML1 gene, which normally codes for one chain of a

heterodimeric transcription factor (core binding factor; CBF), with part of the ETO (eight twenty-one) gene, a transcription factor gene normally expressed in the brain. The resultant fusion gene, AML1-ETO or CBFα-ETO, codes for a chimearic protein expressed on leukaemic cells. This protein is believed to result in the development of leukaemia by interference with the transcription factor activity of the normal AML1 protein.

The subtype AML-M1 was termed "acute myeloblastic leukaemia without maturation" and represents AML in which myeloblasts in the bone marrow show some evidence of granulocyte differentiation (Bennett *et al.*, 1976). These myeloblasts must represent more than 90% of all non-erythroid cells (Bennett *et al.*, 1991). The M2 subtype differs from M1 by the presence of granulocyte maturation to or beyond the promyelocyte stage in at least a proportion of cells. In order to be classified as M2 subtype, it was suggested that myeloblasts and promyelocytes together must represent fewer than 90% but more than 30% of all non-erythroid cells in the bone marrow.

The M3 subtype, known as hypergranular promyelocytic leukaemia or acute promyelocytic leukaemia (APML) is characterised by the presence of abnormal promyelocytes containing heavy cytoplasmic granulation. A variant of M3 (M3v) was described in 1980 (Bennett *et al.*, 1980). This represents an atypical form of M3 in which there is little, as opposed to excessive, promyelocytic granulation. The chromosomal translocation t(15;17)(q22;q21) is found in most patients with the M3 or M3v AML subtype (Bain, 1999).

Myelomonocytic leukaemia, denoted AML-M4, is characterised by both granulocytic and monocytic differentiation. To be categorised as AML-M4, blast cells must constitute more than 30% of all nucleated cells and between 30 and 89% of nucleated cells must be myeloblasts, promyelocytes or myelocytes. This subtype of AML is similar to M2 except that more than 20% of all nucleated cells must be monocytes or promonocytes. A variant of M4, termed AML-M4 with eosinophilia (AML-M4Eo), was described in 1985 (Bennett *et al.*, 1985). In this variant, abnormal eosinophils are present, constituting up to 5% of all non-erythroid nucleated cells. These eosinophils vary from normal in that they contain abnormal basophilic granules that react

positively with chloroacetate esterase and periodic acid Schiff's stains. The AML M4Eo subtype is characteristically associated with inv.16 (Bain, 1999).

The presence of more than 80% of nucleated cells having the features of monoblasts, promonocytes or monocytes is typical of acute monoblastic leukaemia, or AML-M5. There are two types of M5; poorly differentiated (AML-M5a), in which more than 80% of monocytic cells are monoblasts and differentiated (AML-M5b), in which less than 80% are monoblasts, the remainder being promonocytes and monocytes. Erythroleukaemia, or AML-M6, is characterised by more than 50% of nucleated cells being erythroblasts. These cells often have multi-lobed and multi-sized nuclei and are usually distinguishable from myeloblasts.

In 1985, a new FAB subtype was introduced, termed AML-M7. In this subtype, more than 30% of nucleated bone marrow cells are undifferentiated blast cells of the megakaryocyte lineage, identified by expression of platelet-specific antigens or by demonstration of the platelet peroxidase reaction in electron micrographs (Bennett *et al.*, 1985). This leukaemia subtype is commonly associated with the chromosomal translocation t(1;22)(p13;q13).

In 1991, yet another subtype of AML, AML-M0, was described. This is also known as minimally differentiated acute myeloid leukaemia. It cannot be diagnosed on a morphological basis alone but, in addition, requires enzyme cytochemical and immunocytochemical evaluation. Leukaemic blast cells representing M0 are typically myeloperoxidase and Sudan Black negative and negative for most B- and T-cell markers, except for CD4 or CD7, which may be expressed in some cases. The blast cells of AML-M0 are identified as myeloid by their expression of CD13 or CD33 and often also CD11b (Bennett *et al.*, 1991).

1.7.2 Clinical features of acute myeloid leukaemia

The excessive proliferation of abnormal leukocytes causes a massive decrease of normal haemopoietic cells in the marrow, resulting in the majority of clinical symptoms associated with acute leukaemias. Other symptoms are caused by the infiltration of malignant leukaemic cells into other tissues and organs (Haen, 1995).

Most patients with acute leukaemias present with anaemia, with decreased haemoglobin levels resulting from suppression of erythropoiesis and increased destruction of red blood cells by an enlarged liver. A lack of functional neutrophils in these patients increases both susceptibility to and severity of, infections. Abnormal platelet production can result in increased risk of haemorrhage in such patients, with risk of severe internal bleeding if platelet count is not maintained (Haen, 1995).

1.7.3 Therapy for acute myeloid leukaemia

The primary consideration when determining treatment options for patients presenting with AML, is patient age. All AML patients included in this study (see Chapter 7) were adults and therefore therapy for childhood AML will not be discussed. Adult patients are assessed on the basis of their age (usually grouped as either above or below the age of 60) and health status to determine their suitability for intensive chemotherapy. With the Southampton University Hospitals NHS Trust (SUHT), adult patients are treated for AML in accordance with Medical Research Council (MRC) clinical trial protocols (Medical Research Council UKALL XI Trial Protocol, 1992; Medical Research Council MRC AML12AD trial protocol, 1994). The majority of patients included in this study have been treated under the Medical Research Council Twelfth Acute Myeloid Leukaemia Trial in Adults (MRC AML 12AD).

Treatment with chemotherapy is divided into two main stages. Induction chemotherapy to achieve remission and, once remission has been achieved, consolidation chemotherapy to maintain remission. Induction chemotherapy for the MRC AML12AD trial involves patients being randomised to one of two induction regimens. Regimen one consists of two courses of treatment with cytarabine, daunorubicin and etoposide (ADE). Regimen two consists of two courses of treatment with mitozantrone, cytarabine and etoposide (MAE), with or without G-CSF support. Bone marrow trephine biopsies are taken between the two courses of induction therapy and after both induction courses are complete, to assess whether complete remission has been achieved.

Once patients have completed both induction courses of chemotherapy, they then, if in complete remission, receive one course of consolidation chemotherapy in order to

eradicate any residual leukaemic cells. Consolidation therapy consists of M-amsacrine, cytarabine and etoposide (MACE). Should complete remission not be achieved at this stage, further courses of chemotherapy are implicated. Patients are then randomised into receiving one of four further treatment options. Regimens one and two consist of bone marrow transplantation, with or without a single prior course of idarubicin, cytosine arabinoside and etoposide (ICE). Regimens three and four consist of a single course of mitozantrone and ara-C (MIDAC), with or without a prior course of ICE. Further bone marrow trephine biopsies are taken between each subsequent course of therapy.

1.8 Acute Lymphoblastic Leukaemia

1.8.1 Diagnosis and classification of acute lymphoblastic leukaemia

Acute lymphoblastic leukaemia (ALL) is a leukocyte malignancy characterised by excessive proliferation of primitive leukaemic blast cells of lymphoid origin. It is characteristically a disease of childhood, with 60-70% of all cases occurring in young children around the age of 4 years and only 20% of cases occurring in adults (Haen, 1995). The original FAB group classified ALL into three subtypes; L1, L2 and L3, according to morphological and cytological features of leukaemic blast cells in the marrow (Bennett *et al.*, 1976). Subtype L1, the most common ALL subtype, is characterised by a predominance of small lymphoblasts with a regular nuclear shape and high nuclear: cytoplasmic ratio. These lymphoblasts are similar in size to lymphocytes and may be misdiagnosed as chronic lymphocytic leukaemia when categorised solely upon morphological features.

In subtypes L2 and L3, lymphoblasts are characteristically large compared with those found in L1. Lymphoblasts in subtype L2, although relatively large, are heterogeneous in size with an irregular nuclear shape containing one or more nucleoli. The nuclear: cytoplasmic ratio is lower than that of L1 with abundant amounts of cytoplasm often present (Bennett *et al.*, 1976). Subtype L3 differs from that of L2 primarily by the presence of regular as opposed to irregular shaped nucleus and cytoplasmic vacuolation. In addition, a range of B-lymphocyte markers are identifiable in most cases of ALL-L3 (Bennett *et al.*, 1976).

However, lymphoblasts from patients with ALL are often not easily distinguishable from lymphocytes or from myeloblasts in AML-M0 or M1 (Farhi & Rosenthal, 2000). More recently, immunophenotyping and cytogenetic analysis has become increasingly valuable in the diagnosis and management of ALL. Not only do these techniques aid in the diagnosis of ALL but they are also useful predictors of therapy outcome and overall prognosis (Farhi & Rosenthal, 2000).

The classification of ALL has thus diverged from the FAB classification and cases of ALL are now frequently classified according to the MIC-M classification (Bain, 1999) based upon cell lineage differentiation and subsequent cytogenetic findings. These subgroups include; B-cell ALL (B-ALL), T-cell ALL (T-ALL), common ALL (c-ALL) and unclassified ALL (u-ALL). Within each category, further sub-classifications are made according to cytogenetic abnormalities. Immunophenotypic studies of bone marrow trephine biopsy sections have demonstrated that antibodies targeting CD10, CD79a, CD20 and βF1 are particularly useful in distinguishing between c-ALL and T-ALL (Toth *et al.*, 1999). Antibodies against CD79a have been shown to be reactive in the majority of B-ALL and CD3 in T-ALL (Pileri *et al.*, 1999). Cytogenetic abnormalities associated with B-ALL commonly contain translocations involving immunoglobulin genes and those associated with T-ALL commonly affect T-cell receptor genes (Bain, 1999).

1.8.2 Clinical features of acute lymphoblastic leukaemia

The majority of symptoms in patients with acute lymphoblastic leukaemia are common to those found in patients with acute myeloid leukaemia, described in section 1.7.2. However, malignant lymphoblasts often infiltrate other organs, most frequently lymph nodes, causing lymphadenopathy (enlargement of the lymph nodes) but also liver, spleen, skin, bones, kidney and the membranes surrounding the brain, causing meningeal leukaemia (Haen, 1995). Consequently, symptoms associated with ALL are very much dependent upon the organ infiltrated.

1.8.3 Therapy for acute lymphoblastic leukaemia

All patients with acute lymphoid leukaemia included in this study were between the ages of 4 and 14 years and therefore only treatment for childhood ALL will be discussed. Similar to patients with AML, patients treated within SUHT for ALL are treated according to Medical Research Council clinical trials (Medical Research Council UKALL XI Trial Protocol, 1992). Patients included in this study received treatment during the period of 1992 and 1998 and as a result were all treated under the Medical Research Council Eleventh Acute Lymphoblastic Leukaemia Trial UKALL XI for Children.

Treatment for ALL under the UK ALL XI trial protocol consists of three major components. The first stage of therapy, remission induction, was aimed at achieving complete remission over a 4-week period. Patients with ALL differ from those with AML by the increased risk of infiltration of leukaemic cells into the central nervous system (CNS). During the treatment period, patients are randomised to either receive cranial radiation therapy, or high-dose intra-venous and intrathecal methotrexate chemotherapy to prevent CNS disease. The third stage of therapy, termed maintenance therapy, is carried out over a two-year period to prevent subsequent disease relapse (Medical Research Council UKALL XI Trial Protocol, 1992).

Remission induction in the UKALL XI trial consists of asparaginase, vincristine and prednisolone treatment for the first 4 weeks followed by an intensification treatment period at weeks 5 and 20 comprising daunorubicin and etoposide. Bone marrow trephine biopsies are obtained at weeks 1, 2, 5, 12 and 20. Patients are again randomised to receive a further 5-day block of intensification therapy a) soon after remission induction, b) after 5 months, c) both soon and after 5 months or d) not at all, to determine whether intensification therapy given in remission increases the likelihood of event-free survival (Medical Research Council UKALL XI Trial Protocol, 1992).

1.9 Effects of Radiation and Chemotherapy on the Bone Marrow

1.9.1 Effects of radiation therapy on the bone marrow

The bone marrow stroma offers essential support for haemopoietic activity through its physical ability to support haemopoietic progenitor cells and by production and release of essential cytokines and growth factors. The bone marrow stroma is also an important source of extracellular matrix components (Zuckerman *et al.*, 1989), as discussed earlier. Ionising radiation is often used in the treatment of various haematological malignancies, such as ALL discussed in section 1.8.3 and prior to bone marrow transplantation. Such therapy can result in abnormal haemopoietic reconstitution and it has been suggested that this may be mediated by irreversible damage to the bone marrow micro-environment (Sacks *et al.*, 1978).

In a study in 1986 (Laver *et al.*, 1986), investigating the *in vitro* effects of radiation on stromal progenitor cells, it was shown that irradiation of marrow cell suspensions resulted in the inability to generate adherent fibroblastic cell layers. This loss of proliferation of stromal cells occurred in a dose-dependent manner and the level of radiation that resulted in total loss of adherent cells was relatively low at 240 rad. Laver and co-workers (1986) also carried out co-culture experiments in which autologous marrow cells were cultured with highly irradiated stromal layers (up to 1320 rad). Stromal cultures in these experiments sustained production of CFU-GM, suggesting that stromal progenitors maintain their ability to support haemopoietic progenitors after irradiation, at least in terms of cytokine production. Although the proliferative capacity of these stromal progenitors is very radiosensitive, it seems that their functional properties, in terms of their ability to support haemopoiesis, are not impaired. This contrasts with a previous study by Tavassoli and colleagues (1982) who demonstrated a loss in the ability of stromal layers to support haemopoiesis after irradiation of just 500 rad (Tavassoli, 1982).

With regard to bone marrow transplantation, it would appear that, for donor haemopoietic cells to engraft successfully, host stroma must be able to maintain its ability to support donor cells. Since, in most cases, most transplantation occurs successfully after conditioning regimes involving radiotherapy, it would seem that

stroma is quite resistant to irradiation *in vivo*. Host stromal cells that survive irradiation presumably go on to proliferate and replace those that have been killed or critically injured since the resultant stroma, following allogeneic marrow transplantation, is usually of host and not donor origin (Simmons *et al.*, 1987).

1.9.2 Effects of chemotherapeutic agents on the bone marrow

The manner in which some chemotherapeutic agents affect haemopoiesis differs from the effects of radiation therapy. One of the most detrimental effects of many chemotherapeutic drugs *in vitro* is to decrease the ability of adherent stromal cell layers to support haemopoiesis. Many chemotherapeutic agents result in myelosuppression and may cause myelodysplasia and secondary malignancies. Such agents include nitrosureas such as 1,3-bis-(2-chloroethyl)-1-nitrosurea (BCNU) (Uhlman *et al.*, 1991), busulfan and cyclophosphamide (Fried & Adler, 1985). Most chemotherapeutic agents are cell-cycle specific and are usually more detrimental to rapidly proliferating haemopoietic cells than the more slowly proliferating stromal cells. However, these drugs have been shown to affect the stroma by impairing its ability to support the proliferation of haemopoietic stem cells (Fried & Adler, 1985). The mechanisms underlying this effect are not clear, although they may involve a decrease in the production of essential cytokines such as IL-3 and GM-CSF by stromal cells.

In addition to the therapy of a wide range of malignancies, low-dose chemotherapy and/or growth factor regimes are used prior to haemopoietic transplantation. These agents are used in conditioning regimes prior to mobilisation of haemopoietic stem cells from the bone marrow into the peripheral blood, for peripheral blood stem cell (PBSC) transplantation and in preparation for bone marrow allografting.

1.10 Bone Marrow Transplantation

1.10.1 Types of transplantation

The majority of chemotherapeutic agents used for the treatment of haematological malignancies are myelosuppressive and only limited doses can be given without excessive bone marrow toxicity. Bone marrow or stem cell transplantation is carried out to allow greater, more myelo-ablative, doses of chemotherapy to be given in order to cure the malignancy. The first successful bone marrow transplantations were carried out in the 1970s using either autologous (patients' own), syngeneic (identical sibling), or allogeneic (another individual's) bone marrow cells. In the 1980s, PBSC was first used as an alternative to bone marrow stem cells for transplantation (Dannie, 1996). The use of PBSC for transplantation is increasingly being used in preference to bone marrow, especially for autologous transplants. In Europe, in 1997 alone, 11 021 patients received autologous stem cells from peripheral blood compared with just 829 receiving stem cells from bone marrow (European Blood and Marrow Transplantation Handbook, 2000).

Haemopoietic stem cells are mobilised from bone marrow into peripheral blood using low-dose chemotherapy regimes and certain growth factors such as GM-CSF (Barr *et al.*, 1975). The mechanisms by which mobilisation occurs are poorly understood but are believed to involve disruption of cyto-adhesive interactions that normally exist between stem cells and bone marrow stroma (To *et al.*, 1997).

Peripheral blood cells are harvested by leucopheresis to separate mononuclear cells from erythrocytes and platelets. In some cases, haemopoietic stem cells are enriched in the mononuclear cell population on the basis of CD34 expression. This decreases the number of cells that need to be re-infused for successful engraftment and also reduces toxicity associated with exposure to the cryopreservative, dimethyl sulphoxide (DMSO) (Stroncek *et al.*, 1991), since the volume re-infused is minimised. Harvested mononuclear cells, with or without enrichment for CD34 positive cells, may be stored by cryopreservation, sometimes for many years, until required for transplantation.

1.11 AIMS AND OBJECTIVES

The aims and objectives of this study are outlined below:

1.11.1 Aims

1. To improve characterisation of bone marrow stromal cell types, with particular reference to L-NGFR-expressing cells, in order to shed light on their origins and functions.
2. To investigate stromal cell immunophenotypic changes in selected diseases of interest, in order to examine possible pathogenic roles for stroma in such diseases.

1.11.2 Objectives

1. To isolate, using magnetic activated cell sorting (MACS), individual cellular components of human bone marrow stroma. This includes stromal fibroblasts, macrophages and endothelial cells. Haemopoietic components of bone marrow were also isolated by the same methodology.
2. To study the growth characteristics of these cells in culture and, after growth to confluence, to characterise the immunophenotype of these cells in order to investigate their possible origins and functions. Of particular interest, antibodies reactive with L-NGFR were used to isolate stromal fibroblasts and investigate the characteristics of these cells prior to, during and after growth to confluence.
3. To investigate synthesis of L-NGFR mRNA in the above cell types and to determine whether mRNA synthesis corresponds to receptor expression.
4. To study, using immunohistochemistry, the cellular changes that occur in bone marrow stroma of patients with idiopathic myelofibrosis (IMF) and acute leukaemias, compared with normal bone marrow, to further our understanding of the pathogenesis of these diseases. In particular, to investigate the relationship between expression of α-smooth muscle actin and L-NGFR by stromal fibroblasts in these patients.
5. To study, using immunohistochemistry, the stromal cell changes that occur following chemotherapeutic treatment for acute leukaemias, to investigate the effects of these agents on bone marrow stromal cells and to elucidate possible

mechanisms by which these agents might result in variations in haemopoietic regrowth that occur following such chemotherapy.

6. To investigate whether mobilised peripheral blood stem cells contain cells capable of generating stromal layers under human long-term bone marrow culture conditions or cells that express antigens representative of bone marrow stromal cell populations.

2. METHODS

PART A: RNA METHODS

2.1 HANDLING RNA

It is vitally important when extracting and handling ribonucleic acid (RNA) that all possible steps are carried out to both inhibit and prevent contamination by enzymes responsible for the degradation of RNA (ribonucleases; RNases). These enzymes are very stable, highly active enzymes and only minute amounts are sufficient to destroy RNA. Gloves were worn at all times during RNA handling procedures. Although gloves prevent RNase contamination from the skin, they themselves are not RNase free. To minimise contamination from RNases on the gloves, they were changed regularly throughout all RNA handling methods.

When extracting or handling RNA, sterile disposable plastic-ware was used where possible. When this was not possible, such as with gel and blotting apparatus, all plastic-ware was pre-treated by overnight incubation with a solution of 0.5M sodium hydroxide (NaOH). This was rinsed several times the following day with water treated with 0.1% diethyl pyrocarbonate (DEPC) (see section 2.1.1). All glassware was cleaned with a detergent, in a dishwasher capable of washing at high temperatures (80°C) and rinsed several times using distilled or ultra-pure water. It was then baked at, or above, 160°C for at least three hours before use. Oven baking ensured that no RNases remain on the surface of the glassware.

2.1.1 Preparation of solutions

Autoclaving alone is not sufficient to inactivate all RNases. All reagent solutions were therefore prepared using water treated with 0.1% DEPC. This is a powerful inhibitor of RNases, which inactivates them by covalent modification (ethoxyformylation) of histidine residues (Melchior & Fahrney, 1970). Ultra-pure water, prepared by reverse osmosis (RO water) was treated with 1ml DEPC/litre followed by vigorous shaking to disperse the DEPC. The solution was left for 12 hours at room temperature followed by autoclaving. The DEPC-treated water was then stored at 4°C and used to prepare all solutions involved in RNA preparation.

2.1.2 Tissue storage

Prior to RNA extraction, all tissue was stored frozen, in small quantities, in liquid nitrogen. Specimens (cells or tissue samples) were frozen in liquid nitrogen as soon as possible after collection to minimise degradation of RNA by intracellular RNases. Cell samples were then stored frozen in liquid nitrogen to maintain inactivity of RNases. Following RNA extraction from tissues or cells, the RNA was stored dissolved in sterile water, in a suitable sterile container at between -20°C and -70°C.

2.2 RNA Extraction

2.2.1 RNA isolation kits

A number of commercial kits are available for the isolation of total RNA. These are advantageous over traditional phenol/chloroform methods for RNA isolation because they are simple, quick and efficient to use. A comparison of effectiveness was carried out between two such commercially available kits. These were the QIAGEN RNeasy mini kit (Qiagen Ltd.) and the PROMEGA SV total RNA Isolation system (Promega Corp., USA). The QIAGEN kit was found to yield a greater amount of RNA from equivalent sources. The method for RNA extraction using the QIAGEN kit is described in detail in Appendix A1.4.

2.2.2 Tissue lysis and homogenisation

Small pieces of frozen tissue were disrupted using a pestle and mortar. The tissue pieces, stored frozen in liquid nitrogen, were ground to a powder in the presence of additional liquid nitrogen. As the liquid nitrogen evaporated, more was added to prevent thawing of the tissue. When the tissue was ground to a fine powder, the suspension of tissue and liquid nitrogen was transferred to a sterile bijou container and the liquid nitrogen allowed to evaporate. Lysis buffer (Buffer RLT (as supplied); Qiagen Ltd, UK) containing guanidium isothiocyanate (GITC) was added immediately to lyse the powdered tissue and prevent RNA degradation by intracellular RNases. The lysate was homogenised by loading onto a QIAshredder (Qiagen Ltd., UK).

Cell cultures were prepared for homogenisation by aspiration of the supernatant medium immediately prior to addition of GITC-containing lysis buffer to the adherent cells on the growth surface. The lysate was repeatedly pipetted to eliminate any clumps of cell fragments and then transferred to a QIAshredder (Qiagen Ltd., UK) for homogenisation.

Frozen cells were thawed as described in section 2.12.3 and centrifuged to form a pellet. The supernatant was removed, by vacuum aspiration and GITC-containing lysis buffer added directly to the pelleted cells. The lysate was pipetted, as described above, to disperse cell fragments before being transferred to a QIAshredder (Qiagen Ltd., UK) for homogenisation.

The tissue/cell lysate was pipetted onto a QIAshredder and then subjected to centrifugation. The QIAshredder contains a biopolymer membrane, which acts to homogenise the lysate during centrifugation. The homogenised lysate was eluted into a sterile microcentrifuge tube.

2.2.3 RNA extraction

To the eluate obtained from homogenisation, an equal volume of 70% ethanol was added and mixed well by pipetting. Ethanol optimises the conditions under which RNA will bind to the membrane of the RNeasy spin column. The solution was then applied to an RNeasy spin column and centrifuged at 10,000rpm for 15 seconds, during which RNA in the sample bound the membrane of the RNeasy spin column. Buffer RW1, a wash buffer from the RNeasy kit, was added to the column and centrifuged as before for 15 seconds to remove any contaminants from the membrane. Two further washes were carried out using kit buffer RPE diluted 1:4 in 100% ethanol and centrifuged as above, the latter centrifugation step being carried out at 16,000 rpm for 2 minutes to dry the membrane. This is necessary because residual ethanol prevents elution of RNA from the membrane. The RNA was eluted in RNase-free water into a new sterile collection tube by centrifugation at 10,000 rpm for 1 minute.

2.3 RNA QUANTITATION AND PURITY

2.3.1 Calculation of RNA concentration

The concentration of RNA extracted from cells was determined by measuring the optical density of the RNA sample at 260nm (OD_{260}). The RNA concentration was calculated from the OD_{260} using the following equation:

$$[RNA](\mu g/ml) = \frac{A \times 40 \times DF}{\text{Path length correction}}$$

Where A = Absorbance
40 = RNA coefficient (If A = 1, [ssRNA] = 40μg/ml)
DF = Dilution factor at 260nm

2.3.2 OD_{260}/OD_{280} ratio

Measuring the ratio between the absorbance at 260nm (OD_{260}) and 280nm (OD_{280}) in a spectrophotometer was used to determine the purity of the extracted RNA. The absorbance at 280nm was used to measure the concentration of protein contamination in the sample. A 260/280nm ratio of 1.7 to 2.1 represents acceptable protein contamination and a value of 2.0 is considered to represent pure RNA with no protein contamination. Readings above or below this range indicate unacceptable protein contamination.

2.3.3 OD_{260}/OD_{230} ratio

Low RNA yields, reflected by a low OD reading at 260nm, may be a result of GITC or β-mercaptoethanol contamination. This was determined by measuring the absorbance at 230nm in addition to 260nm and calculating the ratio. A 260/230nm ratio outside the range of 1.8 to 2.2 is indicative that GITC or β-mercaptoethanol is still present from the lysis buffer. This can be overcome by precipitation of the RNA with an RNase free acetate salt and ethanol.

2.4 RNA Integrity

2.4.1 Formaldehyde-agarose gel electrophoresis

Optical density measurements within the acceptable range demonstrate that sufficiently pure RNA has been extracted. They do not, however, demonstrate that this RNA is of adequate integrity and that it has not been degraded during the extraction process. Gel electrophoresis was used to separate nucleic acids on the basis of their size. Two common types of gel are polyacrylamide and formaldehyde-agarose gels. These gels differ in pore size within the gel and thus the size of macromolecules that they can separate varies. Formaldehyde-agarose gels are generally used to separate larger macromolecules than polyacrylamide gels. To achieve a sufficiently large pore size with polyacrylamide gels for the separation of large macromolecules (>200kD) would require them to be made with a consistency too soft to be practical. Formaldehyde-agarose gels are useful in that they can separate molecules up to 50,000kD while having a manageable consistency for easy handling. Nucleic acids have a net negative charge and when an electrical current is passed through the gel-running buffer, they migrate towards the anode. They are filtered through the pores of the gel and smaller molecules travel faster than larger ones. As a result, smaller molecules travel further through the gel than do larger ones.

All gel equipment and glassware was treated as described in section 2.1. The gel tank, gel boat and comb were assembled ready for addition of the gel solution. A 1% agarose gel was prepared by weighing 0.5g agarose into 45ml DEPC-treated water. This was dissolved by heating. After allowing partial cooling, 5ml of 10x 3-(N-Morpholino)propanesulphonic acid (MOPS) and 2.5ml of formaldehyde were added. The gel solution was then poured into the gel tank and left to set at room temperature. This method is described in detail in Appendix A1.5.

2.4.2 RNA samples

RNA samples were prepared by the addition of the volume of sample that contained 2μg RNA and an equal volume of load buffer. The load buffer comprised formamide, 10x MOPS, 37% formaldehyde, bromophenol blue (10% w/v stock solution), DEPC-water and glycerol. This was prepared as described in Appendix 1. Addition of load

buffer to the RNA sample was followed by vortexing, pulse centrifugation and heating at 60°C for 15 minutes. Ethidium bromide (1mg/ml) was then added to the RNA samples for visualisation under ultra-violet light. Ethidium bromide interacts with nucleic acids and yields an increased fluorescence relative to unbound dye due to intercalation of its planar groups with the stacked nucleic acid bases (Sambrook *et al.*, 1989). Once the gel had set, the gel comb was removed and the samples pipetted into the wells of the gel and run at 100V for 60 minutes.

2.4.3 Interpretation of RNA integrity gels

When viewed with an ultra-violet light source, ribosomal RNA species appear as sharp bands on the gel. The upper 28s ribosomal RNA band (in human or mouse RNA) should be present in approximately twice the amount of 18s (lower) ribosomal RNA band. If the bands appear as a smear towards smaller-sized RNA species, it is likely that the RNA has degraded during preparation. Likewise, if the bands appear reversed in quantity, (i.e. the 18s RNA band appears to contain approximately twice the amount of RNA as that contained in the 28s RNA band) this indicates degraded RNA, since 28s ribosomal RNA degrades into an 18s-like ribosomal RNA species.

2.5 OLIGONUCLEOTIDE PROBES

2.5.1 Probe design

Tumour necrosis factor receptor family mRNA sequences were obtained from the National Centre for Biotechnology Information (NCBI). Oswel Research Products Ltd. generated possible antisense oligonucleotide sequences and these were screened against the NCBI databases to ensure total specificity for the receptor so that no cross-reactivity would occur between mRNA for different proteins. The chosen oligonucleotide probes were synthesised by Oswel Research Products Ltd. Their position within the mRNA sequence is shown in Appendix 3.

Each oligonucleotide used was 30 bases in length and was 5`-labelled with biotin. Probe cocktails containing 2 to 4 oligonucleotide probes targeting different areas within the receptor mRNA sequence were used as an alternative to single probes in order to enhance the sensitivity of detection. Sense probes, which were

complementary to antisense probes were also synthesised as above and used as negative controls. All oligonucleotides were purified by high performance liquid chromatography (HPLC) following synthesis.

2.5.2 Biotin labelling of oligonucleotides

Oligonucleotides produced by Oswel Research Products Ltd. were labelled at the time of synthesis with one biotin molecule per oligonucleotide at the 5`-terminus. Oligonucleotides consisting of 25mers of dT and dA, used as positive and negative controls respectively, were purchased unlabelled and required labelling with biotin before use (Appendix A1.1).

Unlabelled probes were 3` end-labelled with biotin as described in Appendix 1 by incubation of the oligonucleotide with a biotin-labelled deoxynucleotide (biotin-16-dUTP). End-labelling was catalysed by the enzyme terminal deoxynucleotidyl transferase (TdT), the reagents being incubated together for 2 to 3 hours in a waterbath at 37°C. Addition of ethylenediaminetetra-acetic acid (EDTA) terminated the labelling reaction. Biotin-labelled oligonucleotide probes were purified from unlabelled probe fragments and unattached biotin molecules by separation through a sephadex column. Sephadex columns were prepared as described in detail in Appendix 1. Briefly, they were prepared by filling sterile syringes with sephadex beads and equilibrating with TE buffer. Aliquots of purified probe were then frozen and stored at -20°C. Biotin labelling efficiency of oligonucleotides by the method described above varies and must be checked prior to use of probes for *in situ* hybridisation (ISH) or Northern blot analysis. Dot-blot hybridisation of the oligonucleotides was carried out to confirm adequate labelling, as described in Appendix 1.2.

2.6 IN SITU HYBRIDISATION

In situ hybridisation (ISH) is a method that may be used to demonstrate the presence and intracellular location of nucleic acid sequences within intact tissue sections. This technique is advantageous over many molecular methods since prior tissue disruption and nucleic acid extraction is not required. It permits localisation of nucleic acid sequences to specific cell types within tissue sections.

Biotinylated oligonucleotide probes, synthesised and prepared as described above, were used to demonstrate the presence of messenger RNA (mRNA) encoding low-affinity nerve growth factor receptor (L-NGFR) within bone marrow trephine (BMT) biopsy sections. Binding of biotinylated probe to BMT sections was detected using a three-stage immunoperoxidase technique with streptavidin-biotinylated alkaline phosphatase complexes and the chromogen Fast Red (Appendix A1.3).

2.6.1 Pre-treatments for ISH

Cytocentrifuge preparations and human long-term bone marrow cultures (hLTBMC) were air-dried and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 10 minutes at room temperature. A rinse in 1x PBS and two rinses in DEPC-H_2O were followed by dehydration through graded alcohols from 70%-100% and air-drying for 15 minutes. Patches of cells on each slide were encircled using a hydrophobic pen to ensure adequate retention of reagents in contact with the cells.

Paraffin-embedded tissue sections were de-waxed in xylene, an organic solvent and re-hydrated through graded alcohols from 100% to 70%. Sections were then rinsed briefly with DEPC-H_2O and subjected to acid hydrolysis using 0.2N hydrochloric acid (HCl). This was followed by a detergent wash (0.3% triton-x-100 in PBS) to permeabilise cell membranes and proteolytic digestion in proteinase K (2.5μg/ml in 0.05M tris-HCl) to reduce RNA-protein cross-links. Slides were then rinsed in 0.2% glycine in PBS followed by two rinses in PBS. They were then fixed in 0.4% paraformaldehyde for 20 minutes and rinsed in PBS and DEPC-H_2O.

2.6.2 Hybridisation

Slides were pre-hybridised with ISH hybridisation buffer, prepared as described in Appendix 2, for one hour at the hybridisation temperature. During this time, thawed aliquots of probe solution were added to fresh hybridisation buffer to give the desired probe concentration. Hybridisation was carried out overnight at the hybridisation temperature using 50μl probe hybridisation solution followed by application of a cover-slip to ensure even distribution of hybridisation solution across the section. With hLTBMC, cells were not cover-slipped, to prevent mechanical dislodgement of the cells and an increased volume (100μl) of probe hybridisation solution was used to

allow for any evaporation. Slides were then subjected to stringency washes by rinsing in varying concentrations of standard saline citrate solutions (SSC) and at varying temperatures. Stringency washes are important to minimise non-specific probe binding.

2.6.3 Oligonucleotide probe detection

Sections were laid onto a humidified staining tray, rinsed with the detergent 0.1% triton-x-100 in tris buffered saline (TBS) for 15 minutes and then incubated in streptavidin solution diluted 1:1000 in TBS. Slides were then rinsed in PBS to remove unbound streptavidin and incubated in biotinylated alkaline phosphatase solution diluted 1:1000 in TBS, for 30 minutes. Where increased sensitivity was required, slides were re-incubated sequentially with streptavidin and biotinylated alkaline phosphatase, as above, to increase the amount of bound alkaline phosphatase available for conversion of the Fast Red substrate to coloured product. Slides were rinsed in TBS and incubated for 10 minutes with veronal acetate buffer (pH 9.2) to equilibrate. This was drained from the slides and replaced with fast red substrate solution in veronal acetate buffer for 15-20 minutes. Slides were returned to a staining rack and rinsed in DEPC-H_2O followed by tap water before being counterstained with Mayer`s haematoxylin. They were then soaked in running tap water to remove excess haematoxylin counterstain. Mayer`s haematoxylin is an aqueous stain. It was used instead of the more usual Harris` haematoxylin as the latter is alcohol-based and the Fast Red salt is soluble in alcohol. Slides were mounted in Crystal-mount (Biomeda Corp., Foster City, CA), an aqueous mountant and dried overnight. They were then mounted again with DPX on top of the crystal mount (double-mounted) followed by cover-slipping. Double-mounting enhances the optical clarity of sections for microscopy after use of aqueous stains.

2.7 NORTHERN BLOT ANALYSIS

Northern blot analysis is a useful method for the demonstration of mRNA in selected cells or tissues; e.g., following cell separation techniques such as magnetic-activated cell sorting (MACS). It cannot, however, be used to localise the production of mRNA to specific cell types within tissue sections, as it requires prior disruption of the

tissue/cells and subsequent RNA extraction. The recent availability of chemiluminescent detection systems, such as CDP-star (Tropix Inc. Massachusetts, USA), has permitted the use of non-isotopic methods to study mRNA. Such methods are advantageous because they generate faster results and avoid the need to use radioisotopes. CDP-Star, a 1,2-dioxetane substrate for alkaline phosphatase, emits light at 466nm upon enzyme-induced decomposition by the mechanism outlined in Figure 2.1.

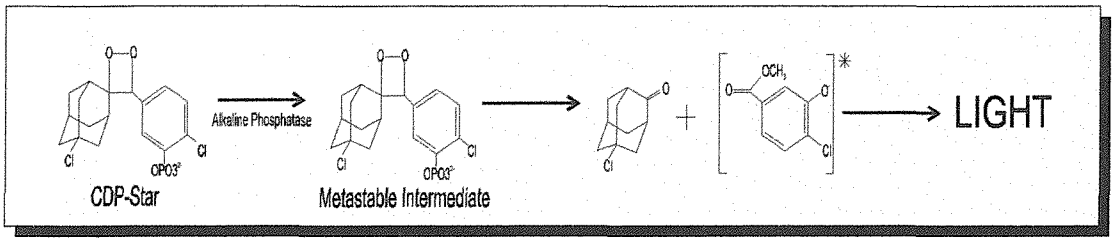


Figure 2.1. Light emission mechanism of CDP- Star

2.7.1 Northern transfer

Transfer of RNA from a formaldehyde-agarose gel to a positively charged nylon membrane was carried out using vacuum blotting equipment treated for RNA handling and described in section 2.1. After the RNA had been run into the gel, it was prepared for transfer onto the nylon membrane as described in Appendix 1.6. The gel was placed onto the vacuum blotter, air bubbles removed and the edges and wells sealed with 1% agarose solution. The vacuum pressure was initiated and maintained at 50mbar. The gel was prepared for transfer of RNA to the membrane by consecutive 7-minute incubations in 0.25M HCl, denaturation solution and neutralising solution. Transfer of RNA to the membrane was carried out in 20x SSC for one hour at room temperature. The blot was rinsed in 20x SSC, blotted dry and fixed by oven baking for 30 minutes at 80°C. Blots were placed between sheets of filter paper in a sealed bag and stored at 4°C until required for use.

2.7.2 Hybridisation of Northern blot

The membrane was hydrated in 0.25M disodium phosphate (pH 7.2) and pre-hybridised in Northern blot hybridisation buffer for one hour at the hybridisation temperature. Hybridisation was carried out in hybridisation solution containing

biotinylated probe (40pmole/20ml) for 2 hours at the hybridisation temperature. The membrane was stringency washed to remove unbound, or non-specifically bound probe by rinsing in 2x SSC containing 1% sodium dodecyl sulphate (SDS) for 2 x 5 minutes at room temperature. This solution was drained and replaced with 1x SSC containing 1% SDS for 2 x 15 minutes at the hybridisation temperature. Following this, the membrane was washed at room temperature in 1x SSC for 2 x 5 minutes.

2.7.3 Chemiluminescent detection

Non-specific protein binding was inhibited by two brief rinses and a 10-minute incubation in blocking buffer. The membrane was then incubated with *Avidx-AP* conjugate supplied with the CDP-star kit, diluted 1:5000 in blocking buffer, for 20 minutes. This was followed by a 5-minute wash in blocking buffer and 3 x 5-minute washes in wash buffer. The membrane was washed twice, for 2 minutes each, in assay buffer, drained and placed on Saran wrap (cling film). A thin layer of CDP-star chemiluminescent solution was pipetted onto the membrane that was then incubated for 10 minutes in the dark. The membrane was wrapped in cling film and placed in direct contact with standard X-ray film for between 1 and 30 minutes. The optimal length of time cannot be predicted and has to be determined by trial and error for each blot, as it is dependent upon the chemiluminescent intensity. The latter is determined by the amount of specific RNA present.

PART B: CELL CULTURE METHODS

2.8 BONE MARROW ASPIRATE BIOPSIES

Bone marrow aspirate samples used in this study were obtained from patients with a wide range of haematological conditions. Control samples considered "normal" were obtained from patients having bone marrow aspirated as part of staging procedures for lymphomas found to have no bone marrow involvement. Bone marrow aspirate samples were obtained from the posterior iliac crest or sternum. A volume of 2-3ml was collected into heparinised Iscove's Modified Dulbecco's Medium (Iscove's medium; IM) in sterile universal containers for transport to the laboratory. Bone marrow samples were handled under sterile conditions at all times and all procedures were carried out in a class II culture cabinet to ensure safe handling.

Leukocytes were harvested from bone marrow aspirate samples by density centrifugation over lymphoprep (Nycomed (UK) Ltd, Birmingham, UK) using the method described by Bøyum (1968). Lymphoprep is a solution containing sodium metrizoate and a polysaccharide that agglutinates erythrocytes. Upon centrifugation, erythrocytes aggregate at the marrow/lymphoprep interface and sediment to the bottom of the container (Boyum, 1968) (Figure 2.2). Briefly, bone marrow samples were layered onto lymphoprep and centrifuged at 1970rpm for 30 minutes at room temperature. The interface layer containing leukocytes was harvested and washed by further centrifugation at 1100rpm for 5 minutes at room temperature following dilution in fresh IM (Appendix A1.7).

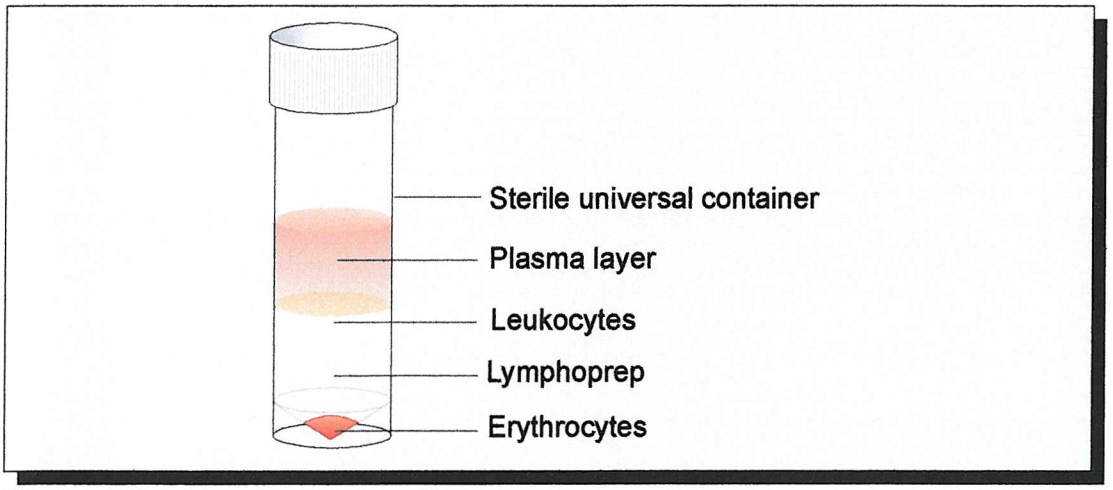


Figure 2.2. Lymphoprep separation of leukocytes

Prior to culture, viable cell numbers were counted by trypan blue exclusion using a standard haemocytometer. Cell number was adjusted to the desired concentration, as described in Appendix 1.8, using freshly prepared cell culture medium. Enriched cell populations, where the desired concentration could not be achieved due to the small number of viable cells obtained, were also cultured in the recommended volume of culture medium for the cell culture vessel used (i.e., at a reduced concentration).

2.9 MAGNETIC-ACTIVATED CELL SORTING

2.9.1 Basic principles

Magnetic-activated cell sorting (MACS) is a process used for the isolation of highly enriched cell populations on the basis of cell surface antigens expression (Miltenyi *et al.*, 1990). The procedure involves labelling the cells immunologically with magnetic microparticles and separation of these cells from within cell suspensions using strong magnetic fields. A strong, high gradient magnetic field is created because the magnetic poles are in very close proximity. This is performed using separation columns, containing plastic-coated ferromagnetic stainless steel wool, placed inside a magnetic field (Figure 2.3). The magnetic microparticles are known as super-paramagnetic which means that they only become magnetised when placed in a magnetic field and therefore bind to the steel-wool in the column. Use of MACS magnetic microparticles has a number of advantages over use of larger magnetic particles, in particular the ability of these particles to be internalised upon culture and their lack of effects on functional activities of target cells. Cell separation systems utilising larger magnetic particles require detachment of the magnetic particle prior to culture and can change the properties of the labelled cells by cross-linking of surface molecules. The use of larger magnetic particles can also result in aggregation of labelled cells due to their high magnetism, which can lead to non-specific loss of labelled cells during separation and result in poor enrichment.

2.9.2 MACS procedure

Leukocytes harvested from aspirated bone marrow samples were incubated with mouse, anti-human, primary monoclonal antibody (reactive with the chosen target antigen) diluted as appropriate in PBS supplemented with 0.5% w/v bovine serum albumin (BSA) to minimise non-specific antibody binding. Goat anti-mouse secondary antiserum conjugated to super-paramagnetic microbeads was then added. Secondary antiserum was diluted as appropriate with PBS, as recommended by Miltenyi Biotec. Labelled cells are retained in the MACS MS+ column (Miltenyi Biotec Ltd. Surrey, UK) while unlabelled cells are eluted as the negative fraction. After three washes with PBS, removal of the column from the magnet de-magnetises the labelled cells, permitting them to be eluted from the column using a plunger and collected as the positive fraction (Figure 2.3). This method is described in Appendix A1.9.

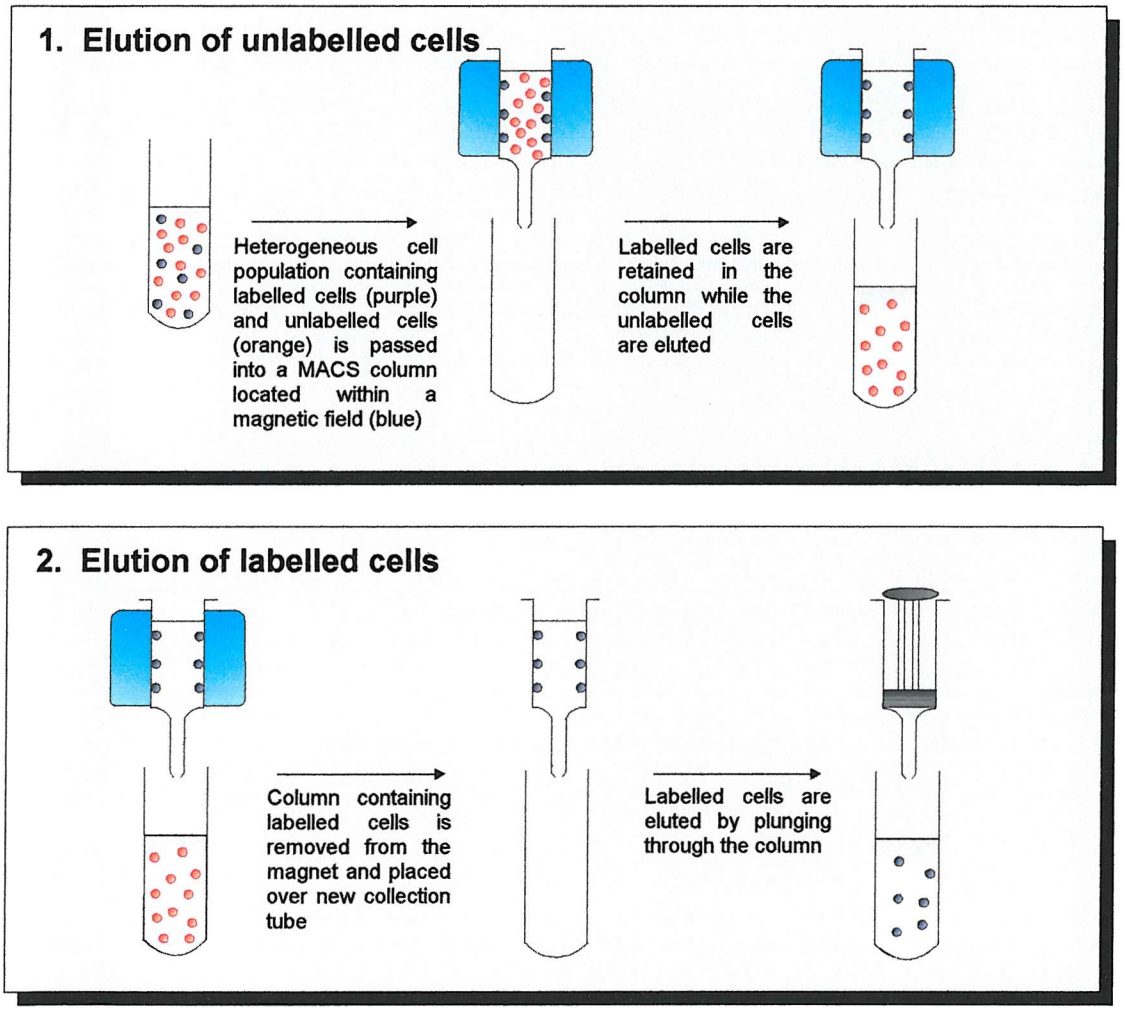


Figure 2.3. Principles of Magnetic Activated Cell Sorting (MACS)

Antibodies used for immunomagnetic cell separation were selected on the basis of their ability to bind antigens expressed by various stromal components of bone marrow. These antibodies were chosen following previous experiments which had shown that these antibodies were specific for antigens expressed by bone marrow stromal cells without cross-reacting with haemopoietic components of marrow or, in the case of CD34, had known cross-reactivity (Wilkins & Jones, 1995a). Other antibodies shown to react with bone marrow stromal cells by other workers include SB10 (Bruder *et al.*, 1997) and HOP26 (Joyner *et al.*, 1997), both of which have been used by these groups to isolate osteogenic components of bone marrow. These antibodies were not available for the current study.

Table 2.1. Details of antibodies used for magnetic cell sorting and subsequent immunocytochemistry

Antibody	Class	Dilution	Antigen	Cell type	Source
Anti-L-NGFR	IgG	1:40	Low-affinity nerve growth factor receptor	Stellate cells	Dako, Denmark
STRO-1	IgM	1:2	STRO-1 antigen	Colony forming unit-fibroblasts	Developmental Studies Hybridoma Bank, University of Iowa, USA
QBEnd10	IgG	1:50	CD34 (Class II)	Endothelial cells, Haemopoietic stem cells	Novocastra, UK
JC70a	IgG	1:10	CD31	Endothelial cells, macrophages	Dako, Denmark
Anti-CD14	IgG	1:100	CD14	Monocytes and their precursor cells	Dako, Denmark
BerMac3	IgG	1:40	CD168	Stroma-adherent macrophages	Dako, Denmark

The antibodies used in this study and their dilutions are shown in Table 2.1. Optimal dilution for each antibody was determined by prior titration in immunohistochemical staining of bone marrow trephine biopsy sections. To ensure that this dilution was also appropriate when applied to aspirated bone marrow cells for separation by MACS, an initial separation was carried out followed by production of cytocentrifuge preparations

from the positive and negative cell fractions. These were visualised by light microscopy following repeat immunolabelling and/or assessment of cytological detail using RapiDiff II stain (Diagnostic Developments, Burscough, Lancs,).

2.9.3 Multi-step, sequential MACS

Multi-step sequential MACS was used to separate multiple cell populations from single bone marrow aspirate samples and to enable prior depletion of one stromal cell type, before enrichment for another. Cells were labelled with primary antibody and secondary antibody-conjugated microbeads, followed by separation as described above. Unbound cells were collected and incubated with a different primary antibody then separated as before by MACS. The resultant eluate from the MACS columns were now depleted of cells expressing the first antigen and enriched for those expressing the second. The purpose of this was to analyse the growth patterns of certain stromal cell populations in the presence or absence of others. For example, this technique was used to determine whether prior depletion of cells expressing L-NGFR before enrichment for cells expressing CD34 affects the ability of CD34-enriched cells to generate stroma in culture.

2.10 CELL CULTURE

2.10.1 Human long-term bone marrow culture conditions

All cells were cultured under standard human long-term bone marrow culture (hLTBMC) conditions (Appendix A1.8) similar to those of the original culture system developed by Dexter and colleagues (Dexter *et al.*, 1977). Cells were cultured at 37°C in 5% CO_2 in air, as no 33°C incubator was available (33°C has been reported as the optimal temperature for haemopoiesis in hLTBMC, at least in murine systems (Dexter *et al.*, 1977)). Culture medium comprised IM containing 500U/ml benzyl penicillin and 200μg/ml streptomycin, supplemented with 10% fetal bovine serum (FBS), 10% horse serum and 10^{-7}M hydrocortisone. After establishment in culture, cells were left for an initial period of two weeks before any change of medium, to minimise disruption while the cells were first growing and becoming adherent to the growth surface. At two weeks, cells were fed by replacement of half of the supernatant volume with freshly prepared hLTBMC medium.

2.10.2 Cell culture vessels for MACS separated cells

A number of different culture vessels were tested for culture of cells selected by MACS (Table 2.2). Initially, attempts were made to culture these cells using 8-well slide flasks (Nunc Lab-tek II slide-chamber: Falcon®, Becton Dickinson (UK) Ltd., UK). The potential advantage of these was that further analyses such as immunocytochemistry could have been performed without prior disruption of the cells from their growth surface. However, attempts to use these for culture of MACS-separated cells were unsuccessful in most cases because the growth surface area is too large for the number of cells available for culture. Culture vessels with a smaller growth surface area were investigated and 96-well flat-bottomed plates were used with success. The disadvantage of these was that they are manufactured from plastic and the cells needed to be transferred, following trypsinisation, to glass slides before immunocytochemical analysis could be performed. This process can result in a loss of viable cells, can lead to a reduction of antigenicity if the antigen is susceptible to trypsin cleavage and disrupts the spatial organisation of the cultured cells.

Table 2.2. Comparison between vessels used for hLTBMC

Culture vessel	Growth surface area	Volume of culture medium required	No. cells required
8-well slide flask	$0.7cm^2$	500µl	$2.5\text{-}5 \times 10^6$
96 well plate	$0.32cm^2$	200µl	5×10^5
24-well cell culture insert	$0.3cm^2$	350µl inside insert 900µl inside well	5×10^5

Cell culture inserts (FALCON, Becton Dickinson (UK) Ltd., UK) were tested and found to be a successful alternative to 96-well plates. The cell culture inserts used consist of polyethylene terephthalate (PET) membranes contained within a plastic housing. The insert is suspended in culture medium and cells are grown on top of the PET membrane. These membranes are advantageous over other cell culture vessels because they are permeable, containing many pores (0.45µm diameter) throughout the membrane thereby allowing diffusion of nutrients to both above and below the growth surface. This potentially provides a more physiologically relevant environment for

growing adherent cells. The 24-well cell culture inserts are advantageous over 96-well plates as they do not require prior trypsinisation before immunocytochemistry can be performed; the insert and membrane can be removed from their housing and from one another in order to mount the membrane directly onto glass slides.

2.11 USE OF CELL LINES

The relatively low abundance of cells expressing L-NGFR within aspirated bone marrow samples and the need to make best use of the clinical bone marrow aspirate samples available, meant that optimisation of some methods used in this study was logistically difficult. Identification and use of cell lines known to express L-NGFR made this more efficient, since methods such as Northern blot analysis and double-staining immunocytochemistry could be optimised using such cell lines.

2.11.1 L-NGFR expression by cell lines

A number of different cell lines believed to express L-NGFR were studied by immunocytochemistry to analyse L-NGFR expression (Table 2.3). Cells were grown to confluence in 75ml cell culture flasks in the cell culture medium described in Table 2.3. The cells were passaged once, following which some were frozen for future analysis, some were re-cultured using 8-well slide flasks and some were used to produce cytocentrifuge preparations. Expression of L-NGFR was determined by immunocytochemical analysis of a) adherent cells in slide flasks following confluent growth and b) the cytocentrifuge preparations.

Table 2.3. Description of cell lines used for analysis of L-NGFR expression

Cell line	Source	Culture medium	Morphology	*L-NGFR expression
SK-Mel-28	Human melanoma	RPMI /2mM Glutamine/10%FCS	Monolayer with polygonal morphology	A: ++ B: +
MM96	Human: Martin bell syndrome	RPMI /2mM Glutamine/10%FCS	Suspension with lymphocyte morphology	A: + B: ++
MeWO	Human malignant melanoma	RPMI /2mM Glutamine/10%FCS	Monolayer with fibroblast morphology	A: +++ B: ++
A875	Human malignant melanoma	RPMI /2mM Glutamine/10%FCS	Monolayer with fibroblast morphology	A: +++ B: +++

* A: L-NGFR expression in cultured cells
B: L-NGFR expression in cytocentrifuge preparations
Both A and B above were visualised by immunocytochemistry (see Table 5.1, Chapter 5)

2.11.2 A875 melanoma cell line

The A875 cell line was originally isolated from a 36-year-old woman undergoing surgery to remove an irregular-shaped mole from the left scapula (Fabricant *et al.*, 1976). Upon pathological examination, invasive malignant melanoma was diagnosed. Over the proceeding two years, despite chemotherapy and irradiation for systemic melanoma, a lesion developed within the right frontal lobe. This was excised and diagnosed as metastatic melanoma. Part of this tumour was sent to the National Cancer Institute (NCI) and used to initiate the A875 cell line.

The A875 cell line was supplied as a kind gift from the NCI and was used with the other cell lines described above for analysis of L-NGFR expression. In their report, Fabricant and colleagues (1976) demonstrated the presence of approximately 7 x 10^5 L-NGF receptors per cell. Immunocytochemical analysis of these cells in comparison with the other cell lines listed, revealed that the A875 cell line demonstrated strongest L-NGFR expression (see Tables 2.3 and 5.1). This cell line was therefore used subsequently for all optimisation studies.

2.12 LIQUID NITROGEN STORAGE OF VIABLE CELLS

2.12.1 Cell freezing

Cells were maintained in a viable state until required for study, by storage in liquid nitrogen (Appendix A1.10). Cells were either stored prior to culture, or cultured, trypsinised from their growth surface (if they were adherent) and then stored. Cells that had been cultured prior to liquid nitrogen storage were detached from their growth surface by trypsinisation using a solution of trypsin (0.05%) and EDTA (0.02%) in Hanks buffered salt solution (HBSS). Initially, HBSS was added to the cells for 10 minutes at room temperature to weaken the bonds holding these cells to their growth surface.

Hanks buffered salt solution is a salt solution deficient in magnesium and calcium. Addition of HBSS to cells results in net diffusion of these ions into solution. Since both calcium and magnesium are inhibitors of trypsin activity and are required for attachment of cells to their growth surface, prior depletion weakens adhesion and renders trypsinisation more successful. Failure to pre-apply HBSS can render trypsinisation unsuccessful and may increase the requirement for repeated application of trypsin. The latter can result in cell damage and reduce the number of viable cells.

The trypsin/EDTA solution was applied to cell cultures for a maximum of 5 minutes at 37°C, to prevent digestion of cell membranes and minimise loss of cell viability. Trypsin cleaves the bonds attaching cells to their growth surface while EDTA, a chelating agent, promotes dis-aggregation of the cells by binding the free calcium and magnesium released into solution from prior addition of HBSS. Trypsin activity was inhibited after 5 minutes by the addition of 10% FBS, a trypsin inhibitor. Cells were frozen at concentrations of up to 10^7 cells per millilitre of freezing medium. Freezing medium consisted of RPMI containing 15% FCS and 10% dimethyl sulphoxide (DMSO). Cells were frozen slowly to –80°C in a sterile cryotube by wrapping in tissue paper, insulating in a polystyrene container and placing in a -80°C freezer. After 24 hours, they were then introduced to liquid nitrogen where they were stored at -196°C until required.

2.12.2 Use of DMSO in freezing medium

Dimethyl sulphoxide (DMSO) is a cryopreservative that penetrates cell membranes to protect against cellular damage due to ice crystal formation and lysis during cell freezing (McGann & Walterson, 1987). When cells are cooled slowly, or frozen at a controlled rate, the extracellular solution usually freezes first. This causes an increase in the solute concentration of the unfrozen extracellular fluid and a resultant osmotic diffusion of water out of the cells into the extracellular fluid. The overall result is cellular dehydration. As the intracellular water content decreases, the cellular components are brought into very close proximity. This can cause repulsive forces, known as hydration forces, to induce membrane stress, cellular damage and ultimately cellular lysis.

Dimethyl sulphoxide, when added to freezing media, increases the intracellular solute concentration. Consequently, upon freezing, the increase in solute concentration of the unfrozen extracellular fluid does not exceed that of the intracellular fluid due to the presence of DMSO. As a result, water does not leave the cell by osmosis and cellular dehydration does not occur, thus preserving the cells from cellular damage. However, the cryopreservative effects of DMSO are dependent on its concentration within the freezing medium as in high concentrations it is itself cytotoxic. Freezing medium typically contains approximately 10% DMSO for successful cryopreservation and minimal toxicity.

2.12.3 Thawing of frozen cells

Frozen aliquots of cells were thawed by incubation in a water-bath pre-heated to 40°C. Upon thawing, cells were transferred immediately to a sterile container containing IM that had been cooled previously to 4°C. Any residual cells were washed from the cryotube by pipetting gently between the two containers. Cells were then washed by centrifugation to remove freezing medium (to avoid DMSO toxicity) and cultured as required.

PART C: IMMUNOHISTOCHEMICAL METHODS

2.13 Bone Marrow Trephine Biopsies

Bone marrow trephine biopsy samples were obtained from patients with myelofibrosis and from patients with acute myeloid leukaemia (AML; subtypes M0-M1) and acute lymphoid leukaemia (ALL) before and after treatment. These were compared with "normal" specimens from patients undergoing biopsy as part of staging procedures for other haematological malignancies, in which no bone marrow involvement was demonstrated. All samples were selected retrospectively from the archival stores of the Pathology Department, Southampton University Hospitals NHS Trust. Methods used are described in detail in Appendix A1.11 and 1.12.

All bone marrow trephine biopsy samples had been obtained from patients under local anaesthesia and sedation if required, using a sterile technique and specially designed, disposable trephine biopsy needles. Biopsy samples were placed immediately in formalin upon collection.

2.13.1 Tissue processing and sectioning

Embedding biopsied tissues in paraffin wax is often used as a convenient and effective means of preparing tissues for histological sectioning and long-term storage. Paraffin wax-embedded blocks permit indefinite storage of the tissue and repeated sectioning when required. Trephine biopsy samples in our department are prepared routinely by fixation in formalin for 24 hours followed by 24-48 hours decalcification in 10% formic acid (in formalin). They are then dehydrated through graded alcohols (70% - 100%) followed by immersion in an organic solvent (xylene). The dehydrated tissue is placed in a suitable mould, which is filled with molten wax and allowed to cool. Sections are cut at 4μm thickness (using a rotary microtome), transferred to 3`aminopropyl-triethoxysilane (APES)-coated glass slides and allowed to dry overnight at 37°C. Sections prepared in this way can be used for tinctorial or immunohistochemical staining immediately and show no deterioration in morphology or antigenicity for at least two weeks, when stored at room temperature in a dry atmosphere.

2.14 Streptavidin-Biotin Complex (ST-ABC) Technique for Immunohistochemistry of Fixed, Paraffin Embedded Tissue Sections

2.14.1 Antibodies

A range of monoclonal antibodies has been used in this study to demonstrate patterns of expression of bone marrow stromal cell-associated antigens from patients with myelofibrosis and patients undergoing therapy for acute leukaemias (AML and ALL). These are shown in Table 2.4.

Table 2.4. Antibodies used in bone marrow trephine biopsy samples

Antibody	Antigen	Cell type	Antigen retrieval method and buffer	Dilution	Source
Anti-L-NGFR	Low affinity nerve growth factor receptor (p75)	Stellate cells	Microwave + citrate	1:40	Dako, Denmark
QBEnd 10	CD34 (Class II)	Haemopoietic progenitor cells and endothelium	Microwave + citrate	1:50	Novocastra, UK
Anti-αSMA	α-smooth muscle actin	Myofibroblasts	Microwave + citrate	1:4000	Sigma, UK
VS38C	P63 rough endoplasmic reticulum-associated antigen	Plasma cells	Microwave + citrate	1:80	Oxford, UK
PGM-1	CD68	Stromal macrophages	Pronase (0.05%)	1:400	Dako, Denmark
MIB-1	Ki-67 nuclear antigen	Proliferating cells	Microwave + citrate	1:500	Coulter, UK

2.14.2 Tissue preparation for immunohistochemistry

Tissue sections, mounted on APES-coated glass slides, as described above, were de-waxed by immersion in xylene followed by rehydration through graded alcohols, from 100% to 70%. Endogenous peroxidase activity was inhibited at this stage by covering

the sections with a solution of 0.5% hydrogen peroxide in methanol for 2x15 minutes. This is longer exposure than is employed with other tissues since a greater level of endogenous peroxidase is present in bone marrow compared with other tissues (due to the presence of large numbers of granulocytes).

2.14.3 Antigen retrieval

Antigenic sites are often "over-fixed" during the fixation process for paraffin-embedded tissue sections and the antigen binding site is consequently unavailable for antibody binding. Antigen retrieval permits access of the antibody to its corresponding binding site on the antigen. For some antigenic targets, retrieval procedures are not required but they were found to be necessary with all antibodies used in this study. As shown in Table 2.4 above, all required microwave wet heat pre-treatment except the PGM-1 antibody, which required proteolytic digestion. Microwave pre-treatment was carried out by immersion of tissue sections in 0.01M citrate buffer (pH6.0) and heating in a microwave oven on medium power for 25 minutes. The microwave oven used was a Panasonic 800-watt unit. Different makes and models of microwave may require different times and settings and this would require prior determination in each case.

Proteolytic pre-treatment was carried out by incubation of the slides in a 0.05% Pronase solution (Dako, Denmark). This solution was prepared freshly, immediately before use by the addition of 0.1ml stock Pronase solution (stored frozen at 10mg/ml) to 1.9ml tris buffered saline solution (TBS). Slides were incubated in Pronase solution for 25 minutes at room temperature, the time having been determined by previous experiments to optimise antigen retrieval conditions for each antibody used.

2.14.4 Primary antibody incubations

After antigen retrieval, slides were laid onto a humidified staining tray and washed gently, twice, with TBS. They were then drained and the primary antibody, diluted to the appropriate concentration in TBS, was applied. Table 2.4 gives details of the working dilution of each primary antibody used. Slides were incubated in human primary antibody overnight at 4°C. Each of the primary antibodies used is a mouse anti-human monoclonal antibody. On the following day, slides were allowed to warm

to room temperature and washed gently, three times, in TBS. Biotinylated anti-mouse secondary antiserum was diluted 1:200 and applied to the slides for 30 minutes, during which time a 1:200 solution of streptavidin plus biotinylated horseradish peroxidase (St-AB-HRP) was prepared and left for 30 minutes to complex. Incubation in secondary antiserum was followed by three gentle washes in TBS. Concentrations of secondary antiserum and St-ABC-HP had been determined by previous experiments in our laboratory.

2.14.5 Antibody detection

Slides were incubated in the prepared St-AB-HRP solution for 30 minutes at room temperature. The use of a biotinylated secondary antiserum, rather than a secondary antiserum directly labelled with horseradish peroxidase (HRP), allows the detection signal to be amplified, since each streptavidin molecule has four binding sites for biotin. Biotin molecules in this complex are conjugated to molecules of HRP, which are subsequently detected by oxidation of the chromogen 3-3` diaminobenzidine tetrahydrochloride (DAB). Oxidation is achieved by the addition of peroxide to peroxidase in the presence of DAB. Following three washes in TBS, slides were incubated in DAB substrate solution, containing substrate buffer, hydrogen peroxide and DAB chromogen, for 10 minutes or until sufficient colour (brown) had developed (Figure 2.4). A rinse in TBS and a wash in running tap water for two minutes followed this.

2.14.6 Quantification of Immunohistochemistry

Bone marrow trephine biopsy sections were analysed semi-quantitatively after immunostaining, using an arbitrary scoring system of 0-3, with increments of 0.5, to record the number of cells staining positive. Similarly, for quantifying the number of blood vessels, these values represent the number of positive vessels and do not represent the intensity of staining. Graphs were plotted showing the variation from normal. All positive values therefore represent increased number of positive cells and all negative values represent decreased number of positive cells compared with normal.

It is important to note that this scoring system does not provide definitive values for the number of positive cells. This method of evaluation was chosen to permit rapid analysis of a large number of biopsies to enable staining patterns and trends to be identified but is relatively insensitive. As a result, minor variations in staining scores may not reliably represent true differences and must be interpreted with caution. Larger variations (<1.0 arbitrary unit), however, are likely to represent true differences in staining patterns.

2.14.7 Statistical Analysis

Differences between the number of CD34-positive leukaemic blast cells in the different AML subtypes were tested for significance using the non-parametric, Mann-Whitney *U*-test with significance at $p<0.05$. This test was used to identify significant differences in CD34 expression between myeloblastic, promyelocytic and myelomonocytic/ monocytic AML subtypes. It is acknowledged that numbers of cases in each of these subtypes is small, but no additional cases were available for study. The results are shown in chapter 7, section 7.5. Statistical analyses were performed using SPSS for Windows version 10.0.5 (SPSS Inc., Chicago, IL).

2.14.8 Counter-staining, dehydration and mounting

Slides were transferred to a staining rack and counter-stained by immersion in Harris' haematoxylin for 2 minutes followed by a rinse in tap water for one minute and differentiation in 1% acid alcohol (1% glacial acetic acid in isopropanol) for 5 seconds. The slides were then left in running tap water for five minutes to blue. Upon incubation of the sections in water, the neutral pH of tap water turns the red, acidic haematoxylin solution to its most stable blue conformation giving the blue appearance of the counter-stain. Excess water was removed by gentle shaking and sections were dehydrated through graded alcohols from 70% to 100% then into xylene. This was followed by DPX mounting and coverslipping. Sections prepared in this way have excellent optical clarity and are essentially permanent specimens, subject to minimal fading if stored in the dark.

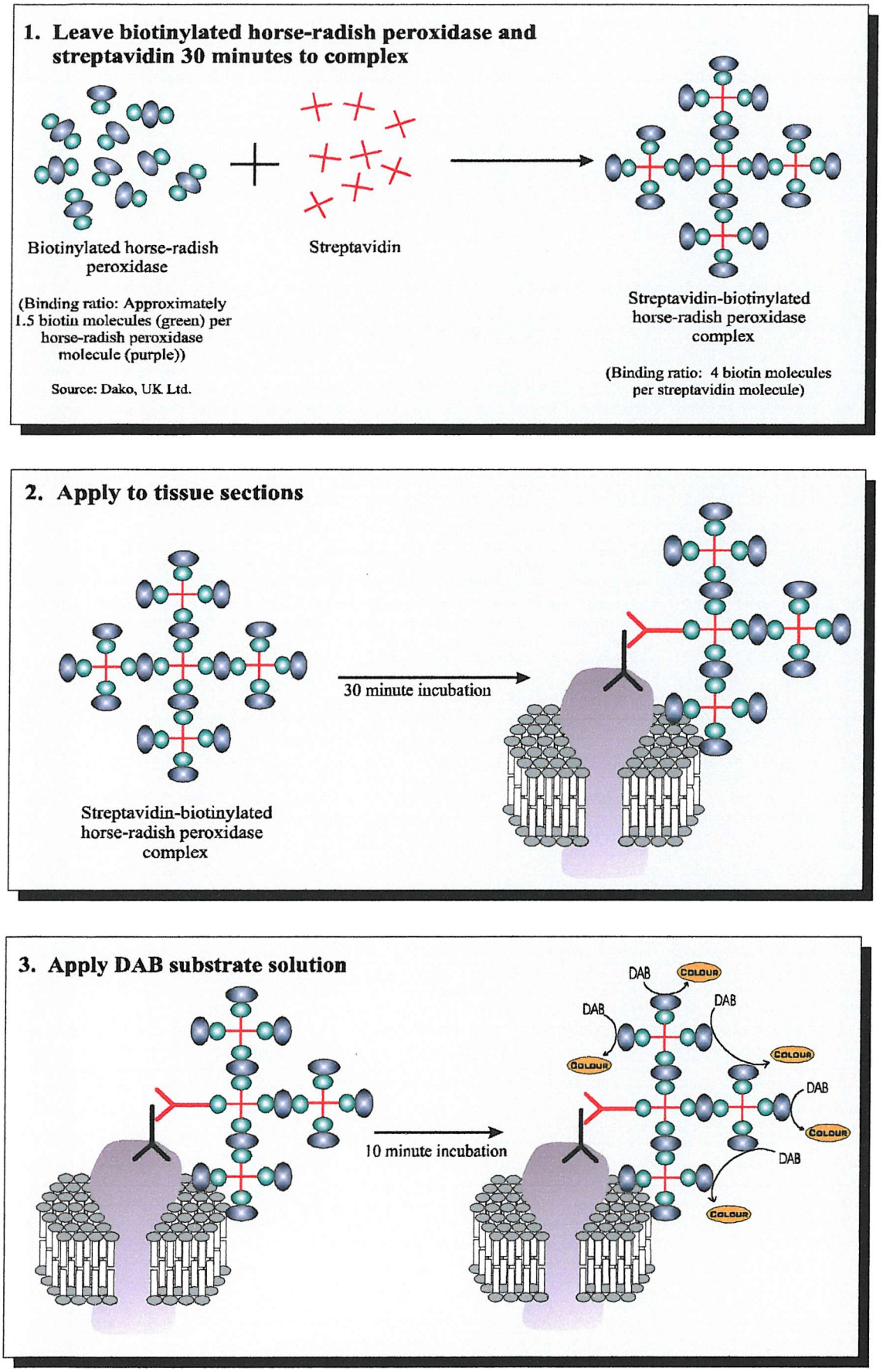


Figure 2.4. Indirect immunoperoxidase technique

2.15 ST-ABC TECHNIQUE FOR CYTOCENTRIFUGE PREPARATIONS AND CULTURED BONE MARROW CELLS

2.15.1 Fixation of cells

Fixation of cytocentrifuge preparations and freshly cultured bone marrow cells differs from that of trephine bone marrow biopsies in that these preparations are much more delicate. Without care, they are subject to loss of morphological detail, especially cells cultured on cell culture inserts. Growth of freshly cultured bone marrow cells was halted by covering with a cold solution of PBS for 10 minutes, followed immediately by fixation. Cytocentrifuge preparations were air-dried before fixation. Optimal fixation was achieved using freshly prepared and cooled 4% paraformaldehyde solution in PBS, pH 7.2, for five minutes at room temperature. See Chapter 3 for details of optimisation experiments.

2.15.2 Inhibition of endogenous biotin/avidin activity

Unlike trephine bone marrow biopsy samples, cultured cells and cytocentrifuge preparations require inhibition of endogenous biotin and avidin activity during immunocytochemistry. Endogenous biotin activity was inhibited by incubation at room temperature in a pre-warmed avidin solution (Vector Laboratories Ltd., Peterborough, UK) for 20 minutes. Endogenous avidin activity was then inhibited by incubation, as before, in a biotin solution (Vector Laboratories Ltd., Peterborough, UK). The latter also ensures saturation of any residual biotin-binding sites remaining after previous incubation with avidin.

2.15.3 Antibody incubations

Prior to incubation in primary antibody, non-specific protein binding was prevented by incubation of the cells in 10% BSA in TBS for 20 minutes. This was followed by a gentle wash in TBS. Primary antibodies used were the same as those used for trephine bone marrow biopsy specimens and applied to the cells at the same dilution for 60 minutes at room temperature. Three washes in TBS were carried out before incubation in biotinylated secondary anti-mouse antiserum, at the same dilution for tissue sections, for 30 minutes at room temperature.

2.15.4 Antibody detection

Antibody detection using St-AB-HRP complexes was carried out as described in section 2.14.5. Preparation of DAB solution was carried out as before but with the addition of 15% sodium azide to inhibit endogenous peroxidase. This endogenous activity was not inhibited using hydrogen peroxide in methanol, as for trephine biopsy sections, because this can result in cell damage in specimens not fixed with formalin. Shorter washes in TBS were used for cultured cells and cytocentrifuge preparations than for trephine bone marrow biopsy sections, as was a shorter rinse in running tap water following DAB incubation, to minimise mechanical damage to the cells.

2.15.5 Counter-staining

Counter-staining of cytocentrifuge preparations was performed using the method described in section 2.14.7. Cultured cells were counter-stained using a similar procedure, differing only in the use of Mayer's haematoxylin solution (rather than Harris') for two minutes. This was followed by a one-minute rinse in running tap water, differentiation in 1% acid alcohol solution and 5 minutes to blue in running tap water. Mayer's haematoxylin was used since mounting was carried out using an aqueous mountant.

2.15.6 Mounting of cell culture inserts

Mounting of cytocentrifuge preparations was carried out as for bone marrow biopsy sections, as described in section 2.14.7. Mounting of cells cultured in glass slide chambers was carried out by application of a few drops of the aqueous mountant, Gel-mount (Biomeda Corp., Foster City, CA) followed by cover-slipping. Dehydration of the cells was not carried out as this was found to cause deterioration of adipocyte morphology. Cell culture inserts were mounted by careful removal of the membrane insert from its plastic housing using a scalpel and forceps. Care was required to ensure correct orientation of the growth surface on the glass slide. One drop of aqueous Gel-mount was applied to an APES-coated glass slide and the membrane was carefully laid onto this. Another drop of Gel-mount was applied directly onto the growth surface and a cover-slip applied. The glass slide was inverted onto the cover-slip and then pressed

firmly onto filter paper to absorb excess mountant and ensure that the membrane was mounted uniformly to prevent loss of optical clarity.

2.16 Double St-ABC Technique for Cytocentrifuge Preparations and Cultured Bone Marrow Cells

2.16.1 Double immunostaining technique

The rarity of stromal cells from clinical samples of aspirated bone marrow has meant that, after MACS separation, only a small number of cultures could be established from each sample. Double immunostaining permits the simultaneous demonstration of two antigens within each bone marrow culture by utilising two primary antibodies, each demonstrated by a different detection system, thereby providing more information from each bone marrow culture. Both antigens were detected using the St-AB-HRP system but with different substrates for each primary antibody (Appendix A1.13). The horseradish peroxidase substrates used were DAB (brown) and Vector VIP (Purple; Vector Laboratories Ltd., Peterborough, UK). These substrates were chosen for these experiments since a good colour contrast is obtained between brown and purple.

The immunostaining procedure for the first antigen was carried out as for St-ABC technique described in section 2.15. Following incubation in the DAB, the cells were washed well, three times, in TBS. The DAB substrate was found to give best colour contrast when used as the first substrate and with the antigen anticipated to be expressed at the highest levels. Vector VIP was therefore used as the second substrate and used to detect the antigen expressed at the lowest levels. The second primary antibody was applied as before for 30 minutes, followed by three TBS washes and incubation in biotinylated secondary antiserum. Prior to incubation in ST-AB-HRP, cells were washed three times in TBS and incubated for 15 minutes in peroxidase inhibitor comprising PBS supplemented with sodium azide and peroxide (see Appendix 2). This step is crucial to prevent cross-reactivity between the two substrates. Following three further washes in TBS, cells were incubated in St-AB-HRP solution diluted 1:200 for 30 minutes, followed by three TBS washes.

2.16.2 Vector VIP detection system

Vector VIP was prepared from a commercially available kit (Vector Laboratories Ltd., Peterborough, UK) and applied to the cells for 15 minutes followed by a rinse in TBS and a wash in running tap water for 5 minutes to remove any excess substrate solution. Nuclear counter-staining in Harris` or Mayer's haematoxylin may be carried out but, due to the similarity in colour between the purple substrate and the blue counterstain, this is not recommended where the specific immunostaining is that of a nuclear antigen. The membrane inserts on which the cells had been cultured were then removed and mounted as described in section 2.15.6.

3. ISOLATION AND CHARACTERISATION OF CELLULAR COMPONENTS OF HUMAN BONE MARROW STROMA

3.1 INTRODUCTION

The cellular and non-cellular constituents of human bone marrow stroma are together responsible for the support and maintenance of normal haemopoiesis (Eaves *et al.*, 1991a). However, the importance of each of the stromal cell types in supporting haemopoietic development remains unclear. Further understanding of the functions of bone marrow stromal cells is reliant upon the ability to isolate and culture these cells successfully *in vitro*. Human studies of bone marrow stromal cells have been hindered by the rarity of these cells in clinical samples of aspirated bone marrow (Wilkins & Jones, 1998). Antigens specific for these stromal cells have been identified previously using immunocytological studies on intact adherent layers of human long-term bone marrow cultures (hLTBMC). Amongst these stromal cells are adherent fibroblasts, which express the low-affinity nerve growth factor receptor (L-NGFR) (Wilkins & Jones, 1995a) and STRO-1 (Simmons & Torok Storb, 1991), haemopoietic and endothelial cells expressing CD34, endothelial cells and macrophages that express CD31 and macrophages plus their precursors expressing CD14 (Wilkins & Jones, 1995a). These are summarised in Table 2.1 (Chapter 2).

Magnetic-activated cell sorting (MACS) (Miltenyi *et al.*, 1990) was used to isolate these stromal cells from clinical samples of aspirated bone marrow. Following enrichment and/or depletion by MACS, cells were cultured for four weeks to assess their growth characteristics and analysed further using immunocytochemical staining techniques. Limited volumes of bone marrow aspirate were available and, due to the rarity of stromal cell populations within these samples, a miniaturised cell culture system needed to be established. A variety of culture vessels were assessed, including 4ml slide flasks, 8-well slide chambers and 96-well plates, of which only the 96-well plates were relatively successful. These supported the growth of the small numbers of stromal cells obtained by MACS but did not permit subsequent immunocytochemistry, as problems resulted from incompatibility between the material from which the plates are produced and the reagents used in immunocytochemistry. Subsequently, cell

cultures were established initially in 96-well plates to increase cell numbers and then passaged into 8-well slide flasks for subsequent immunocytochemistry. This method was largely unsuccessful due to the loss of viable cells during trypsinisation. A recent report (Fedon, 1997) described the advantages of using cell culture inserts for growth of small numbers of cells. The inserts, produced from polyethylene terephthalate (PET), are porous, allowing exchange of nutrients from both above and below the growth surface. Following cell culture on these inserts, cells can be directly immunostained while on the PET membrane, then cut from their housing and mounted onto a glass slide for microscopy. Growth of stromal cells from bone marrow aspirates, separated by MACS, proved to be largely successful using this method for culture and so these inserts were adopted for all subsequent cell culture experiments.

3.2 Optimisation of Immunocytochemical Staining of Cell Culture Inserts

3.2.1 Cell fixation using acetone

Cells were fixed initially by acetone immersion for 20 minutes. A number of problems resulted from the use of acetone as a fixative for bone marrow cells cultured on PET membrane inserts. The material from which the insert housing is produced was found to react with acetone rendering the plastic opaque. Overall cell morphology following immunocytochemistry had deteriorated, raising the possibility that acetone was reacting with the membrane itself (Figure 3.1). Many bone marrow stromal cells, including cells enriched for L-NGFR or CD34 undergo adipogenesis during the culture period of four weeks. This was instantly apparent upon microscopical examination. However, acetone is a lipid extractant and, following immunocytochemistry of the cell culture inserts when acetone was used as the fixative, none of the adipocytes were visible upon microscopy.

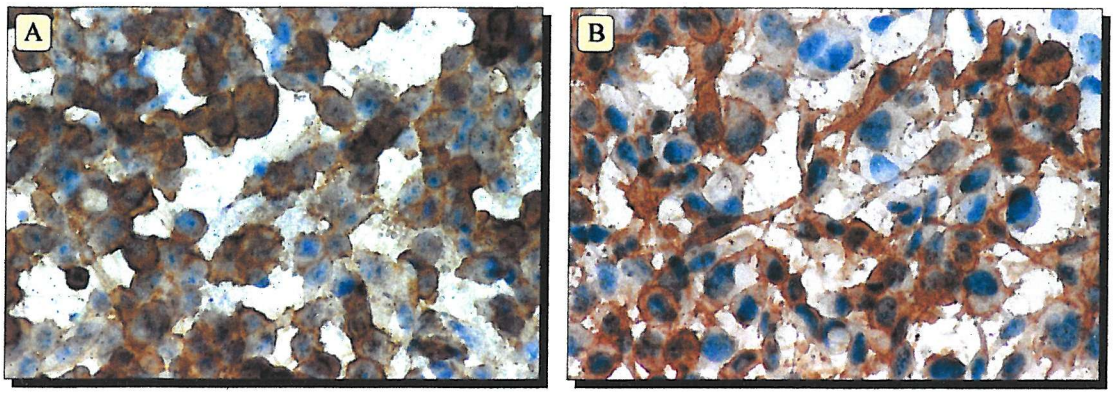


Figure 3.1. Comparison between acetone (A) and paraformaldehyde (B) fixation with cell culture inserts using A875 cell line immunostained for L-NGFR expression. Loss of nuclear morphology has occurred due to exposure to acetone (left). Fixation in 4% paraformaldehyde in PBS for five minutes at room temperature results in preservation of excellent morphological detail (right).

3.2.2 Cell fixation using 4% paraformaldehyde in PBS, pH 7.2

Various fixatives were tested as alternatives to acetone. A solution of 4% paraformaldehyde in PBS, was used finally, since this fixative works by cross-linking proteins to maintain intracellular structure and was considered unlikely to affect adipocyte morphology. The A875 human melanoma cell line was used as a positive control for L-NGFR expression; a titration was carried out to determine optimal fixation times. Cells were grown to confluence and fixed in 4% paraformaldehyde for 1, 3, 5 or 10 minutes. The optimal incubation time in 4% paraformaldehyde was 5 minutes. Fixation for less time resulted in loss of cells from the growth surface during the immunostaining procedure and times of greater than 5 minutes resulted in deterioration of cell integrity and consequent loss of antigenicity. Paraformaldehyde was superior to acetone fixation, as it did not react with the cell culture inserts, preserved excellent cell morphology and did not result in adipocyte loss. A comparison between acetone and paraformaldehyde fixation is shown in Figure 3.1.

3.2.3 Mounting of cell culture inserts

After immunocytochemical staining, most preparations are dehydrated through graded alcohols and mounted using DPX mountant. Bone marrow stromal cell cultures prepared for mounting in this manner resulted in a loss of adipocytes from the culture, possibly due to loss of lipids from the adipocytes during the dehydration process. An aqueous mountant was considered as an alternative since dehydration would not be required. Gel mount (Biomeda Corp., CA) was used successfully for this purpose.

3.3 Multi-Step, Sequential MACS Separations

A technique I have termed multi-step, sequential MACS separations (msMS) was developed to isolate populations of stromal cells, based on their immunophenotype, in order to further characterise these cells. Aspirated bone marrow samples were incubated with a primary antibody e.g. L-NGFR and enriched by MACS (Figure 3.2A). The L-NGFR positive fraction was cultured as required and the negative fraction re-incubated in a different primary antibody e.g. CD34 (Figure 3.2B). The resultant positive fraction contains cells enriched for a L-NGFR-/CD34+ phenotype. Cells were separated by msMS with L-NGFR-/CD34+, L-NGFR+/CD34- and L-NGFR-/CD31+ phenotypes. Using this technique, enrichment varied considerably between aspirated bone marrow samples but typically yielded between 60 and 80% purity for both single MACS and msMS.

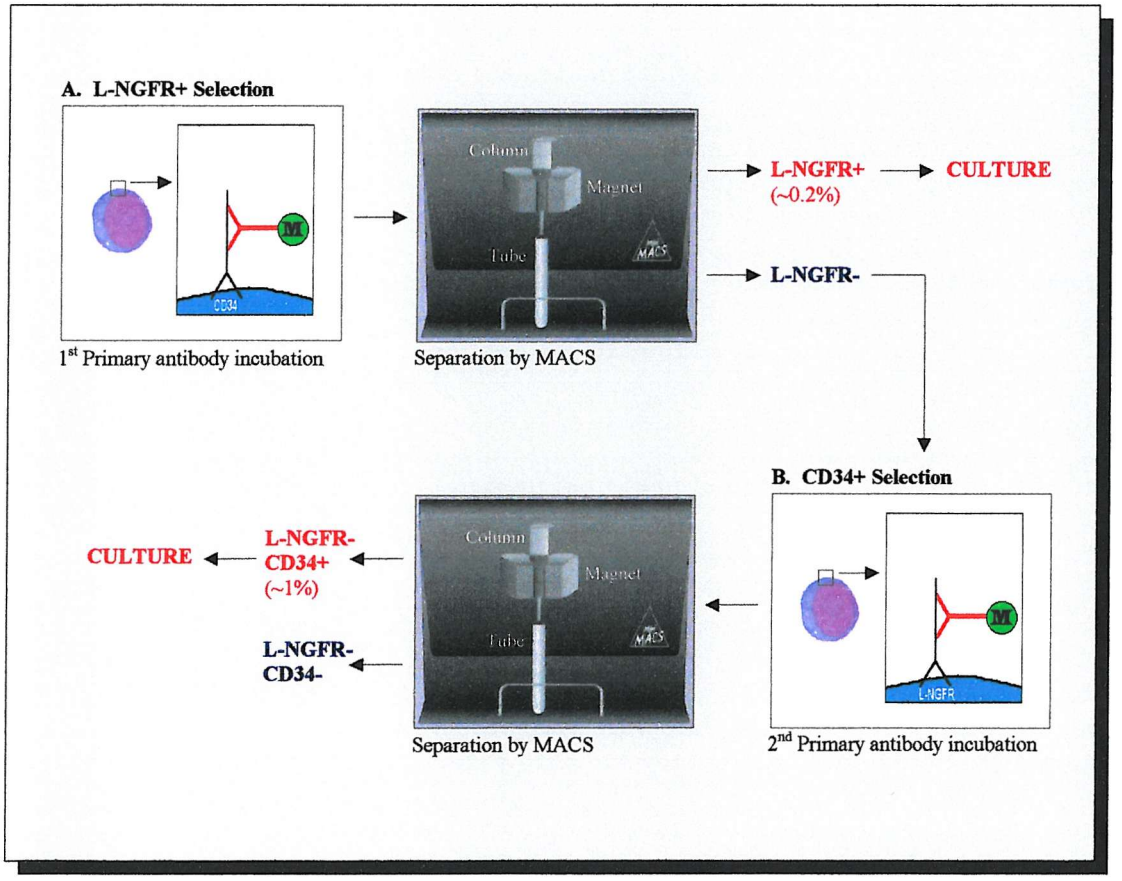


Figure 3.2. Principles of multi-step, sequential MACS. Aspirated bone marrow samples are incubated with a primary antibody e.g. L-NGFR and enriched by MACS (A). The L-NGFR positive fraction is then cultured as required and the negative fraction re-incubated in a different primary antibody e.g. CD34 (Figure 3.2B). The resultant positive fraction contains cells enriched for a L-NGFR-/CD34+ phenotype.

3.4 Isolation and Characterisation of Cellular Components of Bone Marrow Stroma

Cellular components of bone marrow stroma were isolated using antibodies described in section 3.1. Following enrichment and/or depletion by MACS, cells were cultured for four weeks under hLTBMC conditions on polyethylene terephthalate (PET) membranes, as described. Culture growth was then terminated and immunocytochemical analysis performed previously.

3.4.1 Isolation and characterisation of L-NGFR positive stromal cells

Cells isolated by MACS on the basis of L-NGFR expression, analysed morphologically in cytocentrifuge preparations, were a homogeneous population of cells with characteristic morphology (Figure 3.3). They have large nuclei and numerous small, cytoplasmic projections. Following one week in culture, these cells became elongated and were identifiable morphologically as fibroblasts, reaching confluence by the second week in culture.

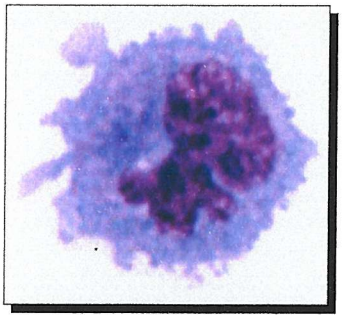


Figure 3.3. Cell separated by MACS on the basis of L-NGFR expression.

By week two, adipogenesis had begun, with adipocytes containing numerous small fat globules. By the fourth week in culture (Figure 3.4), the number of adipocytes had greatly increased and fat globules contained within them had become larger and less numerous. The majority of cells in these cultures were elongated, adherent stromal cells with no evidence of haemopoietic clusters. Only a small number of macrophages were evident (round shape; small nucleus) in comparison to equivalent cultures grown from L-NGFR-depleted cells.

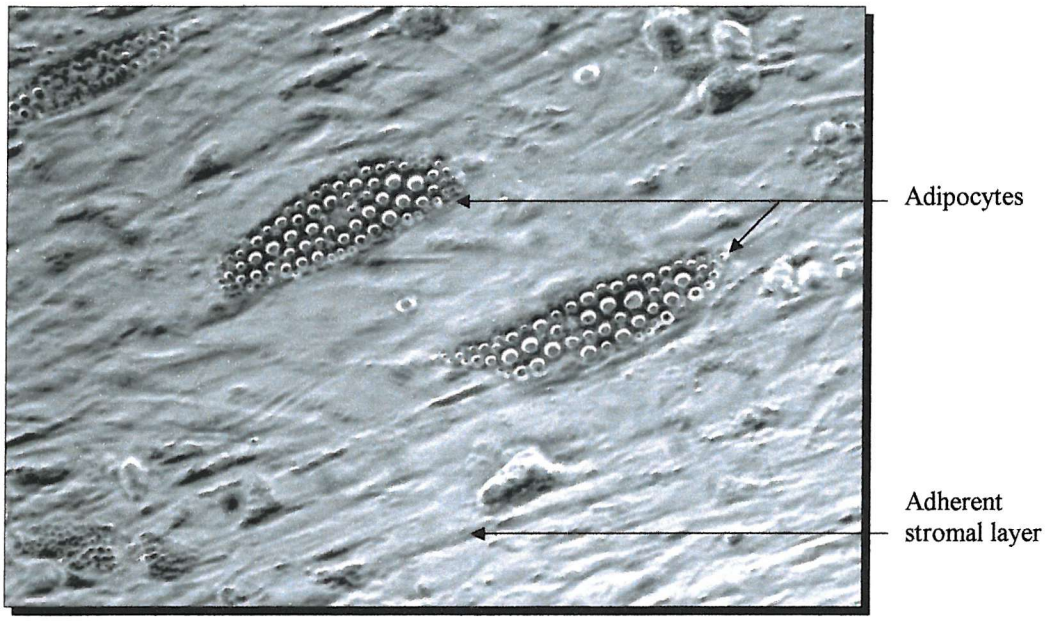


Figure 3.4. Cells cultured for 4 weeks following enrichment for L-NGFR by MACS. Note the adherent stromal cell layer upon which adipocytes, containing numerous fat globules, have formed.

Immunostaining of cell cultures enriched on the basis of L-NGFR expression demonstrated, that after four weeks in culture, a proportion of cells (around 70% of the adherent cells) continued to express L-NGFR. Two morphological variants of L-NGFR positive cells were evident (Figure 3.5A and B). These had either a flattened appearance, with only small dendritic projections or were stellate in appearance with very long cytoplasmic projections. The latter variant was often found in close association with similar L-NGFR positive cells. In addition to cells expressing L-NGFR, there were cells present that expressed α- smooth muscle actin (αSMA). These were characteristically flatter, less dendritic and less numerous (about 30% of adherent cells) than those that expressed L-NGFR (Figure 3.5D). No differences in stromal growth or antigen expression were observed when cells were pre-depleted of cells expressing CD34 prior to enrichment for L-NGFR. It was also evident that a population of cells that expressed L-NGFR contained numerous fat globules within their cytoplasm (Figure 3.5E). These fat globules were usually contained in the flatter, less dendritic variants of L-NGFR positive cells. No fat globules were seen in the cytoplasm of L-NGFR negative cells after culture (Figure 3.5F).

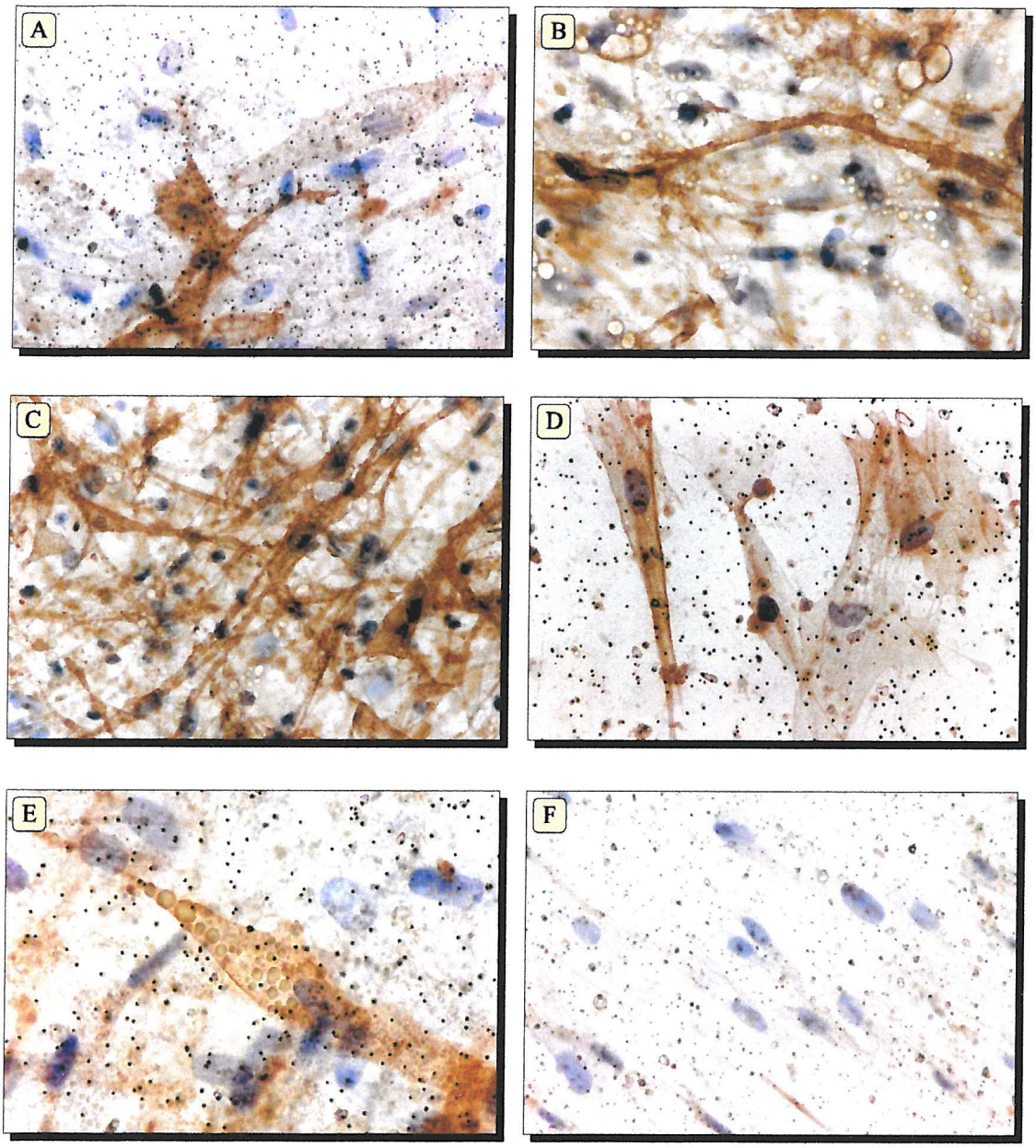


Figure 3.5. Immunostaining of L-NGFR-enriched cultures after 4 weeks growth. The above photographs represent bone marrow aspirate samples separated by MACS on the basis of L-NGFR expression. Enriched cells from each sample were divided and cultured on two separate PET membranes (left and right). The photograph A shows the flattened variant of L-NGFR positive cells; photograph B shows the stellate variant with long dendritic projections. The speckled appearance of some of the photographs is due to the pores on the membrane insert upon which the cells were cultured. This is not apparent on the other photos since these pores are out of the focal plane. The middle photographs show that after 4 weeks in culture, a high proportion of L-NGFR positive cells continued to express L-NGFR (C; approx. 70%) and some expressed α-SMA (D; approx. 30%). The bottom photographs show that, in a proportion of L-NGFR positive cells, fat globules could be seen within their cytoplasm (E). In contrast, adherent cells negative for L-NGFR showed no evidence of adipogenesis (F).

3.4.2 Isolation and characterisation of CD34 positive stromal cells

Cultures generated from CD34-enriched cells contained a heterogeneous population of cells that included both stromal and haemopoietic cell types (Figure 3.6). Within two weeks in culture, an adherent stromal cell layer containing stromal fibroblasts had begun to form. This took up to a week longer than in cultures generated from cells expressing L-NGFR. Adipogenesis also took longer to develop in these cultures than in those grown from L-NGFR positive cells. Depletion of L-NGFR expressing cells prior to enrichment for CD34 had no noticeable effect on the ability of these cells to generate stroma or undergo adipogenesis.

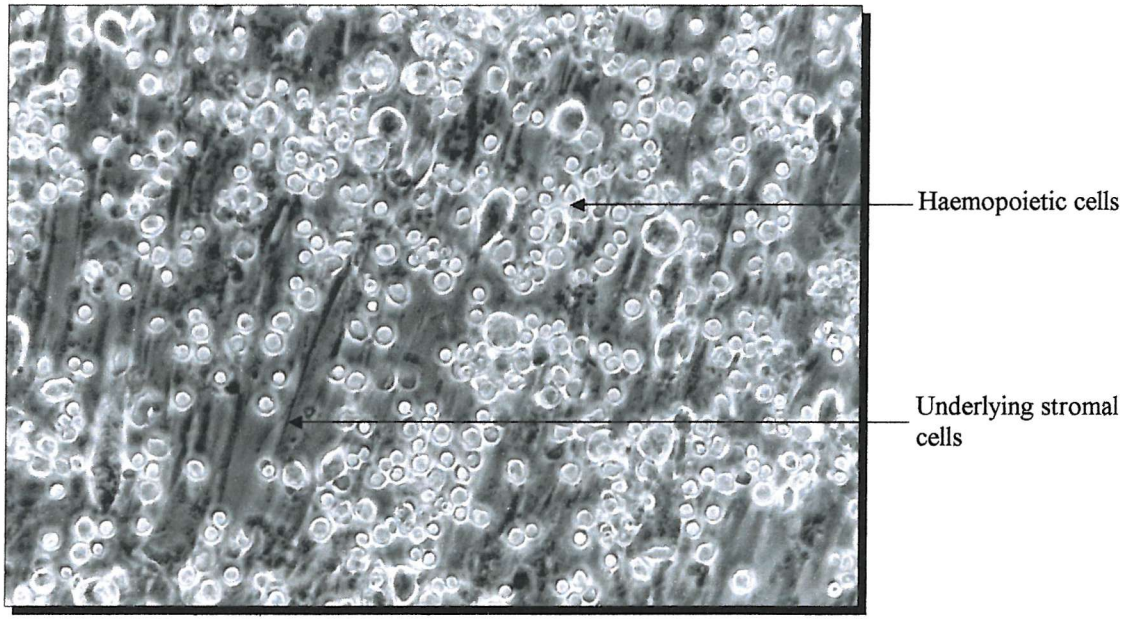


Figure 3.6. CD34-enriched cells after 4 weeks growth in culture. A stromal layer containing cells with fibroblast morphology is seen underlying the extensive number of haemopoietic cells preset.

Cultures grown from CD34-enriched cells were immunostained for L-NGFR and αSMA to elucidate whether expression of these antigens in the stromal layer differed between cultures formed from L-NGFR-enriched cells and those from CD34-enriched cells. As in cultures generated from L-NGFR-enriched cells, only a sub-population of cells expressed L-NGFR. These cells were morphologically similar to those found in L-NGFR-enriched cultures. However, cells expressing αSMA were more numerous in CD34-enriched cultures than in those established from L-NGFR positive cells. These αSMA positive cells had the same polygonal shape as those described in L-NGFR-

enriched cultures. Figure 3.6 shows cultures pre-depleted of L-NGFR positive cells prior to enrichment for CD34 positive cells and immunostained for L-NGFR and αSMA.

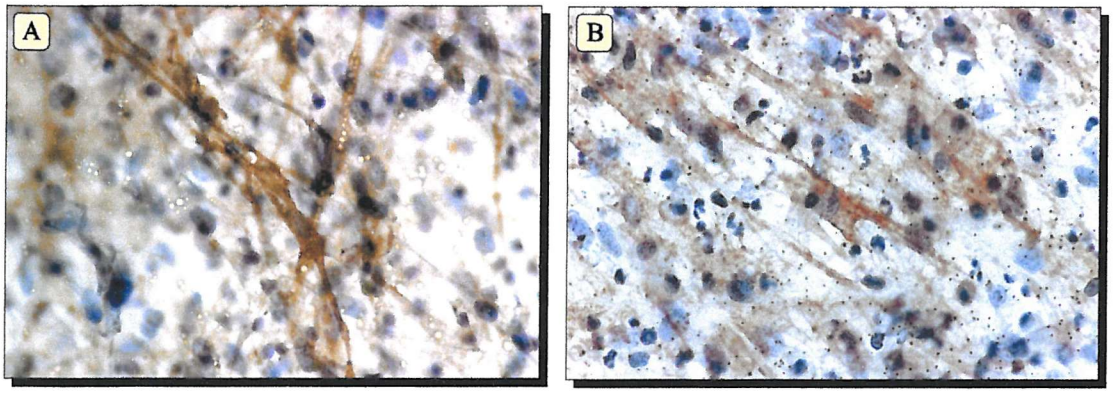


Figure 3.7. Immunostaining of CD34-enriched cultures after 4 weeks growth. The above photographs are from a single bone marrow aspirate sample depleted of cells expressing L-NGFR and enriched for CD34 positive cells by MACS (L-NGFR-/CD34+), then divided and cultured on two separate PET membranes. After 4 weeks in culture, the cells on one membrane were immunostained for L-NGFR (A) and the other for αSMA (B).

3.4.3 Isolation and characterisation of STRO-1 positive stromal cells

Prior to isolation and culture of STRO-1 expressing cells, cytocentrifuge preparations were made from unseparated aspirated bone marrow cells, STRO-1-enriched cells and STRO-1-depleted cells. These were immunostained using antibodies reactive with STRO-1, L-NGFR and CD34. These antibodies were chosen, since the antibody STRO-1 has been shown previously, to react with stromal precursor cells (Simmons & Torok Storb, 1991) and a proportion of stromal precursor cells were shown in sections 3.4.1 and 3.4.2 to express L-NGFR and CD34. In unseparated bone marrow, cells expressing these antigens were rare. Enrichment for STRO-1 resulted in enrichment of a large number of cells identified morphologically as erythroid precursors. After immunostaining of STRO-1-enriched cells, it was evident that a large proportion of STRO-1 positive cells also expressed L-NGFR and CD34.

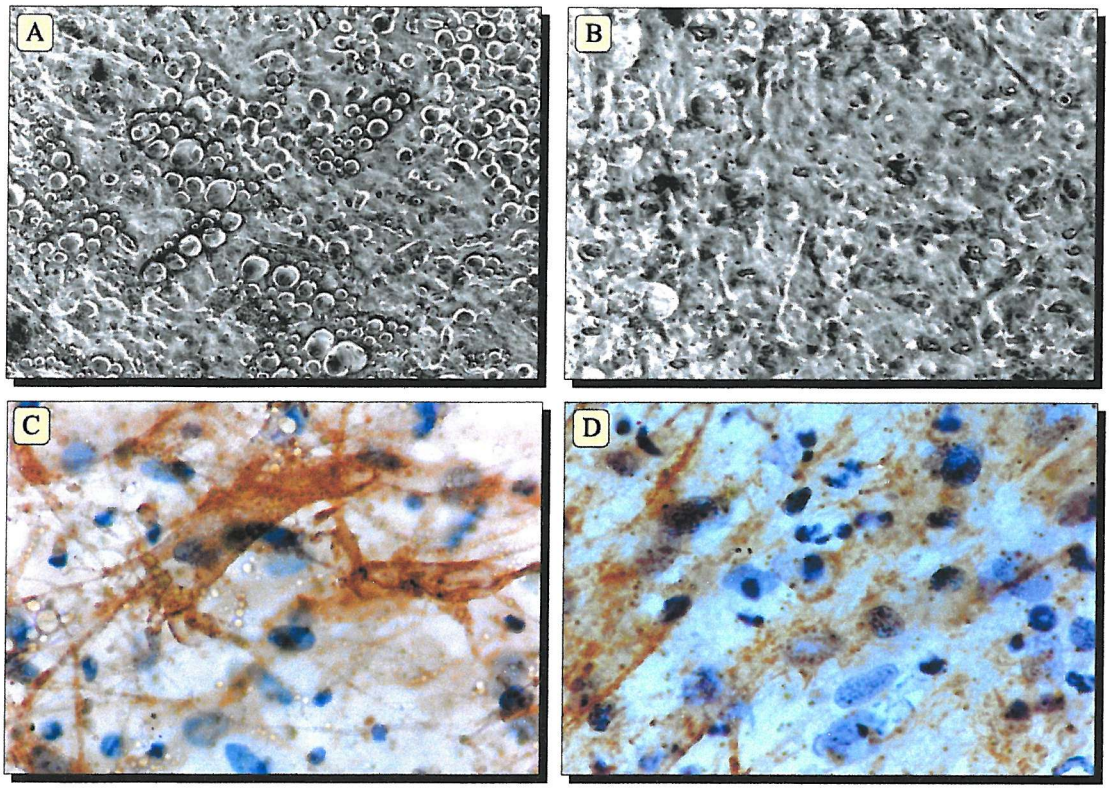


Figure 3.8. Cultures formed from STRO-1 enriched (A) and STRO-1 depleted (B) cells. Cultures from both STRO-1 enriched and STRO-1 depleted cells gave rise to an adherent stromal layer. However, cultures from STRO-1-enriched cells gave rise to highly adipogenic cultures (left) compared with those derived from cells depleted for STRO-1 (right). After 4-weeks in culture, a proportion of cells expressed L-NGFR (C) and αSMA (D).

Cultures derived from STRO-1-enriched cells began to form adherent stromal fibroblasts within one week in culture. Similar to L-NGFR-enriched cells, a confluent stromal layer was present within two weeks in culture. Adipogenesis had begun to occur by week two and continued to increase until the fourth week in culture, when the growth was terminated for subsequent immunocytochemistry. At this time, there were many more adipocytes than equivalent cultures formed from STRO-1-depleted cells (Figure 3.8A and B), although an adherent stromal layer did form in these cultures. This adherent layer was characterised by immunocytochemistry and, similar to cultures derived from L-NGFR positive cells, contained cells that expressed L-NGFR and cells that expressed αSMA (Figure 3.8C and D).

3.4.4 Isolation and characterisation of CD31 and CD14 positive stromal cells

Monoclonal antibodies reactive with CD14 and CD31 were used to isolate precursors of stromal macrophages from aspirated bone marrow samples (Figure 3.9). The latter of these antibodies is also reactive with endothelial cells. Cultures formed from CD31- and CD14-enriched cells both generated stroma within two weeks, similar to cultures formed from CD34-enriched cells. Adipogenesis in these cultures occurred by the third week and was consistently greater in CD31- and CD14-enriched cultures compared with CD31- and CD14-depleted cultures.

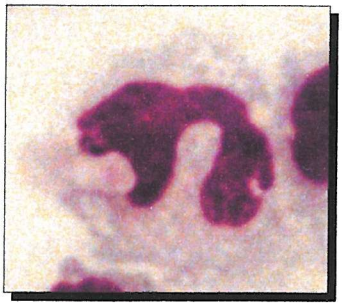


Figure 3.9. Stromal macrophage precursor stained with Diff-Quik following enrichment by MACS using monoclonal antibodies reactive with CD14.

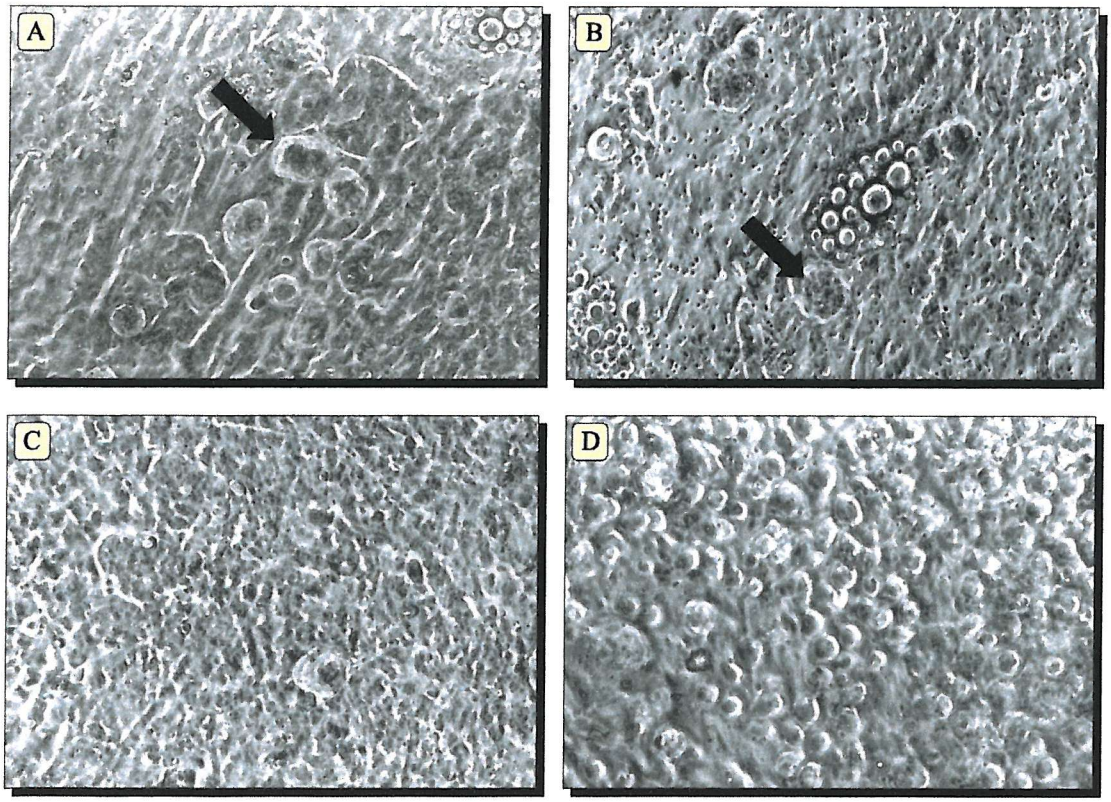


Figure 3.10. 4-week old cultures formed from aspirated bone marrow cells enriched/depleted for CD31 and CD14. An adherent fibroblastic layer with adipogenesis is present in both CD31- (A) and CD14-enriched (B) cultures. These cultures have an abundance of large round macrophages (arrowed) in comparison to CD31- (C) and CD14-depleted (D) cultures. The culture generated from CD14-depleted cells (D) shows a dense population of haemopoietic cells, which may or may not be accompanied by the presence of an adherent stromal layer.

Cultures derived from CD31- and CD14 positive cells differed from L-NGFR and CD34 cultures by the additional presence of an abundant, heterogeneous population of macrophages adherent to the layer(s) of fibroblastic and adipogenic cells (Figure 3.10). In addition, haemopoietic colonies were not observed in any of the CD31 or CD14 cultures.

Cultures were established from L-NGFR-/CD31+ and CD31-/L-NGFR+ cells to determine whether prior removal of cells expressing one antigen, affected the ability of cells expressing the other to generate an adherent stromal layer in culture. Although the L-NGFR-/CD31+ culture did generate an adherent stromal layer, a high number of haemopoietic cells were present. The time-span for the adherent layer to form was similar to that observed with CD31-enriched cells. In contrast to this, the cultures formed from CD31-/L-NGFR+ cells contained almost no haemopoietic cells and an adherent stromal layer took less time to reach confluence in these cultures. Cultures generated from L-NGFR-/CD31- cells contained a relative excess of haemopoietic cells. In these cultures, it was not possible to identify whether an adherent stromal layer had formed beneath the haemopoietic cells.

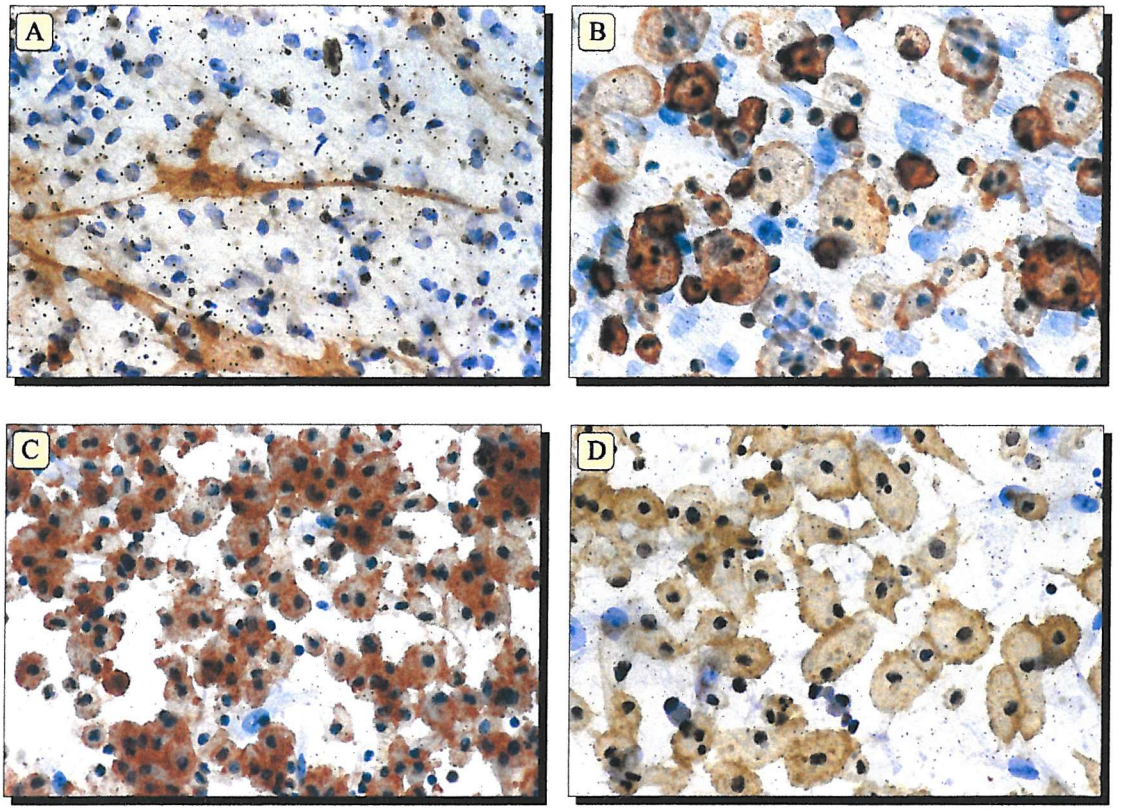


Figure 3.11. Immunostaining of cultures enriched for CD31 (A and B) or depleted for CD31 (C and D). After 4 weeks in culture, a small population of fibroblastic stromal cells express L-NGFR (A). Cultures generated from CD31-enriched cells and immunostained with BerMac3 (B) reveal the presence of two morphological macrophage variants (large and light brown; small and dark brown). In CD31-depleted cultures stained with BerMac3 (D), the smaller, darkly stained variant appears to be absent. Cultures formed from CD31-depleted cells (C) were shown to generate a high number of CD31 positive macrophages during culture.

The adherent fibroblastic stromal layer generated from CD31-enriched cells showed the presence of a population of cells that express L-NGFR (Figure 3.11A). These showed similar morphological features to those generated from L-NGFR- and CD34-enriched cells. Depletion of L-NGFR positive cells prior to enrichment for CD31 did not affect the ability of these cells to generate an adherent stromal layer and undergo adipogenesis.

Cultures from both CD31-enriched and CD31-depleted cell populations were immunostained for the macrophage-associated antigen, recognised by the monoclonal antibody, BerMac3 (CD163) (Pulford *et al.*, 1992). Both types of culture contained two morphological and phenotypic variants of macrophage. There were large macrophages that stained weakly with BerMac 3 and smaller macrophages that stained strongly with BerMac3 (Figure 3.11B). Cultures formed from CD31-enriched cells

contained both large and small macrophages in abundance. Cultures generated from CD31-depleted cells contained predominantly large macrophages staining weakly with BerMac3 (Figure 3.11D). These large macrophages were strongly positive for CD31 after 4 weeks in culture (Figure 3.11C).

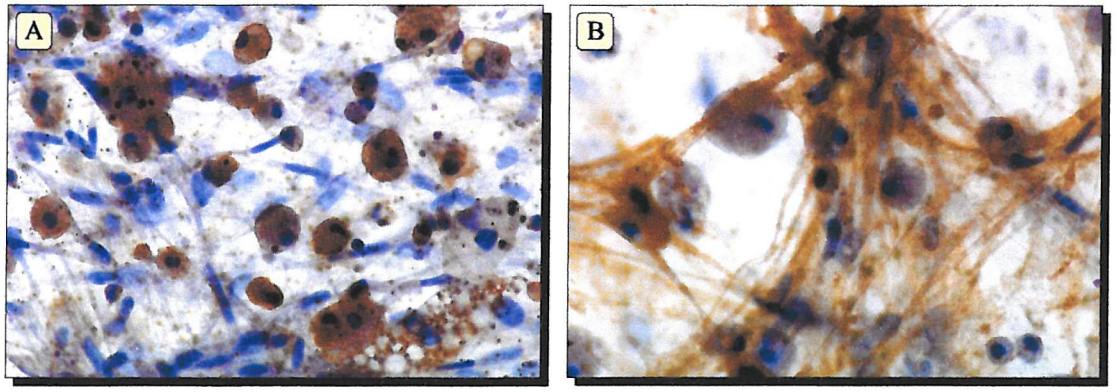


Figure 3.12. Immunostaining of cultures established from CD14-enriched cell populations. Immunostaining with BerMac3 (A) shows a predominance of small macrophages, stained strongly with BerMac3. Photograph B shows that these macrophages are situated within a network of interconnecting stromal fibroblasts, stained positively for L-NGFR.

Similar to cultures derived from CD31-enriched cells, those from CD14-enriched cells contained a large number of macrophages identified with BerMac3 (Figure 3.12A). However, the number of large macrophages, staining weakly with BerMac3, was less and the number of smaller macrophages, staining strongly with BerMac3, was greater in CD14-enriched cultures than in CD31-enriched cultures. Immunostaining also revealed that the adherent layer of CD14-enriched cultures had a greater number of L-NGFR positive cells than CD14-depleted cultures (Figure 3.12B).

3.5 Discussion of Results

The rarity of stromal cells in aspirated bone marrow samples and the limited volume of material available from clinical samples hinder the isolation and culture of human bone marrow stromal cells. Variation between patients also presents reason for caution when analysing results, although the majority of samples were obtained from patients with various types of lymphoma that were found to have no bone marrow involvement.

For these reasons it was necessary to obtain as much information as possible from each sample. Magnetic activated cell sorting (MACS) was carried out to isolate cellular components of bone marrow stroma and multi-step sequential MACS separations (msMS) were carried out to investigate the stromal generating capacity of each of these components in the presence or absence of others. The small number of cells obtained from each sample required the development of a miniaturised cell culture system. For this purpose, porous polyethylene terephthalate (PET) membranes were used. These have a small growth surface area ($0.3cm^2$) and are porous thereby allowing nutrient exchange to both apical and basolateral surfaces creating an environment closer to that found *in vivo*.

Immunocytochemical analysis of cultures grown on PET membranes required optimisation for use with bone marrow stromal cultures to ensure that cellular morphology was not compromised during the immunostaining procedure. Fixation of cells grown on cell culture inserts was found to require a non-acetone based fixative. Acetone reacted with the material from which the cell culture inserts are produced, rendering them opaque. Cultures fixed using acetone were subject to an overall loss of cellular morphology and resulted in the loss of adipocytes from the culture. A solution of 4% paraformaldehyde in PBS, pH 7.2 was used as an alternative to acetone. This works by cross-linking proteins and does not affect adipocytes in the culture nor react with the PET membrane inserts. Fixation was carried out optimally for 5 minutes at room temperature. Dehydration of cell culture inserts for mounting in DPX was also shown to result in adipocyte loss from the culture. To overcome this problem an aqueous mountant was used (Gel mount) that did not require dehydration prior to mounting.

Enrichment by MACS of cells expressing the L-NGFR gave rise in culture to an adherent stromal cell layer with fibroblast morphology. No haemopoietic cells were observed in these cultures. Immunocytochemical analysis revealed that only a small population of cells, after four weeks in culture, continued to express L-NGFR. Two morphological variants of L-NGFR positive cells was observed, consistent with findings from other groups (Cattoretti *et al.*, 1993; Wilkins & Jones, 1995a; Caneva *et al.*, 1997). There were those with long-thin dendritic projections that reached over other cells and were seen in close association with cells of similar morphology. The

other variant of L-NGFR positive cells were flatter and more widely spread. A population of cells derived from L-NGFR-enriched cells were found to express αSMA. This was an unexpected observation as aspirated bone marrow cells have been shown previously to be void of cells expressing αSMA (Wilkins & Jones, 1998). Expression of αSMA may be a true *in vivo* characteristic of these cells or may be due to the culture conditions in which these cells are grown. Immunohistochemical analysis of bone marrow trephine biopsy cores has demonstrated that αSMA is not expressed in non-diseased bone marrow (see Chapter 6) suggesting that expression of αSMA is likely to be due to the culture of these cells *in vitro*. Cells expressing αSMA were similar in morphology to the flat, adherent variant of L-NGFR positive cells. However, it is unclear whether αSMA and L-NGFR are co-expressed or whether they represent separate cell populations. Double immunofluorescence experiments would be required to determine whether these two antigens are co-expressed on the same cell type.

Experiments in which aspirated bone marrow samples were pre-depleted of CD34+ progenitor cells and enriched for L-NGFR were carried out to determine whether prior removal of CD34+ cells affected the ability of L-NGFR+ cells to generate stroma. It is not known whether both stromal and haemopoietic cells are derived from a common precursor or if they originate separately. Cultures derived from CD34-/L-NGFR+ cells generated an adherent stromal layer that was devoid of haemopoietic cells. This suggests that, although CD34 positive progenitor cells are capable of both stromal and haemopoietic reconstitution, there are stromal progenitors present in aspirated bone marrow that do not express CD34 and do express L-NGFR (Figure 9.2, Chapter 9). However, since MACS is an enrichment technique, contamination by CD34+ cells cannot be excluded, although clear differences were observed between cultures formed from CD34-/L-NGFR+ cells and those formed from CD34+ cells.

Stromal cells isolated by MACS for L-NGFR and immunostained for L-NGFR post-culture, showed that within the cytoplasm of the flatter variant of L-NGFR positive cells, fat globules can be seen. In conjunction with this was the observation that in cells that did not express L-NGFR, no fat globules were seen (Figure 3.5). This may suggest that adipocytes are derived from cells that express L-NGFR and that they

reflect the ability of these cells to undergo a transition from stromal fibroblast to adipocyte under certain circumstances (see Figure 9.1, Chapter 9). Consequently, cultures derived from cells with a L-NGFR-/CD34+ phenotype took longer to undergo adipogenesis than those derived of CD34-/L-NGFR+ cells.

Cultures derived from CD34-enriched cells gave rise to a heterogeneous population of cells containing both stromal and haemopoietic elements of bone marrow. The stromal components were similar to those derived from L-NGFR positive cells by containing some cells that expressed L-NGFR and some that expressed αSMA. Depletion of cells expressing L-NGFR prior to enrichment for CD34 was carried out to determine if the cells responsible for the generation of stroma were exclusive to the L-NGFR positive fraction. This was not found to be the case as L-NGFR-/CD34+ cultures formed an adherent stromal layer in all cases.

Cultures formed from STRO-1-enriched cells were similar to those generated from L-NGFR-enriched cells, containing predominantly stromal elements of bone marrow compared with haemopoietic components. However, adipogenesis was more prominent in STRO-1 derived cultures. Cells expressing either STRO-1, L-NGFR and CD34 were shown to be rare in unseparated aspirated bone marrow samples. However, when immunomagnetic separation was carried out for STRO-1-positive cells, it was found that within the STRO-1 positive fraction was a high number of L-NGFR and CD34 positive cells compared with both unseparated cells and STRO-1-depleted cells. It would appear from these results that there are cells present in aspirated bone marrow with STRO-1+/CD34+ and STRO-1+/L-NGFR+ phenotypes. Further studies would need to be carried out to investigate whether there are cells present that co-express each of these antigens, i.e. have a STRO-1+/CD34+/L-NGFR+ phenotype.

Antibodies reactive with CD31 and CD14 were used to isolate stromal macrophages and their precursors. Cultures initiated from either CD31 or CD14 positive cells gave rise to an adherent stromal cell layer with fibroblast morphology. A population of bone marrow progenitor cells that express CD34 have also been shown to express CD31 (Watt *et al.*, 1993) suggesting an explanation for the generation of stroma in these cultures. Two macrophage variants derived from CD31-enriched cells were

shown by immunocytochemistry to express the macrophage-associated protein, BerMac3. There were cells that stained strongly for BerMac3, which were generally smaller than those that expressed BerMac3 weakly. Cultures grown from CD31-depleted cells, when stained for BerMac3 identified only the large macrophage variant, which were also shown to express CD31. Cultures formed from CD14-enriched cells differed by containing predominantly the smaller macrophage variant. It is unknown at present whether these represent different functional variants of macrophage, or perhaps macrophages at different stages of maturation. However, if the latter explanation were true, both types of macrophage would be expected in CD31-depleted cultures. The finding that CD31-depleted cells appear void of the smaller macrophage variant provides a means by which these macrophage variants may be separated in order to investigate their functions in stromal cell cultures.

These results, taken together, may offer additional insight into the origin of stromal and haemopoietic cells. A summary diagram indicating possible origins and differentiation pathways for stromal cells are shown in figure 9.4, Chapter 9. Prior depletion of CD34 positive progenitor cells did not affect the ability of CD34-/L-NGFR+ cells to generate stroma. Likewise, depletion of L-NGFR positive cells did not affect the ability of L-NGFR-/CD34+ cells to generate stroma. These cells give rise in culture to both stromal and haemopoietic cells. These results indicate that there may be progenitor cells present in bone marrow capable of only stromal formation which express L-NGFR and there may be separate cells present that are L-NGFR-/CD34+ that are capable of both stromal and haemopoietic formation.

The relationship between STRO-1, CD34 and L-NGFR in stromal origin is unclear. It is possible STRO-1 positive cells represent one of the earliest stromal progenitor cells as this may explain the formation of a stromal layer in cultures formed from L-NGFR-/CD34+ cells. In addition, since it seems likely from these results that there are cells present with STRO-1+/L-NGFR+ phenotypes, in cultures formed from L-NGFR positive cells it may be these cells responsible for stromal formation. Further studies employing the use of msMS to deplete for STRO-1 positive cells prior to enrichment for L-NGFR and/or CD34 may help to answer these questions.

4. OPTIMISATION OF SINGLE-ENZYME DOUBLE IMMUNOCYTOCHEMISTRY

4.1 INTRODUCTION

Double immunocytochemistry (DIC) is a useful technique for the simultaneous detection of two separate cell types within a heterogeneous population of cells. It was first used in 1968 for the localisation of three simultaneous antigens in rat pituitary tissue (Nakane, 1968). It is an under-rated technique, due to technical difficulties in assurance of staining specificity and prevention of cross-reactivity between immunoenzymatic detection systems. It is not recommended that DIC be used to detect two antigens in the cytoplasm or membranes from the same cell type, due to the possibility of stearic hindrance between the complexes formed from the two detection systems. It is also difficult to distinguish a combination of two colours from two separate coloured products, while being confident that cross-reactivity has not occurred.

This technique should not be considered as an alternative to single immunocytochemistry if the amount of material for study is available in abundance and the two antigens are not expected in the same cell type, as DIC is more time-consuming and technologically more difficult. However, DIC is extremely useful when only a limited quantity of material is available.

The cell culture system described in the previous chapter was optimised for the isolation and culture of bone marrow stromal cells from clinical samples of aspirated bone marrow. The rarity of these cells meant that from any one aspirated bone marrow sample, a maximum of two stromal-cell cultures could be established. Consequently, by using single immunocytochemistry, only two antigens could be detected. Throughout the latter part of this study, DIC was optimised to enable subsequent detection of multiple antigens simultaneously following culture of these rare cell populations. It is hoped that further studies employing this culture system, described in Chapter three, will use DIC to enable more information to be obtained from each aspirated bone marrow sample. The aim of this chapter is to provide a short technical guide describing optimisation of the DIC procedure by alteration of its various stages.

4.2 INITIAL EXPERIMENTS

4.2.1 Choice of detection systems

Double immunocytochemistry may be carried out using either two different enzymes (double-enzyme) or a single enzyme (single-enzyme) with two different detection systems (Mason *et al.*, 1983). The two enzymes commonly used for the conversion of enzyme substrate into coloured product, are horseradish peroxidase and alkaline phosphatase. Many detection kits are available commercially for these enzymes and each varies in the coloured product obtained. When carrying out DIC, it is important to choose detection systems with good colour contrast (for example, brown and purple, or red and blue) bearing in mind the additional colour of the nuclear counterstain, should this be required.

It is common, when carrying out DIC, to use the double-enzyme method with both peroxidase and alkaline phosphatase, to reduce the likelihood of cross-reactivity between detection systems. However, use of alkaline phosphatase tends to yield a more diffuse, less crisp pattern of staining compared with that obtained using peroxidase. As a result, in the methods described here, the single-enzyme method was chosen using peroxidase detection for both antigens. The two enzyme substrates used were 3-3` diaminobenzidine tetrahydrochloride (DAB; brown) and Vector VIP (Vector Laboratories Ltd., Peterborough, UK; purple). Examples of immunostaining carried out using these detection systems are illustrated in Figure 4.1.

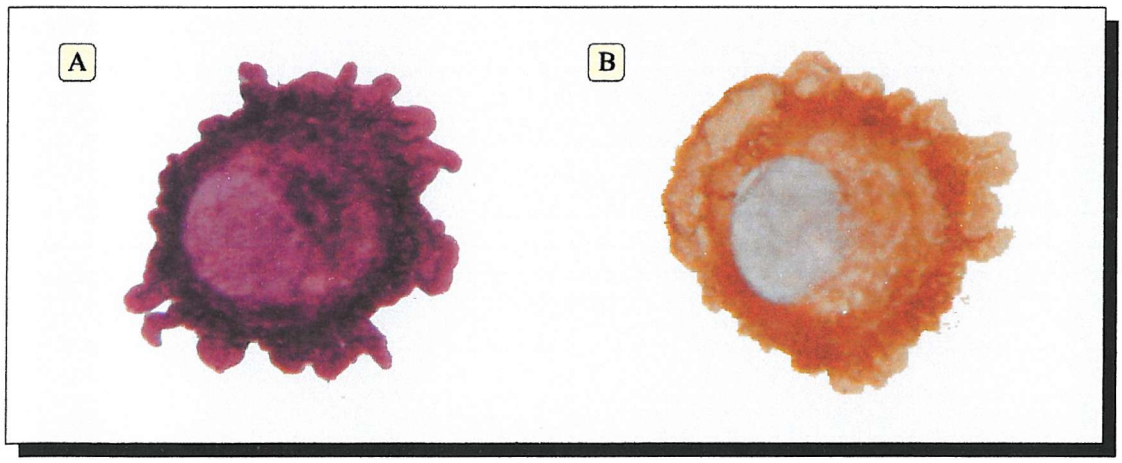


Figure 4.1. Immunocytochemical staining of A875 cells with L-NGFR monoclonal antibody. A875 cells immunostained with L-NGFR monoclonal antibody and detected using: A. Vector VIP (purple) and B. DAB (brown).

4.2.2 Identification of suitable cell lines

Bone marrow aspirate samples were not used for the optimisation of DIC, since these were of more use in the cell culture experiments presented in Chapter three. Instead, two cell lines were used; the human melanoma cell line A875 and the human umbilical vein cell line ECV304, since these were readily available. Single immunocytochemistry was carried out to identify antigens expressed by each of these cell lines (but not shared by both) and to identify those antigens expressed in abundance. High expression of the low-affinity nerve growth factor receptor (L-NGFR) by A875 cells was demonstrated and this antibody was used for their subsequent demonstration in DIC experiments. The ECV304 cell line had been shown previously by colleagues in our laboratory (Dhamrait, 1995) to express the epithelial membrane antigen, EMA. The two cell lines were each immunostained independently with L-NGFR and EMA to ensure that cross-reactivity for either antigen did not occur between them.

Cytocentrifuge preparations, on which DIC was optimised, were prepared from a mixture of the two cell lines described above. Cells from both cell lines were cultured independently, grown to confluence and trypsinised as described in Chapter 2. To ensure that slides contained equal proportions of each cell line, cell numbers were adjusted to the same concentration and an equal volume from each cell line was mixed prior to the production of cytocentrifuge preparations.

4.3 Optimisation Experiments

For initial DIC optimisation experiments, 17 slides were used to include all necessary positive and negative control slides. These are shown in Table 4.1. Slides 1-5 shown in Table 4.1 were used as negative control slides with omission of primary antibodies, with or without omission of enzyme substrate. Slides 6-13 were used as positive control slides, using only one primary antibody, to ensure that single immunocytochemistry was successful (slides 6, 7, 10 and 11) and to ensure that cross-reactivity between substrates did not occur (slides 8, 9, 12 and 13). Slides 14-17 were used for DIC using both primary antibodies and both detection systems.

Table 4.1. Slides used in double immunocytochemistry optimisation experiments

Slide	1st Primary antibody	Enzyme substrate	2nd Primary antibody	Enzyme substrate
1	----------	----------	----------	----------
2	----------	DAB	----------	----------
3	----------	VIP	----------	----------
4	----------	DAB	----------	VIP
5	----------	VIP	----------	DAB
6	L-NGFR	DAB	----------	----------
7	L-NGFR	VIP	----------	----------
8	L-NGFR	DAB	----------	VIP
9	L-NGFR	VIP	----------	DAB
10	EMA	DAB	----------	----------
11	EMA	VIP	----------	----------
12	EMA	DAB	----------	VIP
13	EMA	VIP	----------	DAB
14	L-NGFR	DAB	EMA	VIP
15	L-NGFR	VIP	EMA	DAB
16	EMA	DAB	L-NGFR	VIP
17	EMA	VIP	L-NGFR	DAB

4.3.1 Initial Methods and Results

In all experiments carried out, cells were fixed in 4% paraformaldehyde in PBS for 5 minutes, as determined previously as being optimal for immunocytochemistry in the cell culture systems used in this study (section 3.2.2). Immunostaining was carried out in 2 stages, using a method adapted from Mason and colleagues, (1983). Each stage of the procedure consisted of single immunostaining as described in sections 2.16.1-4, with different primary antibodies for each stage. After the first stage of immunostaining, cells were washed (3x2 minutes) in phosphate buffered saline (PBS)

and incubated as described previously in avidin and biotin solutions, followed by the second stage of immunostaining. Counterstaining was carried out only at the end of the second stage of immunostaining.

Initial results obtained with the method described above were satisfactory for single immunostaining, except that an unacceptable level of background staining was observed in some slides. Slides used for DIC were unsatisfactory due to cross-reactivity between substrates. This indicated that, at some stage during the first round of immunostaining, binding sites were not saturated.

A number of possible explanations were considered for this cross-reactivity. Firstly, it is possible that the secondary antiserum, used in the first round of staining, was not saturating the primary antibody. This would result in secondary antiserum, used in the second round of staining, binding to the first primary antibody. Secondly, it is possible that the streptavidin-biotinylated horseradish peroxidase (St-AB-HRP), used for the second round of staining, was binding to the secondary antiserum used in the first round. Thirdly, it was possible that active peroxidase had remained after the first round of staining, resulting in the conversion of second substrate to coloured product at the site of the first reaction.

4.3.2 Optimisation of bovine serum albumin concentration to prevent non-specific protein binding

Bovine serum albumin (BSA) is widely used to prevent non-specific binding of antibody to cellular proteins. It binds non-specifically to cell proteins with relatively high affinity, so that only antibodies specific for their target antigen are sufficient to displace BSA from this binding site. Insufficient concentrations of BSA thus permit antibodies to bind non-specifically. To eliminate background staining a titration was carried out. Initially, a 1% solution of BSA had been used (1mg/ml). For the titration experiment, single immunocytochemistry was carried out using BSA at concentrations of 1%, 5% and 10%. The results are summarised in Table 4.2.

Table 4.2. Titration of BSA concentration to eliminate background staining

BSA concentration	Background staining	Effect on specific antibody staining
No BSA	High level throughout slide	Difficult to distinguish specific staining
1mg/ml (1%)	Some in some areas on slide	None
5mg/ml (5%)	None	None
10mg/ml (10%)	None	None

Elimination of background staining was observed when either a 5- or 10-% BSA solution was used, with no detrimental effects on specific antibody staining. Due to the technical difficulties in optimising DIC, it was decided that 10% BSA would be used for subsequent DIC experiments. Following DIC using 10% BSA, although background staining was no longer a problem, cross-reactivity between substrates was still occurring.

4.3.3 Effects of overnight incubation in secondary antiserum

Following the elimination of background staining, the problem of cross-reactivity was investigated. The first hypothesis as to the cause of this was that addition of secondary antiserum was not saturating the primary antibody during the first round of staining. Consequently, the secondary antiserum added for the second round of immunostaining was binding to the first primary antibody.

Secondary antiserum was initially added for 30 minutes at room temperature. An experiment was carried out in which incubation in secondary antiserum was increased to overnight at 4°C. It was anticipated that this would be sufficient to ensure saturation of all binding sites on the primary antibody. However, this had no effect on cross-reactivity, suggesting that another explanation was likely.

4.3.4 Optimisation of avidin/biotin incubation times

During single immunocytochemistry, 20-minute sequential incubations in biotin and avidin solutions are carried out to block endogenous avidin and biotin. Although this incubation time is usually sufficient, it is possible that during DIC it is inadequate to ensure that avidin and biotin from the St-AB-HRP complex are sufficiently blocked. A titration was carried out to determine whether increasing these incubation times would prevent the cross-reactivity that was occurring with DIC. The results are summarised in Table 4.3.

Table 4.3. Titration of incubation times in avidin and biotin solutions

Incubation time/mins	Effect on background staining when used in single immunocytochemistry	Effect on cross-reactivity when used in DIC
20	No Background	None
30	No Background	None
40	Some Background	None
60	More Background	None

The increase in incubation times in avidin and biotin solutions had no effect on the cross-reactivity that was occurring in DIC experiments. However, although it was observed that 20 and 30-minute incubations were suitable to inhibit endogenous avidin and biotin, further increases in incubation times had a negative effect. Incubation times in excess of 40 minutes resulted in an increase in background staining.

4.3.5 Inhibition of peroxidase activity

During DIC, if active peroxidase remained after the first reaction, it is likely that the second enzyme substrate would be converted to coloured product at the site of the first reaction, giving rise to a mixture of two coloured products and hence cross-reactivity. To eliminate this, a suitable recipe for inhibition of peroxidase was located and carried out as recommended. The peroxidase inhibitor comprised 15ml PBS, 150µl sodium azide (15%) and 500µl hydrogen peroxide (30%). This solution was added to the cells for 15 minutes at room temperature, prior to incubation in St-AB-HRP complexes.

This was to ensure that endogenous peroxidase was neither present nor active from the first round of DIC.

Excellent results were obtained using this peroxide inhibitor and cross-reactivity did not occur in any of the slides (Figure 4.2 A and B). It was observed, however, that when using DAB as the second substrate, the staining intensity was considerably weaker than when used as the first substrate (Figure 4.2 C and D). To investigate the cause of this further, a titration was carried out to determine the optimal incubation time in enzyme substrate.

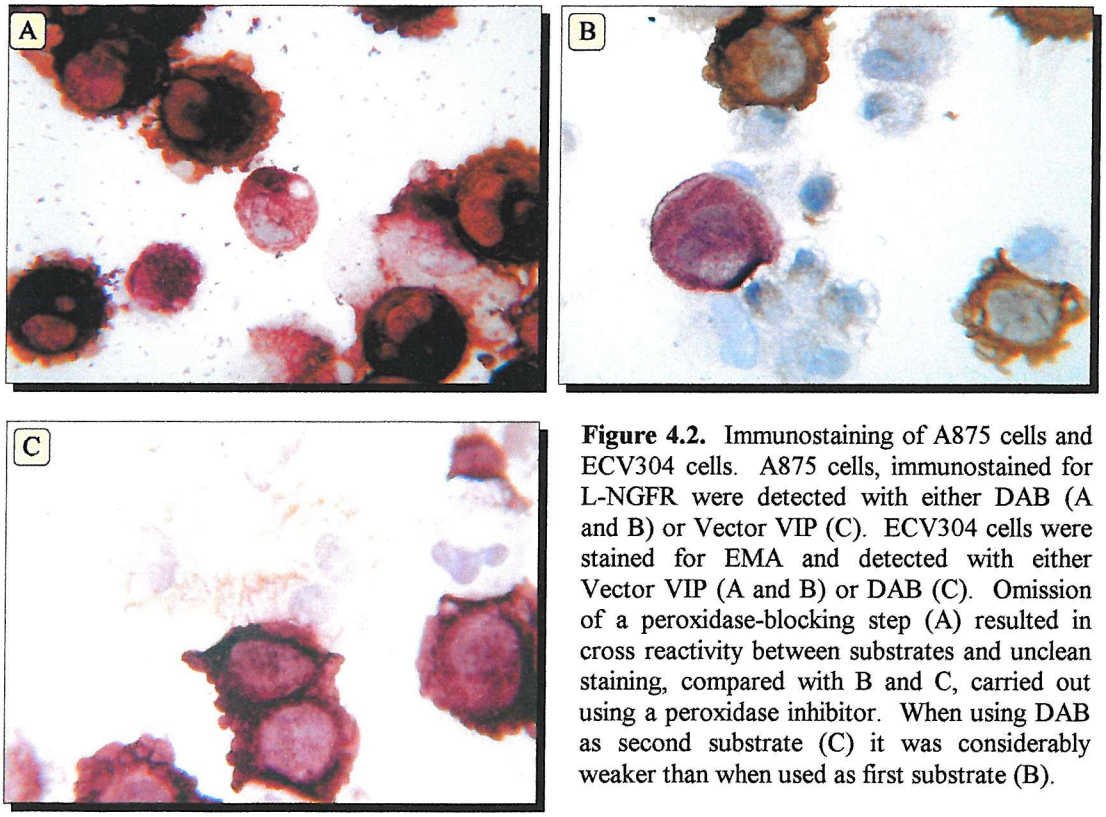


Figure 4.2. Immunostaining of A875 cells and ECV304 cells. A875 cells, immunostained for L-NGFR were detected with either DAB (A and B) or Vector VIP (C). ECV304 cells were stained for EMA and detected with either Vector VIP (A and B) or DAB (C). Omission of a peroxidase-blocking step (A) resulted in cross reactivity between substrates and unclean staining, compared with B and C, carried out using a peroxidase inhibitor. When using DAB as second substrate (C) it was considerably weaker than when used as first substrate (B).

4.3.6 Optimisation of incubation times in enzyme substrate

When carrying out single immunocytochemistry, the enzyme substrate is usually added to the slides for 10-20 minutes. A titration was carried out to determine the optimal incubation period since, with DIC, the colour intensity developed by the enzyme substrate was usually weaker when used for the second round of staining (Figure 4.2B and C). Slides were incubated in enzyme substrate for 20, 40 or 60 minutes. The results are summarised in Table 4.4.

Table 4.4. Titration of enzyme substrate (DAB) incubation times

Incubation time/mins	Staining intensity of DAB when used as second substrate	Amount of non-specific DAB staining
20	Weak	None
40	Weak	None
60	Weak	None

The intensity of specific staining when using DAB as the second substrate did not appear to increase when the substrate incubation time was increased. It was consistently weaker when used as the second substrate. The reasons for this are unclear but may be a result of stearic hindrance caused by the VIP substrate if used for the first round of staining. It is therefore concluded that when carrying out DIC, DAB should always be used as the first substrate and that a 20-minute incubation is sufficient. Further increases in incubation time, although not detrimental, are not beneficial. It was interesting to note that longer incubation times did not result in non-specific staining.

4.4 Summary of DIC Optimisation

The experiments described here have investigated the effects of altering various stages of the DIC procedure. Using the modifications described here, DIC is an effective technique for detecting antigens expressed independently by two separate cell types within a heterogeneous population of cells. The optimised method is described in detail in Appendix 1 and a summary Table describing the effects of these changes is shown in Table 4.5.

In 1979, Sternberger and Joseph reported that, in DIC, the use of DAB to label and detect the first primary antibody results in the masking of unoccupied antigen binding sites on the primary antibody and the blockage of residual enzyme activity (Sternberger & Joseph, 1979). However, in this study, it was found that this was not the case and an additional enzyme blocking stage was fundamental to successful staining. In the optimised procedure described here, DIC was carried out as for single immunocytochemistry, with the addition of a blocking stage prior to the addition of St-

AB-HRP complexes during the second round of staining. In section 4.3.5, peroxidase was inhibited prior to St-AB-HRP incubation in both first and second rounds of DIC. Although this was probably not essential, it ensured that any endogenous peroxidase activity was inhibited.

Table 4.5. Summary of alterations to DIC procedure and their effects

Stage of DIC procedure	Titration	Amount of background staining	Effect on cross-reactivity in DIC	Suitable Conditions for DIC
Blocking of avidin/biotin	20mins	0	----------	Yes
	30mins	0	----------	Yes
	40mins	+	----------	No
	60mins	++	----------	No
Blocking of Non-specific protein binding with BSA	20mins	+++	----------	No
	1mg/ml (1%)	+	----------	No
	5mg/ml (5%)	0	----------	Yes
	10mg/ml (10%)	0	----------	Yes
Peroxidase inhibition	With peroxidase inhibitor	----------	No cross-reactivity	Yes
	Without peroxidase inhibitor	----------	Cross-reactivity	No
Incubation in enzyme substrate	20mins	0	----------	Yes
	40mins	0	----------	Yes
	60mins	0	----------	Yes

Blocking of endogenous avidin and biotin activity was optimal at 20-30 minutes. However, it is important to note that incubation times in excess of these resulted in increased levels of background staining. Blocking of non-specific protein binding, following incubation with avidin and biotin solutions, was found to be most effective when using 5-10% BSA, compared with using 1% BSA. Since many immunostaining protocols use 1% BSA as a standard ingredient, it is useful to consider increasing this if background staining is a problem.

It was observed that, when carrying out DIC, the peroxidase substrate DAB was best used as the first substrate. The intensity of DAB staining was markedly reduced when used as the second substrate, possibly due to a reduction in antigenicity after two rounds of staining. For the second substrate, Vector VIP was particularly useful since reactivity of this substrate with peroxidase is extremely sensitive and produces a very intense coloured product.

For these reasons, optimal results were obtained when DAB was used as the first enzyme substrate and used to detect the cell type anticipated to be most abundant. Vector VIP was best used as the second substrate and to detect the less abundant cell type, as it is considerably easier to detect an intensely stained purple cell amongst a population of many brown cells, than to detect a weakly stained brown cell amongst a population of intensely stained purple cells.

In summary, this technique was optimised to enable the simultaneous detection of two antigens in cell culture inserts. Although DIC is not recommended for the detection of two antigens in the same cell type, this technique may be used for the identification of two stromal cell types in each cell culture insert thereby doubling the amount of information that can be obtained from each culture. Future experiments using this technique could involve investigation of the interaction between macrophages, using antibodies reactive with CD163, for example, with stromal fibroblasts, using antibodies reactive with L-NGFR. Similarly, changes in proportions/distribution of each of the immunophenotypically defined stromal fibroblastic cell types could be investigated in cultures from patients with idiopathic myelofibrosis or following leukaemia therapy (subject to sufficient numbers of aspirable bone marrow cells).

5. OPTIMISATION OF NORTHERN BLOT ANALYSIS AND SUBSEQUENT DETECTION OF L-NGFR mRNA IN THE A875 HUMAN MELANOMA CELL LINE AND HUMAN LONG-TERM BONE MARROW CULTURES

5.1 INTRODUCTION

A population of stromal fibroblast precursors from aspirated bone marrow samples and their progeny in human long-term bone marrow cultures (hLTBMC) were shown, as described in Chapter 3, to express the low-affinity nerve growth factor receptor (L-NGFR). To demonstrate the presence of L-NGFR mRNA in bone marrow, *in situ* hybridisation (ISH) was used initially, as this technique does not require prior RNA extraction and can be carried out without disruption of cells from their growth surface, thereby enabling morphological assessment of positive cells. Despite numerous optimisation experiments, however, *in situ* hybridisation proved unsuccessful, probably due to insufficient sensitivity of the technique used (Chapter 2, section 2.6). Northern blot analysis was investigated as an alternative to ISH, as this is a more sensitive technique. However, it does require prior extraction of RNA and hence disruption of cells from their growth surface.

The aim of the experiments described in this chapter was to optimise Northern blot analysis for the detection of L-NGFR mRNA in bone marrow stromal cells and to investigate whether L-NGFR mRNA synthesis in hLTBMC correlates with receptor protein expression. Immunocytochemical analysis of aspirated bone marrow samples has demonstrated previously that only a small percentage of cells express L-NGFR (Wilkins & Jones, 1998). For this reason, the first stage was to identify suitable cell lines that expressed high levels of L-NGFR, in which to optimise the Northern blot procedure. It was then important to determine the optimal conditions required for the formaldehyde-agarose gel electrophoresis and subsequent detection of RNA on positively charged nylon membranes to ensure that sufficient sensitivity could be achieved to detect small quantities of L-NGFR mRNA. Once these optimisation experiments had been carried out, Northern blot analysis could then be carried out to detected L-NGFR mRNA in hLTBMC.

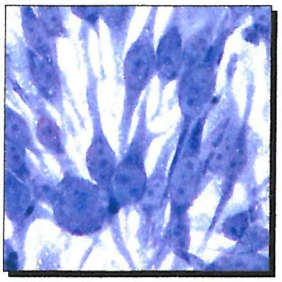
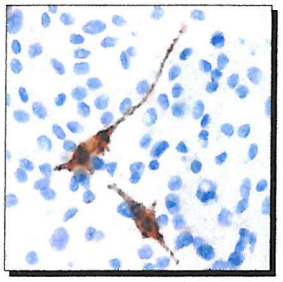
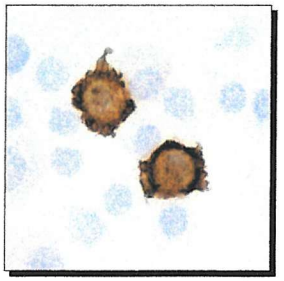
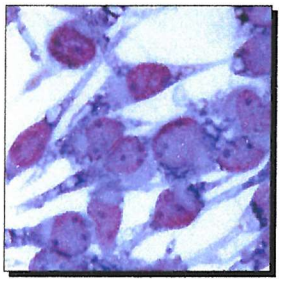
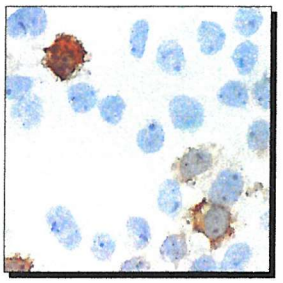
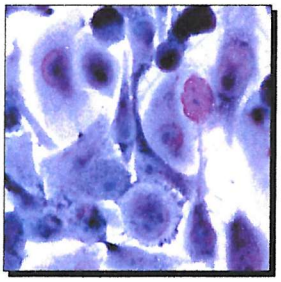
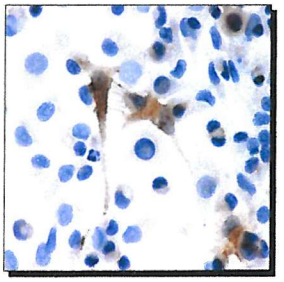
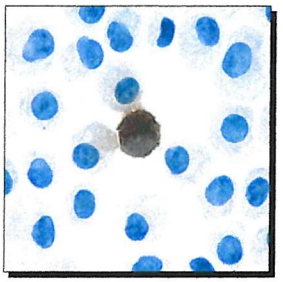
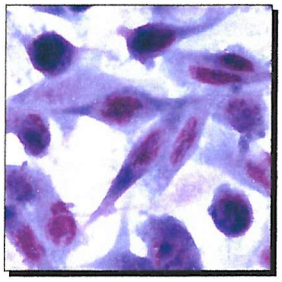
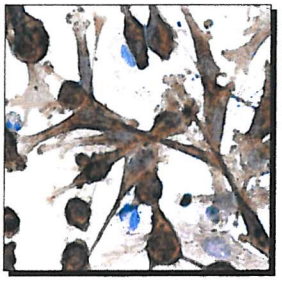
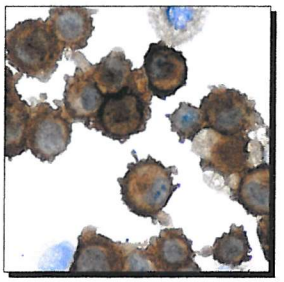
5.2 NORTHERN BLOT OPTIMISATION

5.2.1 Validating L-NGFR expression by cell lines

To permit successful optimisation of Northern blot analysis, in view of the small amount of material available from each aspirated bone marrow specimen and the small number of aspirated samples available, cell lines expressing the L-NGFR were sought. A number of cell lines were screened for abundant L-NGFR expression. These included MM96, MeW0, SKMel 28 and A875. Each of these cell lines was grown in triplicate to assess their growth characteristics and expression of L-NGFR (section 2.11, Chapter 2).

Following growth to confluence, one culture of each cell line was stained for morphological assessment and another was used for immunocytochemical analysis of L-NGFR expression, performed directly on the undisturbed growth surface. In the third culture, cells were grown to confluence, trypsinised to detach adherent cells from the growth surface and cytocentrifuge preparations were produced (section 2.11). The latter were also assessed by immunocytochemistry for L-NGFR expression, to determine whether trypsinisation affected the ability to detect L-NGFR by this technique. The results of these experiments are shown in Table 5.1.

Table 5.1. Growth characteristics and L-NGFR expression of cell lines

Cell line	Growth in culture	L-NGFR expression following 4 weeks in culture	L-NGFR expression in cytocentrifuge preparations
MM96			
MeW0			
SKMel28			
A875			

Each of the above cell lines was grown in triplicate to assess growth characteristics and L-NGFR expression. Cells were grown to confluence and either a) stained for morphological evaluation (column 1) or b) stained using immunocytochemistry to analyse L-NGFR expression post-culture (column 2). To determine the effects of trypsinisation on L-NGFR expression, confluent cells were trypsinised and then cytocentrifuge preparations made. These were also stained for L-NGFR (column 3). The A875 human melanoma cell line was found to have the most abundant L-NGFR expression. Expression of L-NGFR was not affected by prior trypsinisation of cells from their growth surface.

5.2.2 Optimisation of conditions required for 1% Formaldehyde-agarose integrity gel

A comparison was made to determine the optimal ratio between RNA sample and load buffer volume and the optimal ethidium bromide concentration required for a 1% formaldehyde-agarose integrity gel, to obtain the most sensitive visualisation under ultra-violet light (Table 5.2 and Figure 5.1).

Table 5.2. Volume of RNA, load buffer and ethidium bromide added to the 1% formaldehyde-agarose integrity gel

Lane	Volume of RNA sample containing 2μg RNA/μl	Load buffer volume/μl	Ethidium bromide volume/μl (1mg/ml)
1	2.9	2.9 (1x)	1
2	2.9	7.25 (2.5x)	1
3	2.9	14.5 (5x)	1
4	5.0	5 (1x)	1
5	5.0	12.5 (2.5x)	1
6	5.0	25 (5x)	1
7	2.9	2.9 (1x)	2
8	5.0	5 (1x)	2

RNA was extracted from the cell line MM96, using the QIAGEN RNeasy Mini Kit (Qiagen Ltd., UK) (section 2.2). The extracted RNA concentration, determined by optical density measurements, was 0.69μg/μl. All lanes of the integrity gel were loaded with 2μg RNA. Lanes 1, 2, 3 and 7 contained 2μg RNA at the extracted concentration (a volume of 2.9μl) and were compared with lanes 4, 5, 6 and 8, containing 2μg RNA diluted to 5μl with DEPC-treated H_2O. The volume of load buffer added in each lane was either: 1) a volume equal to the RNA volume, containing either 2.9μl (lane 1) or 5μl (lane 4); 2) two-and-a-half times the RNA volume (lanes 2 and 5) or 3) five times the RNA volume (lanes 3 and 6). Lanes 7 and 8 contained equal volumes of load buffer and RNA solution but contained 2μl ethidium bromide (1mg/ml) compared with 1μl in lanes 1 to 6.

Figure 5.1. Formaldehyde-agarose gel electrophoresis to demonstrate effects of RNA volume, load buffer volume and ethidium bromide concentration. Lanes 1-3 and 7 contain 2μg RNA in 2.9μl and lanes 4-6 and 8 contain 2μg RNA diluted to 5μl with DEPC-H_2O. Lanes 1,4,7 and 8 contain equal volumes of RNA and load buffer, lanes 2 and 5 contain 2.5x more load buffer than RNA and lanes 3 and 6 contain 5x more load buffer than RNA. Lanes 1-6 contain 1μl ethidium bromide (1mg/ml) and lanes 7 and 8 contain 2μl ethidium bromide (same concentration).

All lanes demonstrate that the extracted RNA was of good integrity and had not been degraded during the extraction process. The two most intense bands represent, from top to bottom, 28s and 18s ribosomal RNA (rRNA) of 5.0kb and 1.9kb respectively. Non-degraded RNA of good integrity has 28s rRNA present in approximately twice the amount of 18s rRNA as shown above. The 28s rRNA degrades into an RNA species of similar size to that of 18s rRNA and thus with degraded RNA the intensity of the bands is reversed.

Lanes 1-3 and 4-6 demonstrate that, as the ratio between RNA and load buffer volume increases, the band intensity decreases. The dilution of 2μg RNA to 5μl with DEPC-H_2O (lanes 4, 5, 6 and 8) resulted in a reduction in definition and clarity of the bands compared with those containing 2μg RNA in the extracted volume of 2.9μl (lanes 1, 2, 3 and 7). Lanes 7 and 8, contained twice the concentration of ethidium bromide and, although brighter than the other lanes, resulted in an excessive increase in background staining of the gel. Therefore, the optimal combination was chosen to be an equal volume of load buffer and RNA solution, without prior dilution of the latter and the addition of 1μl ethidium bromide.

5.2.3 Dot-blot hybridisation to test hybridisation procedure and chemiluminescent detection protocols

Hybridisation and chemiluminescent detection of biotinylated oligonucleotide probes was tested using a dot-blot hybridisation procedure (described in Appendix 1). Increasing amounts of RNA, extracted from the human melanoma cell line A875, were

blotted directly onto a positively charged nylon membrane and hybridised with 5 picomoles/ml of antisense biotinylated probe cocktail specific for L-NGFR (see section 2.5, Chapter 2 for more details). In addition, 5µl biotinylated probe cocktail (10ng/µl) was blotted directly onto the membrane to ensure that, if hybridisation was not satisfactory, the effectiveness of the CDP-Star, chemiluminescent detection system (Tropix Inc. Massachusetts, USA) could still be demonstrated. The results are shown below in Figure 5.2.

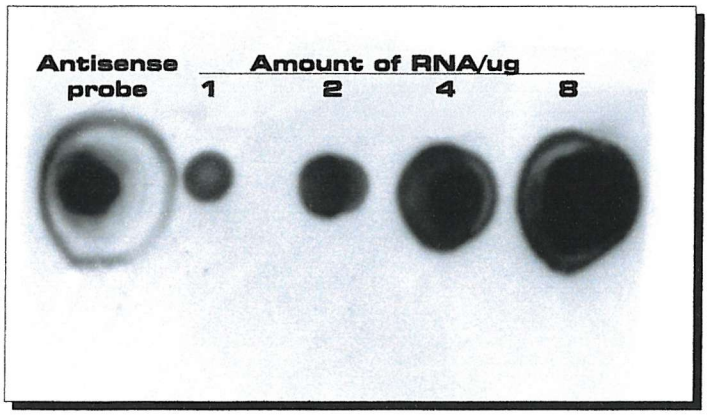


Figure 5.2. RNA dot-blot hybridisation to determine the effectiveness of hybridisation and chemiluminescent detection protocols. RNA and antisense probe was blotted directly onto positively charged nylon membrane to ensure that a) the probes were hybridising to RNA on the membrane and b) that should hybridisation be unsuccessful, the chemiluminescent detection of antisense probe could be tested.

From the blot above (Figure 5.2), it can be seen that the antisense biotinylated probe, blotted directly onto the membrane, was detected satisfactorily by the CDP-Star detection system. The RNA blotted directly onto the membrane shows no intensity variation but the dot size correlates positively with sample volume loaded onto the membrane. This does not therefore demonstrate the optimal amount of RNA required for detection on the membrane but does demonstrate that the hybridisation and chemiluminescent detection of antisense probe to the RNA on the membrane was successful over the range of RNA concentrations tested.

5.2.4 Optimisation of RNA quantity to load onto 1% formaldehyde-agarose gel for Northern Transfer

A range of RNA quantities was used to determine the optimal amount of RNA required to load into a 1% formaldehyde-agarose gel for gel electrophoresis and transfer to positively charged nylon membrane for subsequent Northern blot analysis. The RNA quantities used were 1, 2, 5 and 10µg. The hybridisation temperature had

not yet been optimised and a temperature of 55°C was recommended as a good starting point (Tropix Inc. Massachusetts, USA, Chemiluminescent detection protocol). The results (not shown) demonstrated non-specific hybridisation of L-NGFR probe to RNA, as shown by the presence of smeared bands throughout the blot. The results did show, however, that 1 and 2μg RNA were insufficient for optimal detection and that 10μg appeared excessive. All further experiments in which Northern transfer was to be carried out used 5μg RNA loaded into the formaldehyde-agarose gel.

5.2.5 Optimisation of hybridisation temperature

The previous experiment suggested that the hybridisation temperature might have been insufficient to prevent non-specific binding of probe to RNA. Alternatively, the hybridisation temperature may have been adequate, with alterations required in stringency washing. The hybridisation temperature was increased to 70°C and 80°C to elucidate the effect of temperature variation on hybridisation (Figure 5.3).

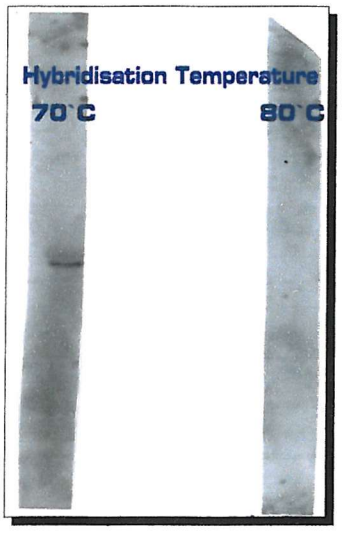


Figure 5.3. Northern blot analysis showing hybridisation and detection of oligonucleotide probes to L-NGFR mRNA in the A875 cell line. Only at 70°C was hybridisation of the probe to the mRNA detected. A single band was measured at 5.8cm from the top of the gel. This band was compared to the RNA markers, which were run alongside and found to represent a band of RNA of approximately 3.5kb. The L-NGFR mRNA is 3.4kb suggesting that the band detected corresponds to mRNA of the L-NGFR.

At 80°C, hybridisation of L-NGFR oligonucleotide probe to RNA was not detected. At 70°C a single band was demonstrated, 5.8cm from the top of the blot. A standard curve was constructed, from RNA size markers, of the distance that each band had travelled through the gel and the size of the RNA to which they corresponded. The band detected from the L-NGFR probe at 5.8cm was determined from the graph to represent a band of RNA of approximately 3.5kb. The L-NGFR mRNA is 3.4kb (3386

bases) suggesting the band obtained is likely to be that of the L-NGFR mRNA. The discrepancy between the two values is likely to have arisen from measurement inaccuracies.

5.2.6 Detection of L-NGFR mRNA in human long-term bone marrow cultures

Mononuclear cells were harvested from aspirated bone marrow samples and cultured under hLTBMC conditions over a 4-week period in 4ml slide flasks. RNA was then extracted as described in Chapter 2. Since L-NGFR is not expressed by all stromal fibroblasts in hLTBMC, Northern blot analysis was carried out using 10μg RNA from the hLTBMC rather then 5μg. This was compared with 5μg A875 RNA, used as a positive control (Figure 5.4) due to abundance of L-NGFR mRNA.

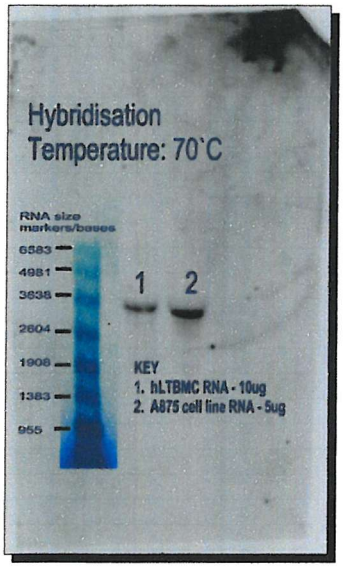


Figure 5.4. Northern blot analysis showing detection of L-NGFR mRNA in hLTBMC and A875 human melanoma cell line. In lane 1, 10μg RNA from hLTBMC was used to ensure the presence of adequate RNA. In lane 2, only 5μg was used due to the abundance of L-NGFR mRNA in A875 cells. The two bands produced from lanes 1 and 2 were compared with the RNA markers and found to represent mRNA of 3.4kb, consistent with mRNA for the L-NGFR.

From the blot (Figure 5.4), it can be seen that both bands (lane 1 and 2) are approximately the same distance from the top of the gel. The distance was compared with the RNA size markers and found to correspond to mRNA of 3.4kb. This is the same as that of the L-NGFR mRNA, in agreement with previous experiments using the A875 cell line.

5.3 DISCUSSION OF RESULTS

In these experiments, Northern blot analysis was optimised for the detection of L-NGFR mRNA using a cocktail of biotinylated oligonucleotide probes and a non-isotopic detection system. The use of non-isotopic detection systems is relatively new in such methods and, besides safety benefits, offers several potential advantages over the use of equivalent isotopic methods. The CDP-star chemiluminescent substrate for alkaline phosphatase used here generates high intensity, prolonged light emission. Rapid results were obtained, with exposure to X-ray film requiring from as little as a few minutes. The maximum light emission is reported to occur within 10-minutes of the addition of substrate (Tropix Inc. Massachusetts, USA, Data sheet TPX 501-002, 1998) and emitted light could still be detected several days later (data not shown). This is advantageous over isotopic methods, which can require exposure times of several days (Engler-Blum *et al.*, 1993).

The A875 cell line was compared with other cell lines and, using immunocytochemical techniques, found to express high amounts of L-NGFR. This was subsequently used for cell culture and Northern blot optimisation as it provided an abundant source of L-NGFR positive cells for study. Optimal conditions for a 1% formaldehyde-agarose integrity gel required 2μg RNA at the extracted concentration and an equal volume of load buffer. Prior dilution of RNA to a volume of 5μl was neither required nor beneficial. Addition of 1μl ethidium bromide (1μg/μl) was found to result in superior visualisation of the bands compared with the addition of 2μl. When carrying out a 1% formaldehyde agarose gel for subsequent Northern transfer onto positively charged nylon membranes, 5μg RNA was optimal for A875 cells, in which specific mRNA was present in abundance. However, for the detection of L-NGFR mRNA in hLTBMC, 10μg was used since L-NGFR mRNA was less abundant than in the A875 cell line.

Hybridisation of L-NGFR oligonucleotide probes to mRNA was optimal at 70°C with stringency washes carried out as described in Chapter 2, part A. The single band obtained by hybridisation at this temperature had an approximate size of 3.4kb and correlates with that expected for L-NGFR mRNA. Further experiments were carried

out to ensure that these results were consistent and this technique was then applied to hLTBMC, demonstrating the presence of L-NGFR in hLTBMC at the mRNA level.

Northern blot analysis has therefore been optimised to permit successful detection of L-NGFR mRNA both in A875 cells and hLTBMC. It would be of interest to conduct further experiments to carry out Northern blot analysis of aspirated bone marrow samples pre- and post immunomagnetic separation for each of the bone marrow stromal cell types and following culture, in parallel with immunostaining. This will determine if any differences exist between L-NGFR mRNA synthesis and receptor expression amongst each of the stromal cell populations in human bone marrow. In the current study, however, the supply of aspirated bone marrow biopsies within the time available was insufficient in order to progress to carry out these experiments.

6. STROMAL ALTERATIONS IN THE DEVELOPMENT OF IDIOPATHIC MYELOFIBROSIS

6.1 INTRODUCTION

Idiopathic myelofibrosis (IMF) is a chronic myeloproliferative disorder characterised by bone marrow fibrosis, splenomegaly and extramedullary haemopoiesis (Reilly, 1997; Weinstein, 1991). It is associated with an increase in megakaryocyte numbers in the bone marrow and premature release of their regulatory cytokines and growth factors (Thiele *et al.*, 1997). Previous studies investigating the pathogenesis of this disorder have involved immunohistochemical analysis of the various extracellular matrix components of myelofibrotic stroma (Lisse *et al.*, 1991) and investigation of the abnormal synthesis and release of regulatory cytokines by neoplastic megakaryocytes (Thiele *et al.*, 1997). The aim of this immunohistochemical study was to investigate the cellular changes that occur in the bone marrow of patients with IMF. Investigation of the relationship between expression of α-smooth muscle actin (αSMA) and the low-affinity nerve growth factor receptor (L-NGFR) by stromal fibroblasts was included.

Bone marrow trephine biopsy sections were obtained and prepared as described in Chapter 2. Trephine biopsies were obtained from "normal" patients (n=14), having bone marrow sampled for investigation of malignancies and subsequently found to have no bone marrow involvement and patients with idiopathic myelofibrosis (n=24). Antibodies reactive with L-NGFR and αSMA were used to identify stromal fibroblasts; anti-CD34 antibodies were used to identify sinusoids, blood vessels and haemopoietic progenitor cells; macrophages were identified using the PGM-1 antibody, specific for CD68. All antibodies used in this study have been previously demonstrated in bone marrow trephine biopsy sections to react with various components of marrow stroma (Wilkins & Jones, 1995a). Bone marrow trephine biopsy sections were analysed semi-quantitatively after immunostaining, using an arbitrary scoring system of 0-3, with increments of 0.5, to record the number of cells staining positive. This is described in more detail in section 2.14.6 (Chapter 2).

6.2 Antigen Expression in Normal Bone Marrow

6.2.1 Stromal fibroblasts

An extensive network of L-NGFR positive dendritic stromal fibroblasts was observed throughout the haemopoietic compartment of the marrow cavity in all but one of the normal patients studied (Figure 6.1A). These stromal fibroblasts did not express αSMA (Figure 6.1B). A single-cell layer of L-NGFR positive stromal fibroblasts was identified surrounding the majority of trabecular margins whereas αSMA positive fibroblasts surrounded only occasional trabecular margins. However, in a number of biopsy sections, trabeculae had become disrupted due to the heat-mediated antigen retrieval techniques required for these antibodies. In these sections, assessment of staining around trabecular margins was not possible. A summary graph of L-NGFR and αSMA expression in normal trephine biopsy samples is shown in Figure 6.3, page 106.

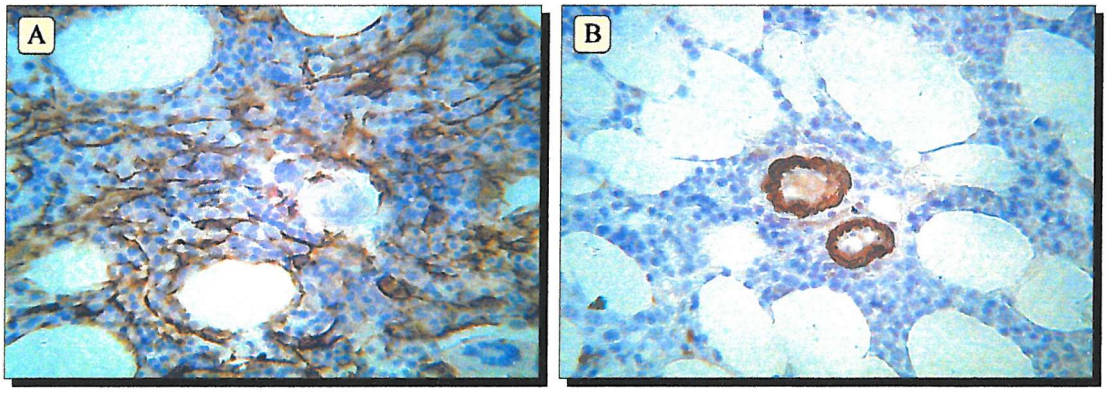


Figure 6.1. Expression of L-NGFR (A) and αSMA (B) in normal trephine bone marrow biopsy sections. An extensive network of L-NGFR positive stromal fibroblasts is seen throughout normal biopsies (A). These stromal fibroblasts are negative for αSMA (B).

6.2.2 Blood vessels, sinusoids and haemopoietic cells

The number of large, arterial blood vessels in normal bone marrow biopsy sections was small. Morphologically, most arterioles were small, round and dilated. These arterioles were composed of an inner endothelial layer, which stained positively for CD34 and a middle layer of smooth muscle cells staining positively for αSMA. Surrounding the αSMA positive cells was a layer of stromal fibroblasts that stained

positively for L-NGFR (Figure 6.2). A layer of CD34 positive endothelial cells also lined thin-walled venous sinusoids. These were more numerous than arterial vessels, usually larger and more irregular. A small number of haemopoietic progenitor cells, identified by their CD34 expression, were also present (Figure 6.3), scattered throughout the biopsy samples; no clustering was observed. These haemopoietic progenitor cells were distinguishable from CD34 positive endothelial cells by virtue of their granular staining pattern.

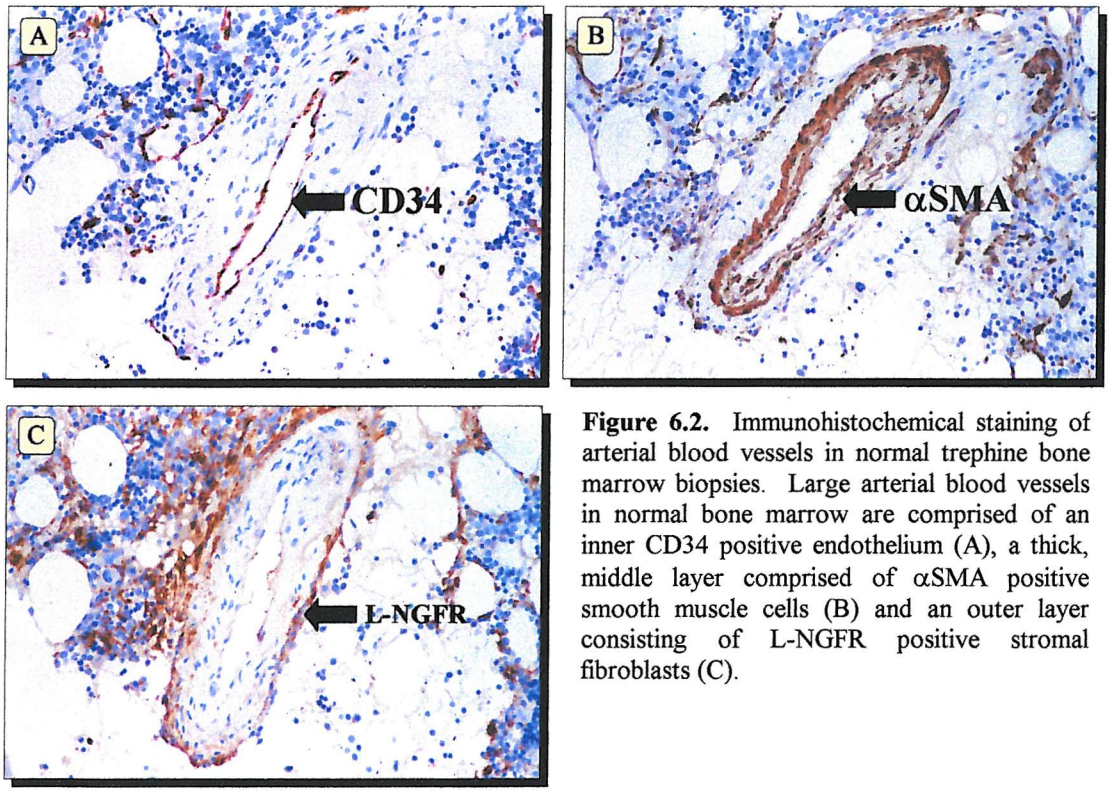


Figure 6.2. Immunohistochemical staining of arterial blood vessels in normal trephine bone marrow biopsies. Large arterial blood vessels in normal bone marrow are comprised of an inner CD34 positive endothelium (A), a thick, middle layer comprised of αSMA positive smooth muscle cells (B) and an outer layer consisting of L-NGFR positive stromal fibroblasts (C).

6.2.3 Stromal macrophages

In all normal bone marrow biopsy sections, the number of macrophages, identified using the PGM-1 antibody, specific for CD68, was high (Figure 6.3). These macrophages were scattered throughout the bone marrow stroma and were unclustered. Morphologically, they were large and most had an irregular, slightly elongated appearance. Although these macrophages contained reasonably granular cytoplasm, lipid debris was seldom observed in macrophages in normal bone marrow samples.

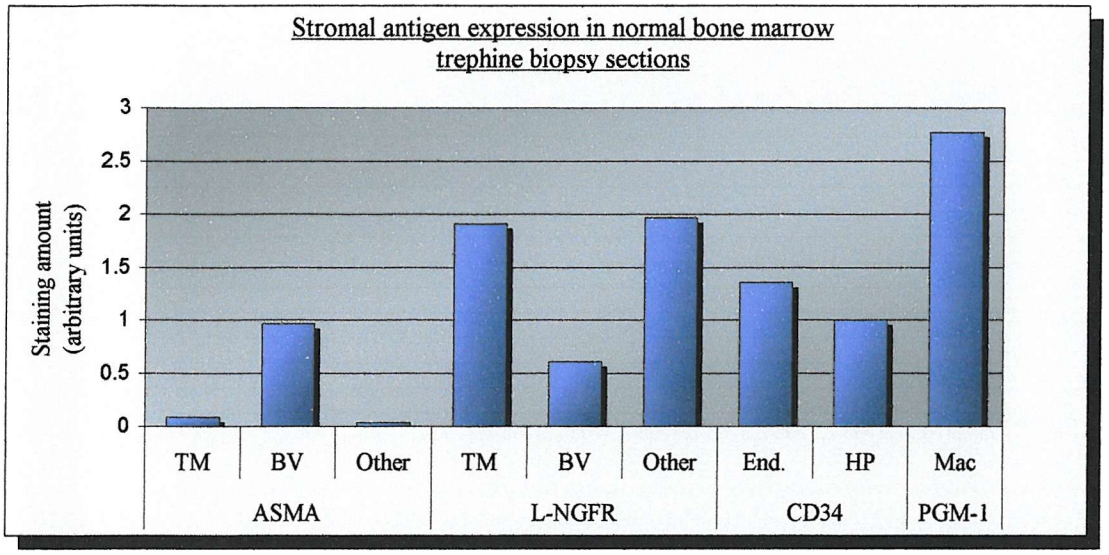


Figure 6.3. Antigen expression in normal bone marrow trephine biopsy samples. Trabecular margins (TM), blood vessels (BV) and other staining of stromal fibroblasts (other) was analysed by anti-αSMA and anti-L-NGFR antibodies. Endothelium (End) and haemopoietic progenitor cells (HP) were stained using anti-CD34 antibody and macrophages (Mac) were stained using PGM-1 antibodies, specific for CD68.

6.3 Antigen Expression in Myelofibrotic Bone Marrow Trephine Biopsy Sections

6.3.1 Stromal fibroblasts

A number of biopsy sections from patients with IMF contained αSMA positive stromal fibroblasts surrounding trabecular margins compared with normal bone marrow in which there were no αSMA positive fibroblasts (Figure 6.5). Throughout the marrow cavity, stromal fibroblasts positive for αSMA were present in 21/24 myelofibrosis patients, compared with only 1/24 of normal patients (Figures 6.4 and 6.5). In a small number of marrow biopsies from myelofibrosis patients, expression of αSMA was restricted to areas of marked fibrosis although, in most cases, αSMA positive stromal fibroblasts were present throughout the entire stroma.

Expression of L-NGFR by stromal fibroblasts in biopsies from myelofibrotic marrow was similar to that observed in normal marrow (Figure 6.6). In some myelofibrosis patients, L-NGFR positive fibroblasts were found only in fibrotic areas and, in a small number of cases, this correlated with areas of high αSMA expression. Between

patients, L-NGFR expression varied; some biopsies contained large numbers of L-NGFR positive fibroblasts throughout the stroma and others contained very few.

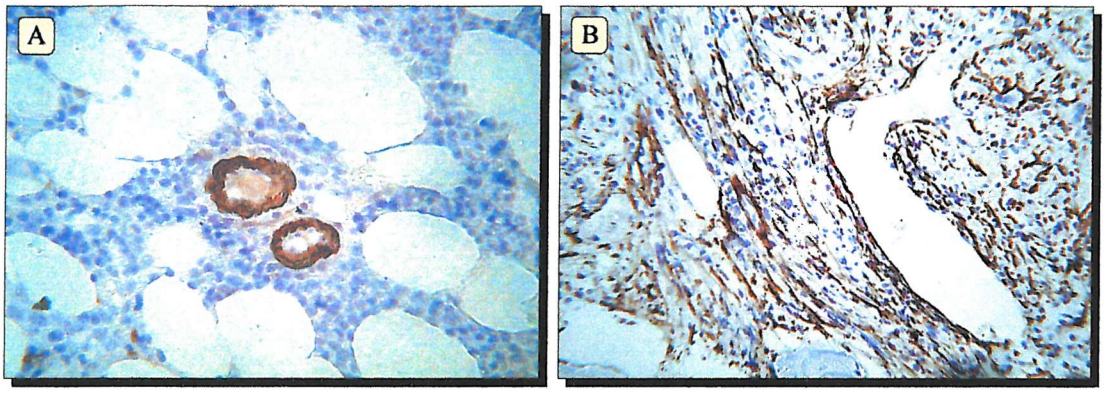
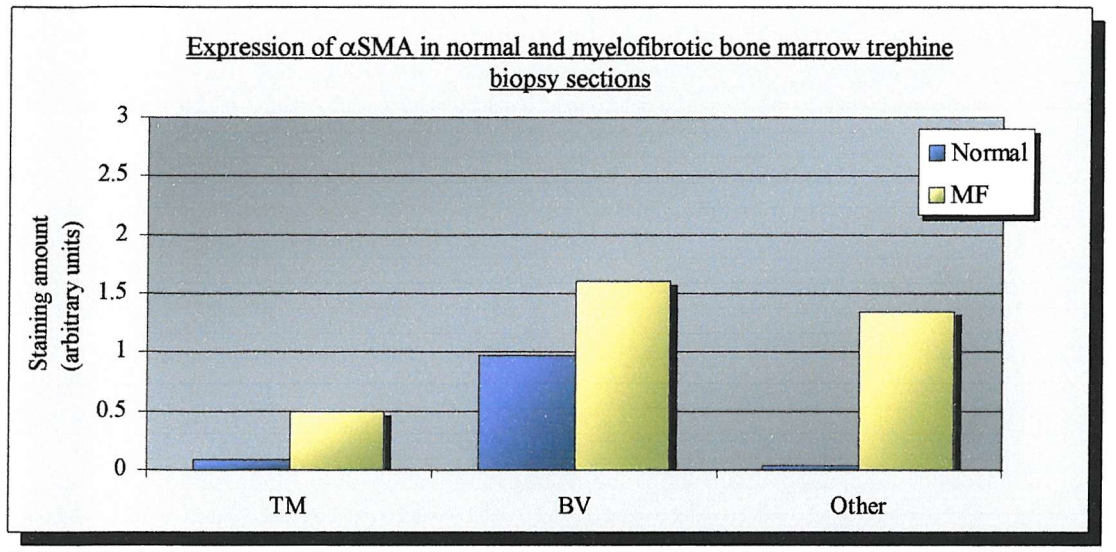
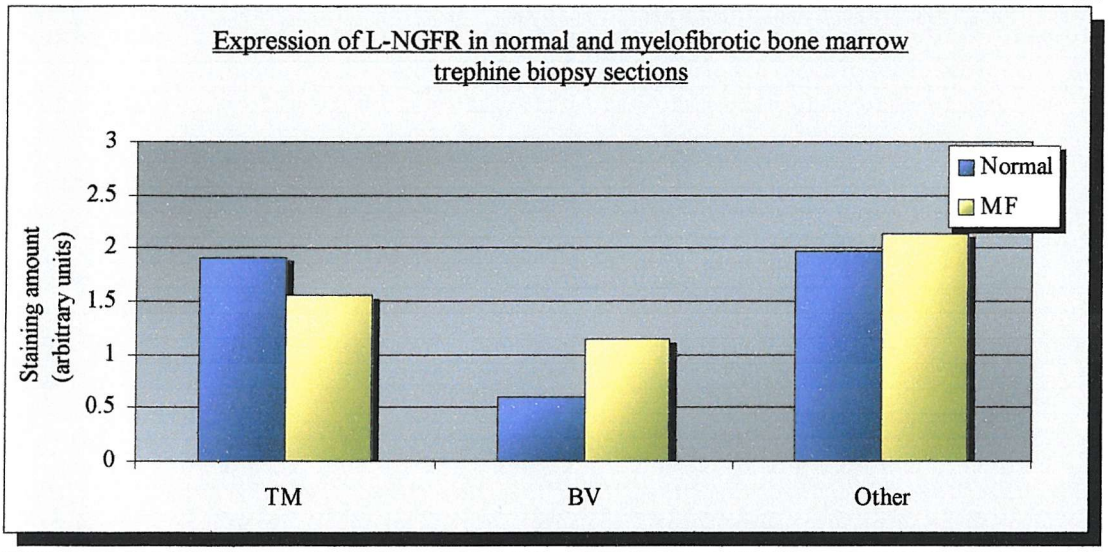


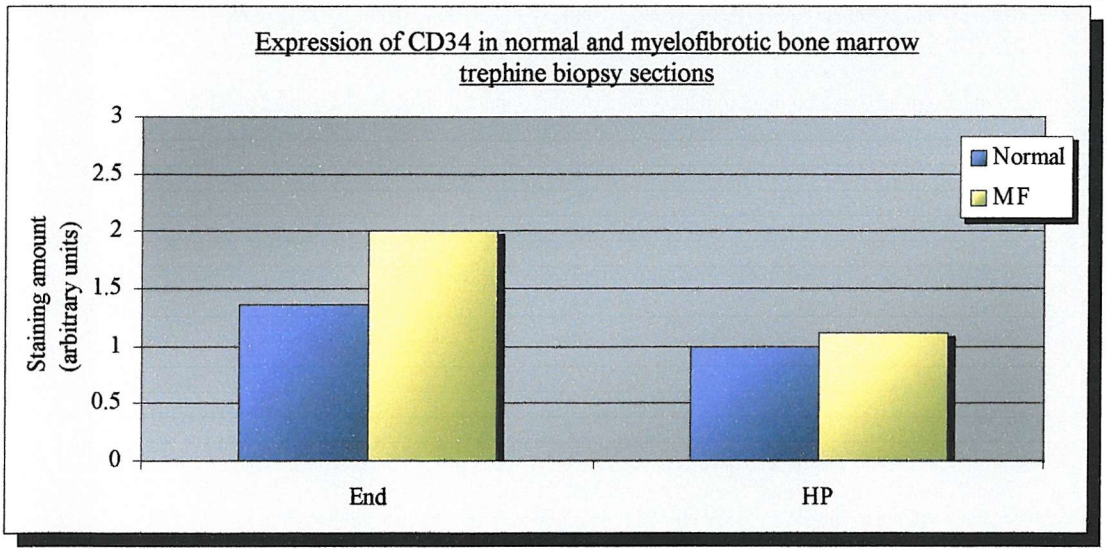
Figure 6.4. Expression of αSMA in normal (A) and myelofibrotic (B) bone marrow. In normal marrow (A), there is no expression of αSMA by stromal fibroblasts. However, in idiopathic myelofibrosis (B), stromal fibroblasts exhibit characteristics of myofibroblasts by the expression of αSMA.

6.3.2 Blood vessels, sinusoids and haemopoietic cells

The number of arterial blood vessels in biopsies from patients with myelofibrosis was, in most cases, increased compared with normal. Sections from some biopsies showed large increases in the number of αSMA positive blood vessels whereas some had essentially normal numbers. A layer of perivascular, L-NGFR positive stromal fibroblasts surrounded the majority of these small arteries and arterioles (Figure 6.2). In myelofibrotic biopsies, the size of the arterial vessels was usually increased and there was an increase in the number of large, distended sinusoids, not observed in normal marrow. A comparison of αSMA staining between sections representing myelofibrosis and normal marrow is shown in Figures 6.4 and 6.5.







Figures 6.5 (top), 6.6 (middle) and 6.7 (bottom). Comparison between trephine bone marrow biopsies in normal and myelofibrosis patients. Antibodies reactive with αSMA and L-NGFR were used to stain trabecular margins (TM), blood vessels (BV) and other stromal fibroblasts (other). Antibodies reactive with CD34 were used to identify endothelium (End) and haemopoietic cells (HP). The number of blood vessels and amount of endothelium was increased in patients with myelofibrosis, although the greatest difference was an increased expression of αSMA by stromal fibroblasts in patients with myelofibrosis.

Figure 6.7 shows the variation observed in CD34 expression in myelofibrotic and normal marrows. There was quite a large variation among myelofibrotic patients, both in staining of blood vessels and sinusoids and in staining of haemopoietic progenitor cells. However, in most biopsies, the number and size of sinusoids was increased. Likewise, the number of CD34 positive haemopoietic cells in some biopsies from myelofibrosis patients was high, while in others there were very few of these cells.

6.3.3 Expression of PGM-1

The number of macrophages present in biopsy sections from myelofibrosis patients was, in most cases, reduced compared with normal controls (Figure 6.8). In some cases, macrophages in myelofibrotic biopsies were of similar size and shape to normal but, in the majority, they were smaller and appeared more dendritic. These macrophages were often clustered in areas of the biopsy containing predominantly fibrotic tissue and frequently contained lipid debris within their cytoplasm.

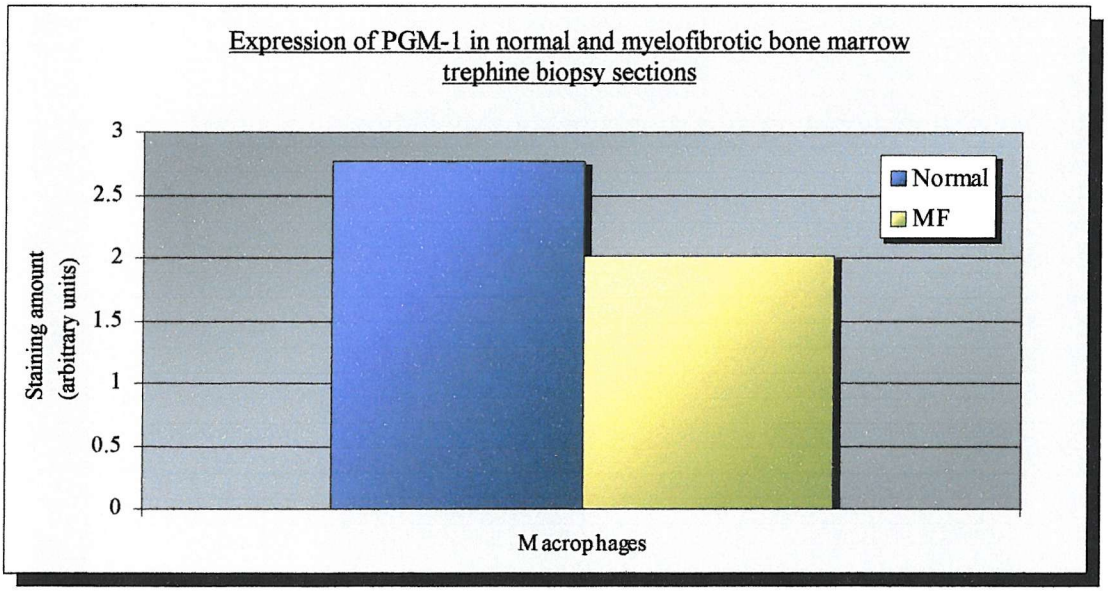


Figure 6.8. Comparison between macrophage numbers in normal and myelofibrotic bone marrow. In myelofibrosis patients, a reduction in numbers of macrophages was evident in most cases. These macrophages were morphologically smaller and more dendritic than those observed in normal patients.

6.4 DISCUSSION OF RESULTS

Idiopathic myelofibrosis (IMF) is a complex disorder arising from an underlying haemopoietic stem cell defect that causes unregulated marrow fibrosis and extramedullary haemopoiesis (Weinstein, 1991). It has been described previously as being characterised by an increase in stromal fibroblasts and endothelial cells, with excess deposition of their extracellular matrix products (Dilly & Jagger, 1990). The network of fibroblastic stromal cells observed in IMF was characterised in this study using antibodies reactive with L-NGFR and αSMA. These antibodies, in addition to anti-CD34 antibodies reactive with stromal endothelium, were used to investigate immunophenotypic changes that occur in the bone marrow stroma of patients with IMF. They also enabled the pathological basis of this disease with respect to stromal elements of bone marrow to be studied further. The method of evaluating the immunocytochemical staining of biopsies used in this chapter was carried out semi-quantitatively as described in section 2.14.6. Although this method of evaluation does not permit accurate identification of small changes in staining (i.e. <1.0 arbitrary unit) changes in staining greater than this can confidently be treated as true differences. This was taken into account when drawing conclusions from these data.

Expression of L-NGFR in stromal fibroblasts was not consistent throughout all IMF cases studied. In some patients, there were high numbers of L-NGFR positive stromal fibroblasts throughout the marrow cavity and in others, there were few. The most apparent and consistent finding was the large increase in expression of αSMA by stromal fibroblasts throughout the biopsy. In contrast to this, αSMA positive stromal fibroblasts were absent from normal biopsies. Previous studies have shown that no cells expressing αSMA are present in normal aspirated bone marrow biopsy samples (Wilkins & Jones, 1998), suggesting that expression of αSMA, at least in adult bone marrow, is exclusive to pathological conditions such as IMF.

It has been suggested that fibrosis in IMF may be caused by cytokines derived from malignant megakaryocytes in the marrow (Thiele *et al.*, 1997). These megakaryocytes are believed to secrete growth factors that regulate the proliferation of stromal fibroblasts, including interleukins 1, 6 and 7 (Mayani *et al.*, 1992). Although not assessed quantitatively in this study, high numbers of megakaryocytes were observed

in the myelofibrotic marrows studied. It is possible that the abnormal secretion of growth factors by malignant megakaryocytes may induce the formation and proliferation of excessive numbers of αSMA positive stromal fibroblasts with myofibroblastic characteristics. Alternatively, the abnormal secretion of growth factors by megakaryocytes may be inducing an immunophenotypic transition of stromal fibroblasts from L-NGFR positive stromal fibroblasts seen in normal marrow, to the αSMA positive myofibroblasts observed in IMF. These αSMA positive stromal fibroblasts are not present in normal adult bone marrow and may therefore represent a dysfunctional component of bone marrow not capable to support haemopoiesis to the same extent as L-NGFR positive stromal fibroblasts present in normal marrow.

The number of arterial blood vessels in IMF was shown to be slightly elevated compared with normal. These arterioles were larger and more distended than those seen in normal marrow. The difference observed in marrow sinusoids from patients with IMF was more noticeable as they were frequently much larger and more abundant than normal. A surrounding layer of αSMA positive cells most frequently identified these. Previous studies investigating IMF have also reported distension of marrow sinusoids (Wolf & Neiman, 1985). There has also been published literature showing that, in patients with myelofibrosis arising from other malignancies such as chronic myeloid leukaemia, these arterial blood vessels are not surrounded by stromal fibroblasts to the same extent as that observed in normal bone marrow (Kuto *et al.*, 1984). However, in this study, the cellular composition of blood vessels identified using antibodies reactive with CD34, αSMA and L-NGFR to identify endothelium, smooth muscle and perivascular stromal fibroblasts, respectively, was not unlike that observed in normal marrow.

The number of macrophages in IMF marrows was, in the majority of cases, decreased compared with normal. These macrophages were generally smaller and morphologically more elongated and dendritic than those found in normal marrow. In many cases, lipid debris within the cytoplasm of macrophages was also evident and commonly associated with reduced numbers of adipocytes in biopsies from patients with IMF. Little is known about the functions and origin of adipocytes in bone

marrow and ingestion of lipid debris by stromal macrophages may suggest that adipocyte abnormalities are associated with IMF.

The decreased numbers of adipocytes and increased numbers of fibroblasts observed in the bone marrow of patients with IMF may be a result of adipocyte apoptosis occurring in association with replacement by stromal fibroblasts. This could account for the adipocyte debris observed in the cytoplasm of macrophages in IMF. However, the amount of lipid debris observed in the macrophage cytoplasm is perhaps insufficient to account fully for the decrease in adipocyte numbers observed in IMF. An alternative explanation may be that adipocytes and fibroblasts within the bone marrow are interchangeable under appropriate stimuli (see Figure 9.1, Chapter 9). Evidence to support this theory comes from Bianco and colleagues (1993). They have shown that increases in adipocyte-rich marrow, such as in hypocellular states, are mirrored by decreases in the number of fibroblastic stromal cells in the marrow (Bianco *et al.*, 1993). Modelled on these data, cytokines and growth factors released by malignant megakaryocytes in IMF may cause adipocytes to de-differentiate into fibroblasts, giving rise to reduced numbers of adipocytes and increased numbers of fibroblasts.

It is well documented that adipocytes may have osteogenic potential (Bennett *et al.*, 1991; Nuttall *et al.*, 1998). However, the mechanism by which this occurs is unclear and it may be that adipocytic and osteoblastic phenotypes are interchangeable. It is possible that, in IMF, some adipocytes differentiate into osteoblasts, which may provide a potential explanation for the increase in new bone formation that occurs in some myelofibrotic bone marrow specimens. However, it is also possible that adipocytes and osteoblasts originate from a common progenitor cell and that, in IMF, adipocyte apoptosis is accompanied by stem cell stimulation to replace them with osteoblasts.

These observations may also indicate a compromised ability of stromal macrophages to phagocytose debris from stromal cell turnover and could implicate macrophages in the pathogenesis of myelofibrosis, as has been suggested (McCarthy, 1985).

7. BONE MARROW STROMA IN PATIENTS WITH ACUTE LEUKAEMIA, BEFORE AND AFTER TREATMENT

7.1 INTRODUCTION

Bone marrow trephine (BMT) biopsy specimens were obtained from patients with acute myeloid leukaemia (AML) and acute lymphoblastic leukaemia (ALL) to investigate, using immunohistochemistry, the phenotypes of stromal cells in these diseases before and after treatment. Control biopsy sections were obtained from patients having bone marrow examination for investigation of malignancies and found to have no bone marrow involvement. Preliminary observations identified a correlation between AML subtype and CD34 expression by leukaemic cells and between AML subtype and the presence of CD34 negative erythroblastic islands in BMT biopsy sections. Further pre-treatment trephine biopsy sections were obtained from patients with AML and studied to quantify these observations and to determine whether these correlations were of significance. Patients included in this study were treated in accordance with Medical Research Council clinical trial protocols as described in Chapter 1. Sequential bone marrow trephine biopsies were obtained from these patients after each course of chemotherapy and compared with pre-treatment biopsies to determine the effects of the chemotherapeutic agents on the phenotypes of bone marrow stromal cells.

Chemotherapy used in the treatment of acute leukaemia and other malignancies, results in suppression of normal haemopoiesis and haemopoietic regeneration following treatment varies among patients (Wilkins *et al.*, 1993). The effects of chemotherapeutic agents on the bone marrow stroma are poorly understood and yet the role of bone marrow stromal cells in supporting functional haemopoiesis is paramount (Singer *et al.*, 1985). The aim of this study was to investigate whether these agents cause injury to bone marrow stromal cells to determine whether this may be responsible for the variations in haemopoietic regrowth that occurs following treatment.

Antibodies used to identify various elements of bone marrow stroma were selected on the basis of previous experiments that had identified stromal antigens in normal bone marrow stroma (Wilkins & Jones, 1995a). Expression of these antibodies in normal bone marrow is shown in Table 2.4, Chapter 2. Bone marrow trephine biopsy sections were analysed semi-quantitatively after immunostaining, using a scoring system of 0-3, with increments of 0.5, to record the number of cells staining positive, as described in section 2.14.6 (Chapter 2). Graphs were plotted showing the variation from normal. For example, if the normal value was 1 and pre- or post-chemotherapy value was 3, a value of 2 would be plotted. All positive values therefore represent increased number of positive cells and all negative values represent decreased number of positive cells compared with normal. This is described in more detail in section 2.14.6 (Chapter 2), and the limitations are acknowledged.

7.2 PATIENT GROUPS

Forty-six patients were investigated, 24 of whom were treated for AML, 8 were treated for ALL and 14 were normal controls. Patients for study were selected on the basis of the availability of adequate archival trephine biopsy material and the number and quality of post-treatment biopsies. All leukaemic patients received at least one course of chemotherapy; the number of post-treatment specimens available for each patient ranged from 1 to 5 (average 2). Specimens representing relapsed disease or taken after transplantation were excluded from study, in order to simplify the assessment of changes potentially due to chemotherapy. One hundred and twelve biopsy specimens were studied in total, of which 32 were taken prior and 66 taken after chemotherapy. In addition, biopsy specimens were obtained from a further 48 untreated AML patients to investigate CD34 expression in AML subtypes. These are discussed in section 7.5.

7.2.1 AML patients

The 24 AML patients whose pre- and post-treatment samples were studied comprised 4 patients from each AML subtype, AML-M0 to AML-M5. Patients with AML-M6 or AML-M7 were excluded from study because examples of these subtypes were rare among the available archival material. The age range of all AML patients was 12 to 72 (mean 56.1). Ages within individual subtypes were as follows: M0, range 12 to 69

(mean 46.5); M1, range 40 to 68 (mean 53.5); M2, range 50 to 72 (mean 61); M3, range 42 to 57 (mean 48); M4, range 56 to 68 (mean 63.3); M5, range 64 to 64 (mean 64).

7.2.2 ALL patients

Eight patients with ALL were studied, of whom 5 had a diagnosis of ALL-L1 and 3 had a diagnosis of ALL-L2. However, ALL is now more commonly subtyped according to the MIC-M classification (Bain, 1999). Information for each of the patients in this study, relating to the MIC-M classification, was not available and so all patients with ALL were regarded as a single group The number of suitable archival biopsy specimens from patients with ALL was less than for AML. This is because the ALL biopsies were predominantly from children and the amount of biopsy material was smaller, rendering many biopsies unsuitable for study. The age range of ALL patients in this study was 4 to 14 (mean 8.5).

7.3 STROMAL ALTERATIONS ASSOCIATED WITH AML AND ITS TREATMENT

7.3.1 Expression of L-NGFR

Trabecular Margins

In normal trephine bone marrow biopsies, a layer of L-NGFR positive stromal fibroblasts was observed surrounding the majority of bony trabeculae. In pre-treatment biopsies from patients with AML, expression of L-NGFR surrounding trabecular margins was slightly reduced in all subtypes compared with normal controls, although this was more noticeable in subtypes M0, M4 and M5 (Figure 7.1). Following chemotherapy, expression of L-NGFR surrounding trabecular margins varied among the AML subtypes. Biopsies from subtypes M0 and M1, M4 and M5 showed an increase in staining following chemotherapy (Figure 7.1) whereas, in biopsies from subtypes M2 and M3, a decrease was seen. However, as discussed in section 6.2.1, staining surrounding trabecular margins could not be assessed in some biopsy sections due to trabecular disruption resulting from the immunostaining technique. Interestingly, it was observed during analysis of trabecular margin staining

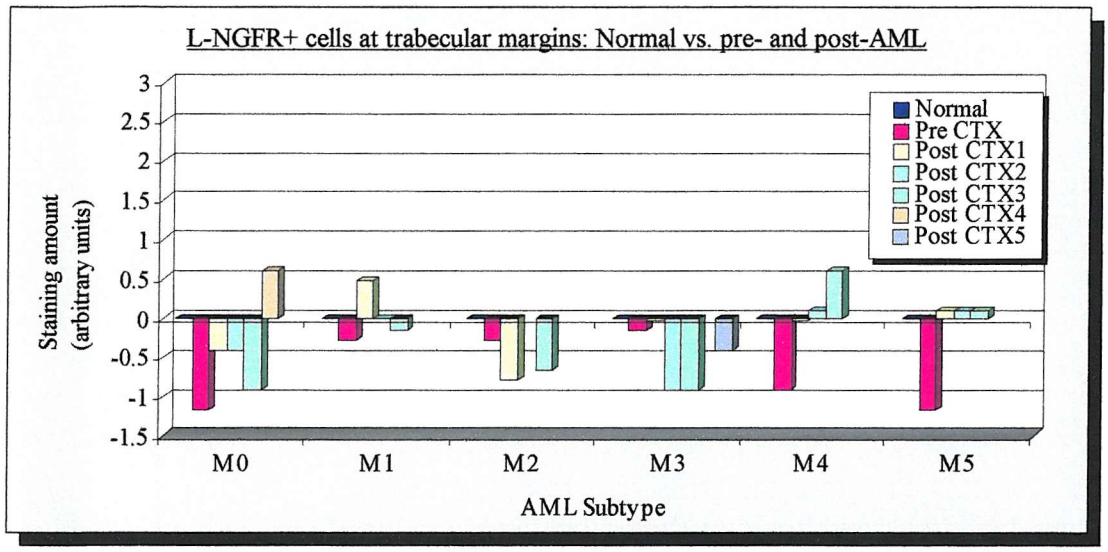
that, from a total of 23 biopsies not assessable due to trabecular disruption, 15 were obtained directly following the patients' second course of chemotherapy.

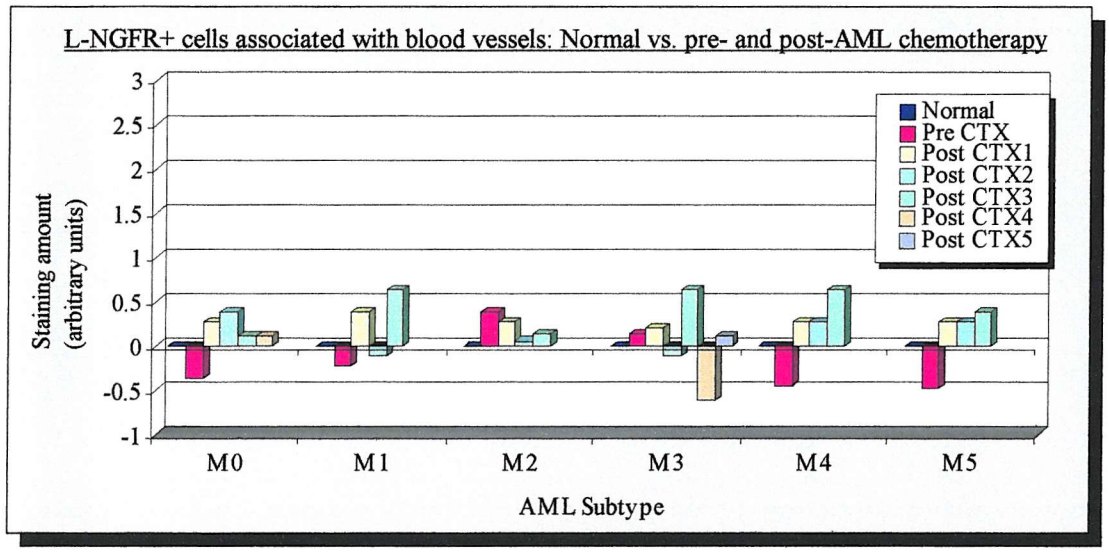
Blood Vessels

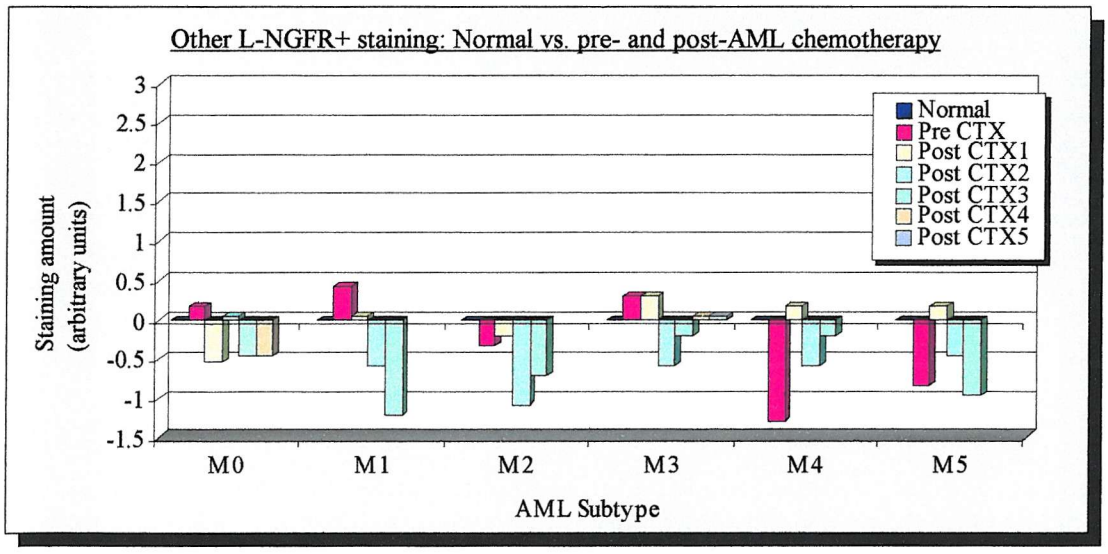
Stromal fibroblasts, expressing L-NGFR, were identified in sections of normal bone marrow surrounding the smooth muscle layer of thick-walled, arterial blood vessels. The number of arterial blood vessels in sections of normal marrow was relatively small. In biopsy specimens from untreated AML patients, the number of blood vessels identified using antibodies reactive with L-NGFR was not considerably different to that observed in normal controls (Figure 7.2). Following chemotherapy, in all AML subtypes except M2, the number of blood vessels with associated L-NGFR positive cells was higher than in bone marrow trephine sections from untreated patients and higher than normal controls. In AML-M2, a slight reduction in the number of blood vessels was observed following treatment although, after 3 courses of treatment, the number of blood vessels remained higher than that observed in normal marrow.

Other L-NGFR positive stromal fibroblasts

In the majority of normal specimens studied, an extensive network of L-NGFR positive stromal cells was distributed throughout the bone marrow stroma (see Figure 6.1A, Chapter 6). The number of L-NGFR positive stromal fibroblasts in biopsy sections from patients with AML M0-M3 was similar to that in normal bone marrow sections (Figure 7.3). However, in subtypes AML-M4 and M5, the amount of L-NGFR staining of stromal fibroblasts was noticeably reduced, with many biopsy specimens containing only a very small number of L-NGFR positive cells. Following treatment for AML, in subtypes M0 to M3, the number of L-NGFR positive stromal fibroblasts was seen to be decreased in nearly all post-chemotherapy biopsy specimens studied. In subtypes AML-M4 and M5, all post-treatment biopsy samples contained an increased number of L-NGFR positive stromal fibroblasts compared with pre-treatment biopsy sections. However, compared with normal biopsies, the number of L-NGFR positive stromal fibroblasts, in all cases, was reduced after multiple courses of chemotherapy (Figure 7.3).







Figures 7.1 (top), 7.2 (middle), 7.3 (bottom). Expression of L-NGFR by stromal fibroblasts surrounding trabecular margins (top), blood vessels (middle) and throughout the bone marrow stroma (bottom) following chemotherapy (CTX) for AML. All values are plotted as a difference from normal values.

7.3.2 Expression of αSMA

Trabecular margins

In normal BMT sections, only occasional trabeculae were incompletely surrounded by αSMA positive stromal fibroblasts. In patients with AML, αSMA expression by fibroblasts surrounding trabeculae was either similar to normal (M2 and M3) or reduced (M0, M1, M4 and M5). Following an initial course of chemotherapy in all cases, irrespective of AML subtype, αSMA expression by cells adjacent to trabeculae was greatly increased (Figure 7.4). Following further courses of chemotherapy, results were more varied as, in all subtypes except M1 and M3, staining adjacent to trabecular margins was increased whereas, in M1 and M3, αSMA expression by cells at this site was decreased. However, even in AML-M1 and M3, there was still more staining than in normal bone marrow.

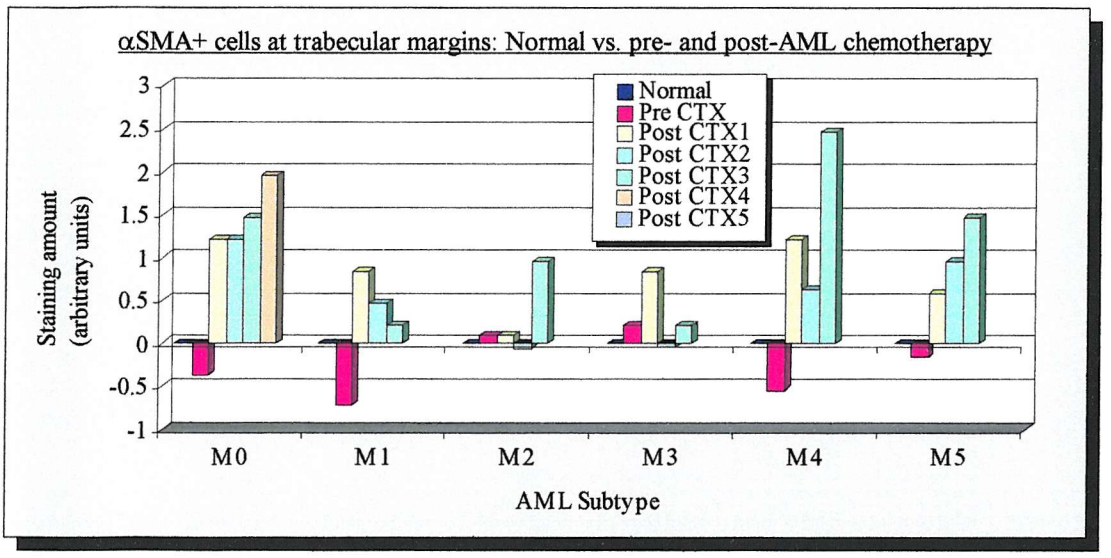
Blood vessels

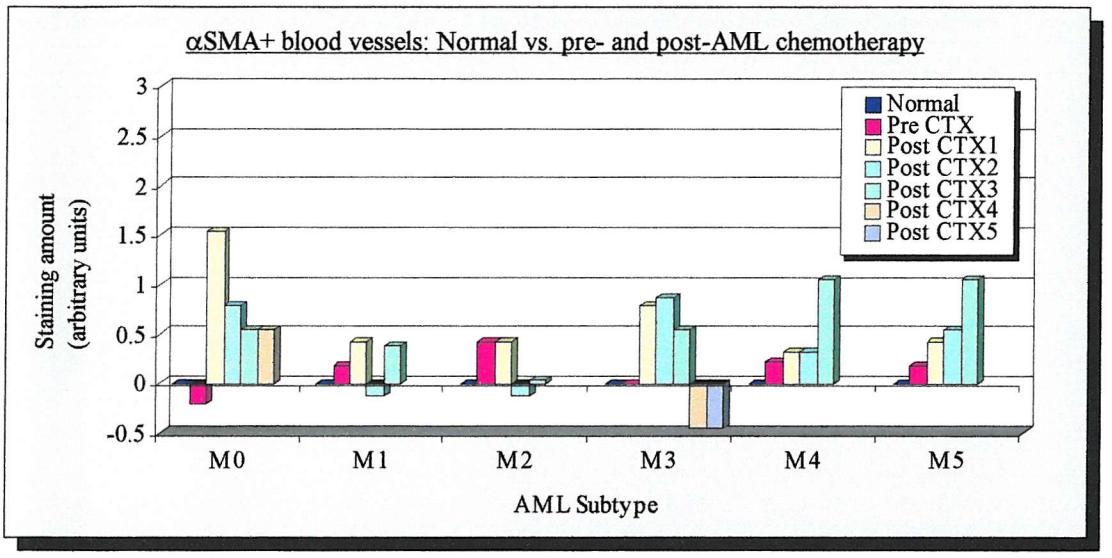
The highest expression of αSMA in normal bone marrow trephine biopsies was identified in the smooth muscle cells of arterial and venous blood vessel walls. Staining of blood vessels by αSMA in AML patients was similar to normal controls although the number of αSMA+ blood vessels was slightly elevated in AML-M2 (Figure 7.5). After the first course of chemotherapy, the number of αSMA+ blood vessels was either the same as pre-treatment (M2 only) or increased. In subtypes M0, M4 and M5, all post-chemotherapy BMT sections showed an increase in αSMA+ vasculature. In subtype M0, this increase was most noticeable after the first course of chemotherapy and declined after subsequent courses whereas, in M4 and M5, this trend was reversed. In subtypes M1 and M2, no trend was observed, as the number of αSMA+ blood vessels fluctuated around the normal levels. In subtype M3, following an initial increase in number of αSMA+ blood vessels after the first course of chemotherapy, the number of αSMA+ blood vessels after 3 courses of chemotherapy was less than normal.

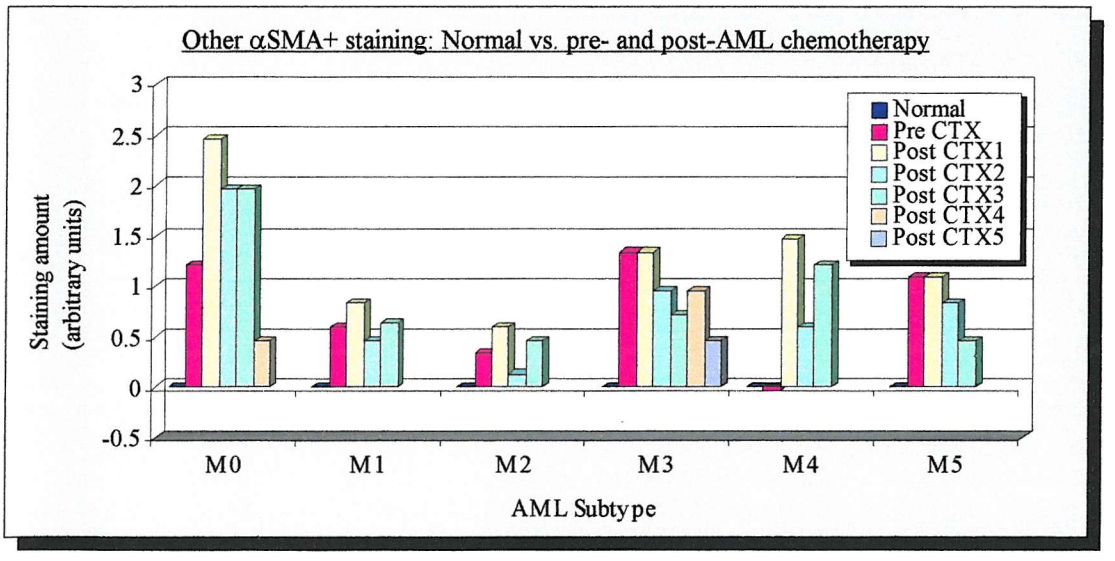
Other αSMA positive stromal fibroblasts

Fibroblasts throughout the bone marrow stroma of normal control BMT sections did not express αSMA (Figure 7.6). In untreated AML patients, αSMA expression in these cells was increased in all subtypes except M4. In the more immature AML

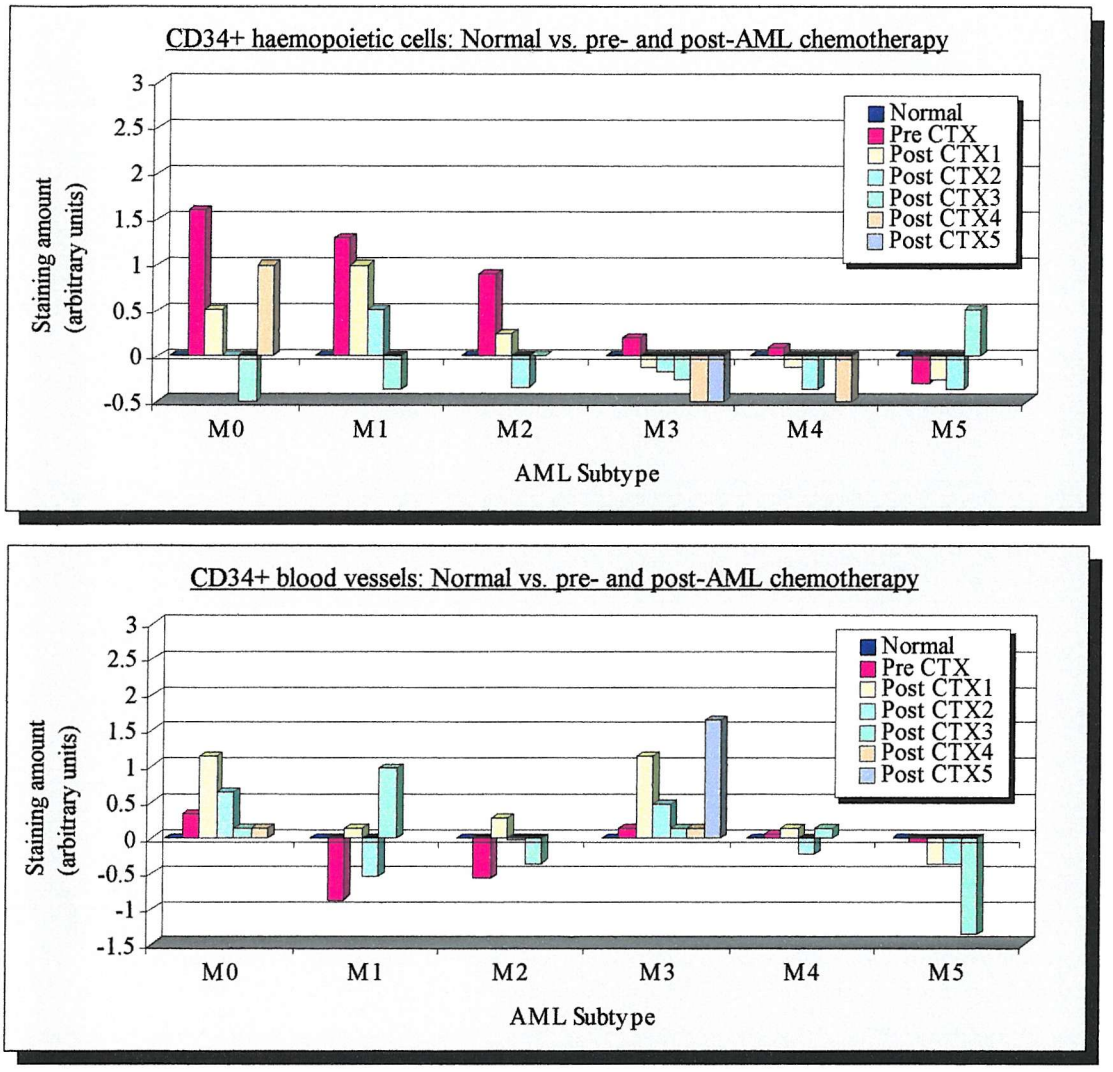
subtypes, AML-M0 to M2, increased αSMA expression was highest in M0 and lowest in M2, although still higher than normal. High numbers of αSMA+ cells were also observed in the bone marrow stroma of patients with AML-M3 and M5. After chemotherapy, the number of αSMA+ cells was either the same as before treatment (M3 and M5) or increased. The most noticeable increase was observed in AML-M4. Subsequent courses of chemotherapy resulted in a progressive decline in stromal αSMA expression towards normal levels. However, in all AML subtypes after multiple courses of chemotherapy, the level of αSMA expression by stromal fibroblasts was increased compared with normal (Figure 7.6).







Figures 7.4 (top), 7.5 (middle), 7.6 (bottom). Expression of αSMA by stromal fibroblasts surrounding trabecular margins (top), blood vessels (middle) and throughout the bone marrow stroma (bottom) following chemotherapy (CTX) for AML. All values are plotted as a difference from normal values.



Figures 7.7 (top), 7.8 (bottom). Expression of CD34 by haemopoietic cells (top) and blood vessels (bottom) following chemotherapy (CTX) for AML. All values are plotted as a difference from normal values.

7.3.3 Expression of CD34

Expression of CD34 in untreated AML patients compared with normal is discussed in detail in section 7.5. Following chemotherapy for AML, in all subtypes except M5, a reduction in the number of CD34 positive haemopoietic cells was observed (Figure 7.7). In subtype AML-M5 a small increase was observed after the first course of chemotherapy. Following multiple course of chemotherapy, in subtypes M1 to M4, the number of CD34 positive haemopoietic cells decreased with each course of chemotherapy. After the third course of chemotherapy, in each of these subtypes, there were fewer CD34 positive haemopoietic cells than in normal marrow. In subtypes M0 and M5, after multiple courses of chemotherapy, the number of haemopoietic cells expressing CD34 was higher than normal.

The number of capillary blood vessels and sinusoids identified using anti-CD34 antibodies post-treatment varied between subtypes (Figure 7.8). In all subtypes except AML-M5, an initial increase was seen after the first course of chemotherapy. After multiple courses of chemotherapy, in subtypes M0, M2, M4 and M5 there was an overall decrease in the number of blood vessels compared with pre-treatment values. However, in subtypes M1 and M3, the number of CD34+ blood vessels had increased.

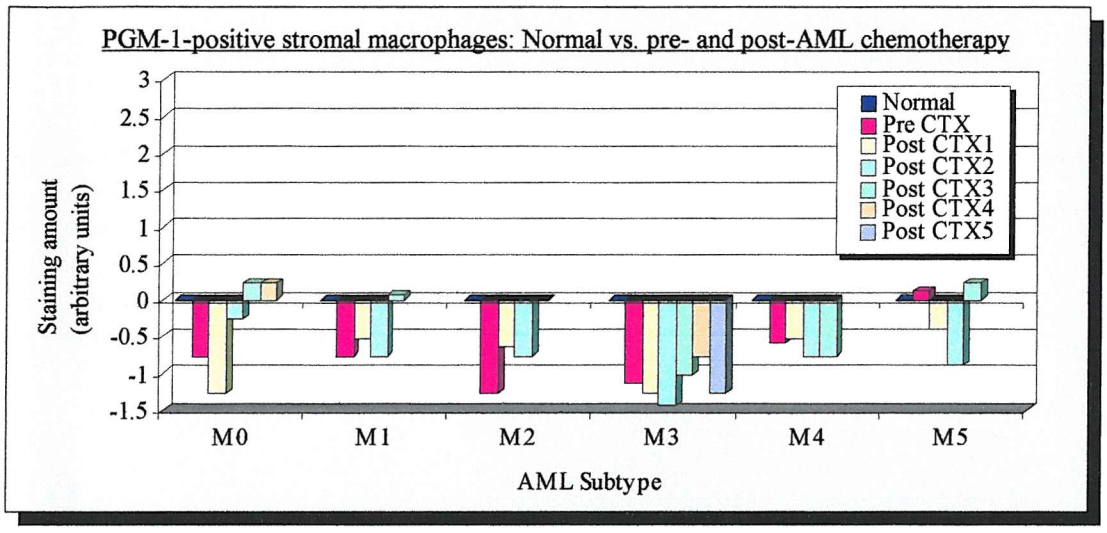


Figure 7.9. Number of CD68 positive stromal macrophages pre- and post- chemotherapy (CTX) for AML. The number of macrophages following chemotherapy is either similar to normal or decreased depending on AML subtype. All values are plotted as a difference from normal values.

7.3.4 Expression of CD68

Macrophages were identified using the monoclonal antibody, PGM-1, which reacts with a monocyte/macrophage-restricted epitope of CD68 (Figure 7.9). In AML subtypes M0-M4, there was a moderate decrease in the number of stromal macrophages compared with normal. In AML-M5, the number of stromal macrophages was slightly elevated. Biopsy sections obtained from patients prior to receiving chemotherapy predominantly contained macrophages that were small with an irregular, dendritic appearance. After the first course of chemotherapy, the macrophage responses among the AML subtypes varied; an initial decrease in macrophage numbers occurred in subtypes M0, M3 and M5 and a slight increase in subtypes M1, M2 and M4. However, after the third course of chemotherapy, in

subtypes M0-M2 and M5, macrophage numbers had returned to normal levels. In the AML-M3 subtype and, to a lesser extent, AML-M4 macrophage numbers continued to be lower than normal after three courses of chemotherapy. In both AML-M3 and M4, macrophage numbers after each course of treatment did not vary considerably. In all cases, regardless of AML subtype, an increase in macrophage size was observed after each course of chemotherapy (Figure 7.10). In addition, an increase in lipid debris was observed in the cytoplasm of these macrophages.

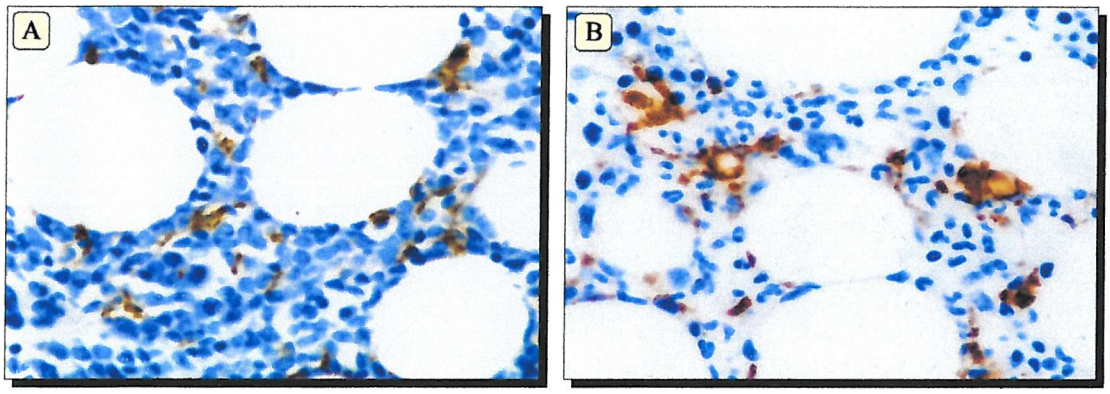


Figure 7.10. Stromal macrophages in patients with AML-M3 before (A) and after (B) treatment. Note the increase in size following treatment and the presence of lipid debris within the cytoplasm of some of these macrophages.

7.3.5 Reactivity of stromal components with monoclonal antibody VS38C

Expression of the rough endoplasmic reticulum-associated antigen (p63) with which VS38C reacts was analysed in plasma cells (Figure 7.12) and fibroblastic stromal cells (Figure 7.13). Prior to chemotherapy, there were a moderate number of plasma cells scattered throughout each biopsy section. After chemotherapy, results varied within AML subtypes and no correlation was observed between AML subtype and plasma cell numbers. However, it was observed that, after chemotherapy, in a large proportion of biopsies from all AML subtypes, there was clustering of osteoblasts around trabecular margins and plasma cells around blood vessels, presumably reactive to marrow injury or cell death, or concurrent infection (Figure 7.11).

The number of VS38C positive stromal fibroblasts prior to chemotherapy was low. After a single course of treatment, in all subtypes except M2, a moderate increase was observed. In all AML subtypes, a further course of chemotherapy resulted in a subsequent decrease in number of VS38C positive stromal cells. The result of further treatment courses varied between subtypes and is summarised in Figure 7.13.

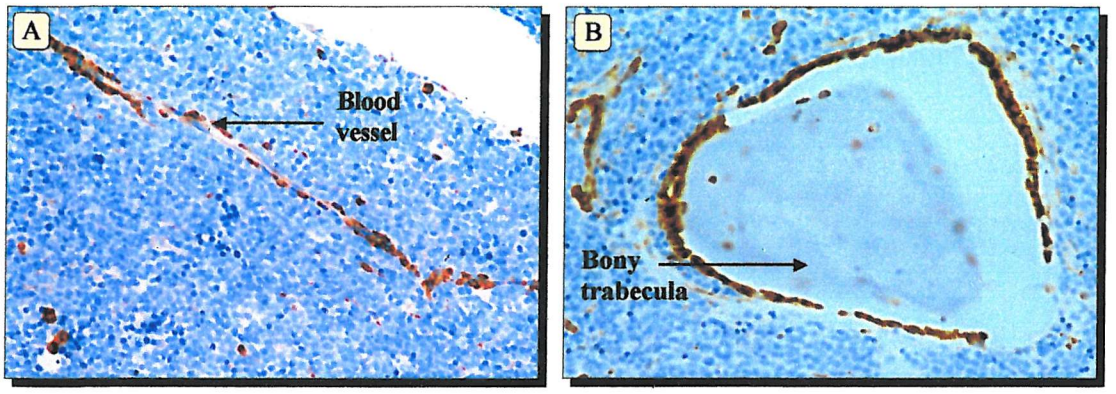
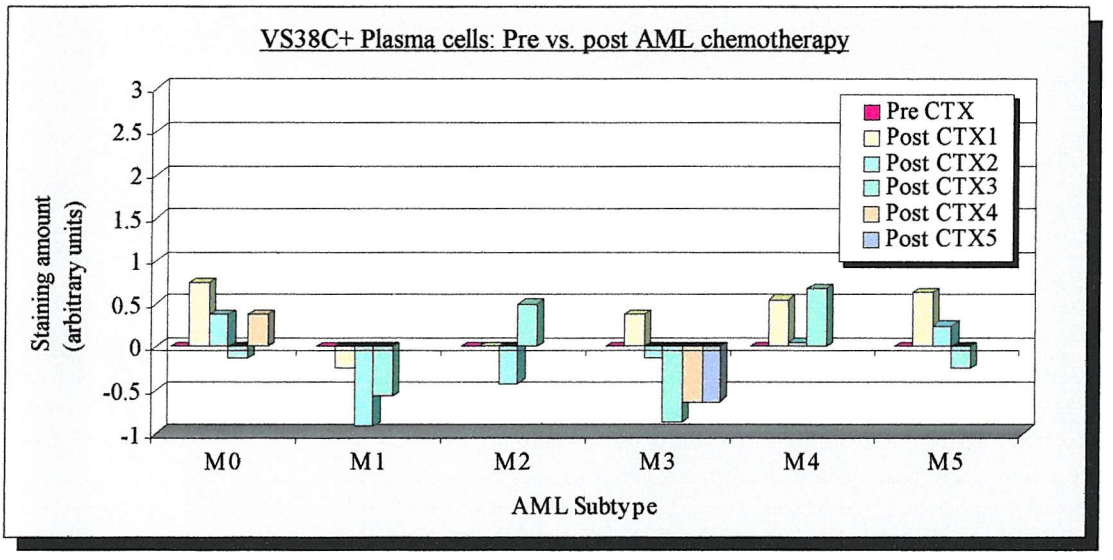


Figure 7.11. Staining of plasma cells (A) and osteoblasts (B) in post-treatment BMT sections. Following chemotherapy for AML, extensive clustering of plasma cells around blood vessels and osteoblasts around trabeculae was observed.



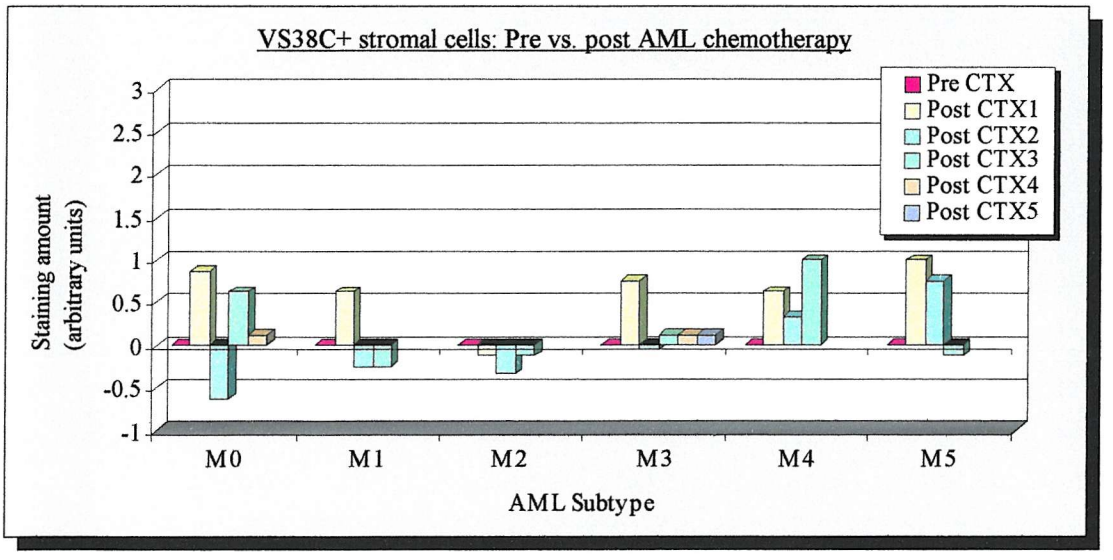
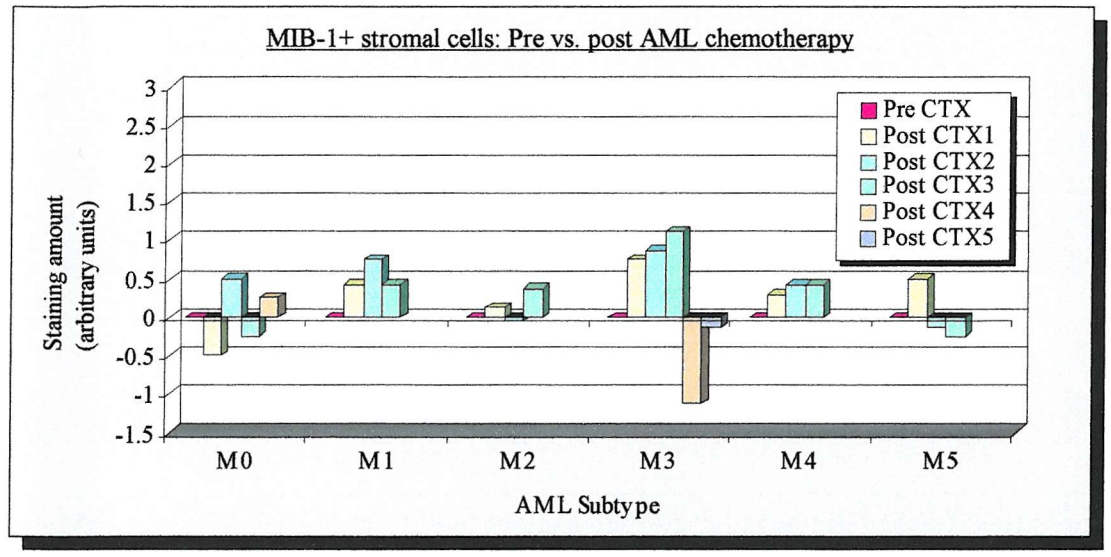


Figure 7.12 (top) and 7.13 (bottom). Expression of VS38C by plasma cells (top) and fibroblastic stromal cells (bottom) in bone marrow trephine biopsy sections obtained pre-and post-chemotherapy (CTX) for AML. All values are plotted as a difference from pre-chemotherapy values.

7.3.6 Expression of Ki67 antigen

The monoclonal antibody MIB-1 was used to detect proliferating haemopoietic and stromal cells expressing the Ki67 antigen. In many biopsy sections, the number of proliferating haemopoietic cells was high relative to proliferating stromal cells. As a result, it was technically difficult to quantify the relative proportion of proliferating stromal cells present in these biopsies. Despite this difficulty, it was observed subjectively that, after chemotherapy, there was an initial increase in the number of proliferating stromal cells in BMT sections from patients of all AML subtypes except M1. After further courses of chemotherapy, there was a tendency for the number of proliferating stromal cells to increase, although this was not always the case (Figure 7.14). In most biopsy sections, the number of proliferating haemopoietic cells prior to chemotherapy was relatively high. After the first course of chemotherapy, a further increase in the number of proliferating haemopoietic cells occurred in all subtypes except AML-M0. In subtypes M0-M2 and M4, after subsequent chemotherapy courses, the number of proliferating haemopoietic cells was still slightly elevated compared with pre-treatment levels (Figure 7.15). In subtypes AML-M3 and M5, sections from the last available post-treatment biopsy specimen contained a slightly decreased number of proliferating cells compared with pre-treatment biopsy sections.



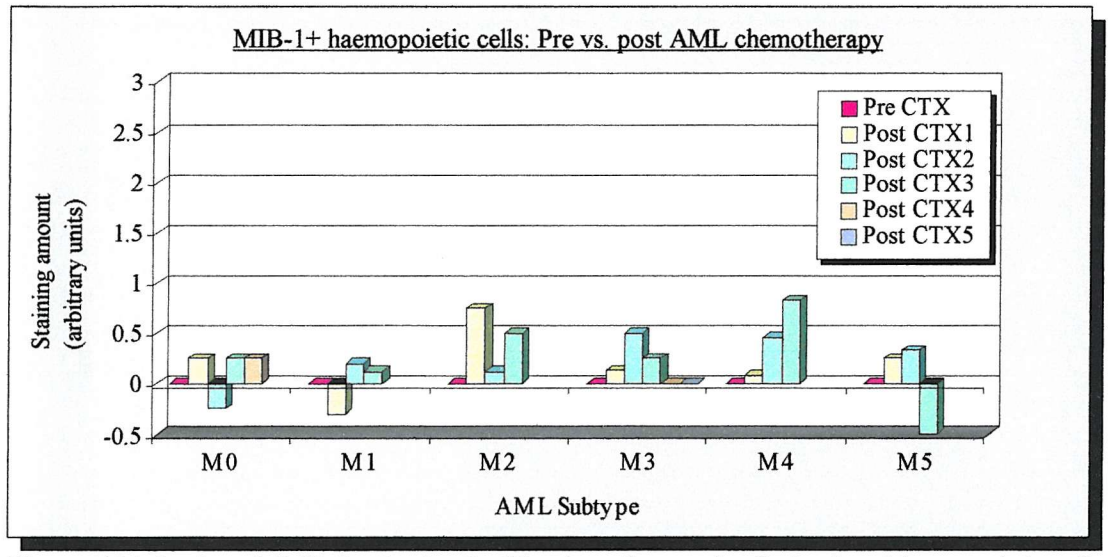


Figure 7.14 (top) and 7.15 (bottom). Proliferation of stromal cells (top) and haemopoietic cells (bottom) before and after chemotherapy (CTX) for AML. All values are plotted as a difference from pre-chemotherapy values.

7.4 Stromal Alterations Associated with ALL and its Treatment

7.4.1 Expression of L-NGFR by stromal fibroblasts

Trabecular margins

Due to the fragile nature of BMT specimens obtained from the young ALL patients used in this study, disruption of the trabeculae occurred in many biopsies. As a result, antigen expression by cells at trabecular margins was not assessed in these patients.

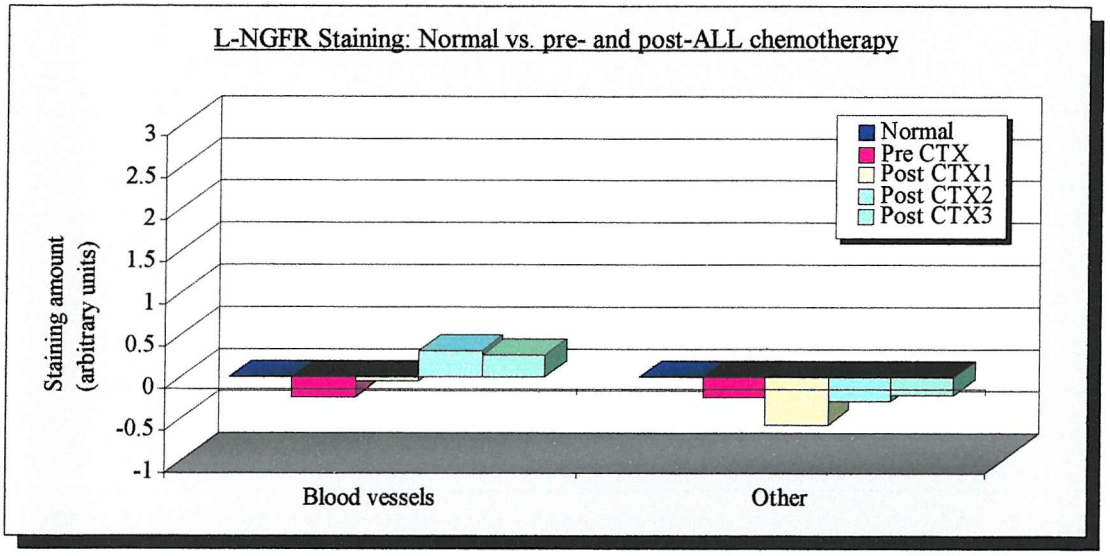


Figure 7.16. Expression of L-NGFR by stromal fibroblasts in bone marrow trephine biopsy sections obtained pre- and post-chemotherapy (CTX) for childhood ALL. Staining was assessed surrounding the outer layer of blood vessels (left) and the stromal fibroblasts found throughout the haemopoietic compartment (right). All values are plotted as a difference from normal values.

Blood vessels

The number of blood vessels surrounded by L-NGFR positive stromal fibroblasts in trephine biopsy sections from patients with ALL was small, with very few or no L-NGFR positive cells in this distribution observed in some biopsies. Overall, there was slightly less L-NGFR expression than seen in normal control sections obtained from adult patients. After the first two courses of chemotherapy, the number of L-NGFR positive cells adjacent to blood vessels had increased slightly (Figure 7.16). After the final course of chemotherapy, the number of L-NGFR+ cells associated with blood vessels was similar to after the second course of chemotherapy and was increased compared with normal control biopsy sections.

Other L-NGFR positive stromal fibroblasts

The number of L-NGFR positive stromal fibroblasts scattered throughout the bone marrow stroma in untreated children with ALL was slightly lower than that in normal BMT sections (Figure 7.16). Following chemotherapy, the number of stromal fibroblasts expressing L-NGFR decreased further. With subsequent courses of chemotherapy, however, the number of L-NGFR positive stromal fibroblasts increased, approaching the levels seen in normal adult marrow.

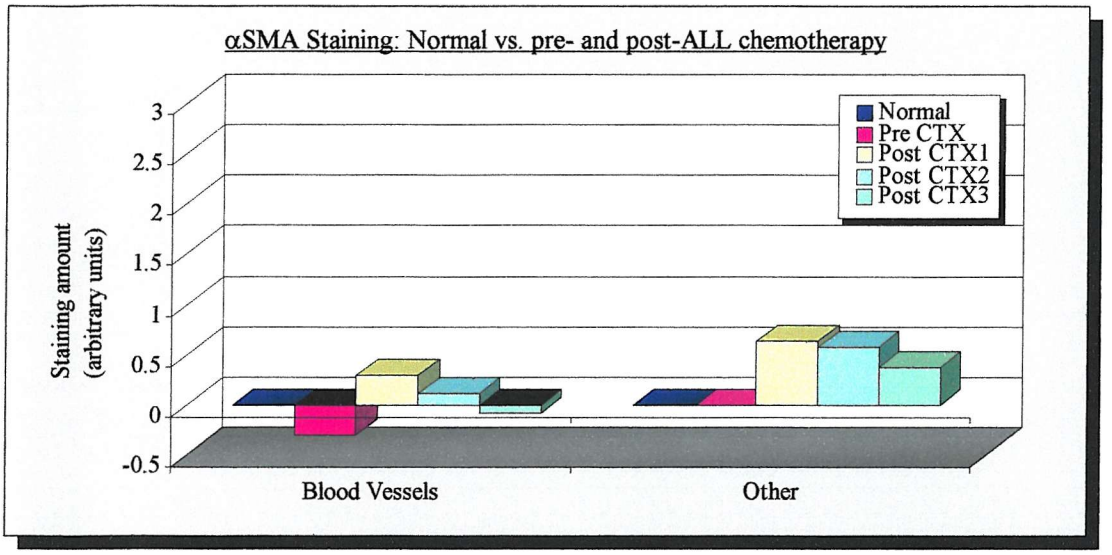


Figure 7.17. Expression of αSMA in bone marrow trephine biopsy sections from patients pre- and post-chemotherapy (CTX) for childhood ALL. Staining was assessed in blood vessels (left) and throughout the haemopoietic compartment (right). All values are plotted as a difference from normal values.

7.4.2 Expression of αSMA by stromal fibroblasts

Blood vessels

All arterial blood vessels contained a layer of αSMA positive smooth muscle cells. The number of these αSMA positive blood vessels was lower in BMT sections from patients with ALL compared with normal controls (Figure 7.17). After an initial course of chemotherapy, the number of αSMA positive blood vessels had increased and was greater than normal. These blood vessels were generally larger than those present in pre-chemotherapy biopsy sections. However, successive courses of chemotherapy resulted in a progressive decrease in the number of αSMA positive blood vessels towards normal levels.

Other αSMA positive stromal fibroblasts

No αSMA positive stromal fibroblasts were seen in biopsy sections obtained from normal patients or patients with untreated childhood ALL. This contrasts with untreated patients with AML, in whose BMT sections a moderate number of αSMA positive stromal fibroblasts were present (Figure 7.17). After chemotherapy, a small number of αSMA positive fibroblasts were observed in some biopsy sections. However, in no case was an extensive network of αSMA positive cells seen

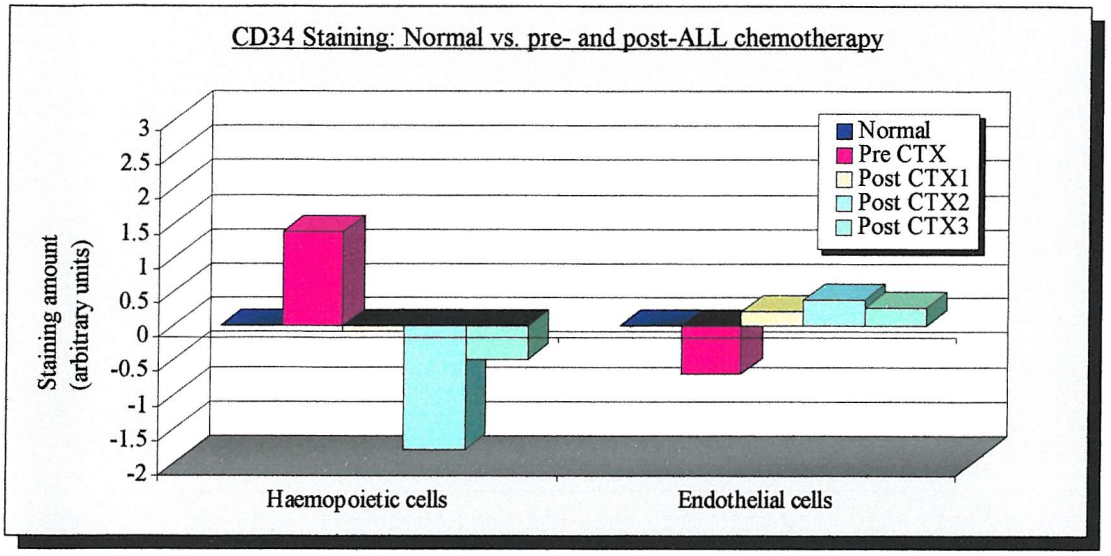


Figure 7.18. Expression of CD34 by haemopoietic cells and endothelial cells in trephine bone marrow biopsy sections from patients pre- and post-chemotherapy (CTX) for childhood ALL. All values are plotted as a difference from normal values.

resembling the network of L-NGFR positive cells that is seen in normal biopsy sections. After three courses of chemotherapy, the number of αSMA positive stromal fibroblasts had decreased in these patients, although there were still a small number in whom sections continued to show a minor population of αSMA positive fibroblasts.

7.4.3 Expression of CD34 by haemopoietic cells, blood vessels and sinusoids

Expression of CD34 by haemopoietic cells in patients treated for ALL was similar to patients with myeloblastic AML. Trephine bone marrow biopsy specimens obtained prior to treatment contained high numbers of CD34 positive haemopoietic cells compared with normal controls (Figure 7.18). This decreased to below normal after the first 2 courses of chemotherapy. After the third course of treatment, the number of CD34 positive haemopoietic cells increased again, approaching the number seen in normal control biopsies. The number of blood vessels in BMT sections from patients with untreated ALL was slightly reduced compared with normal. However, due to predominance of CD34 positive haemopoietic cells, observation of CD34 positive blood vessels was sometimes difficult. Following three courses of chemotherapy, the number of blood vessels observed was slightly greater than normal.

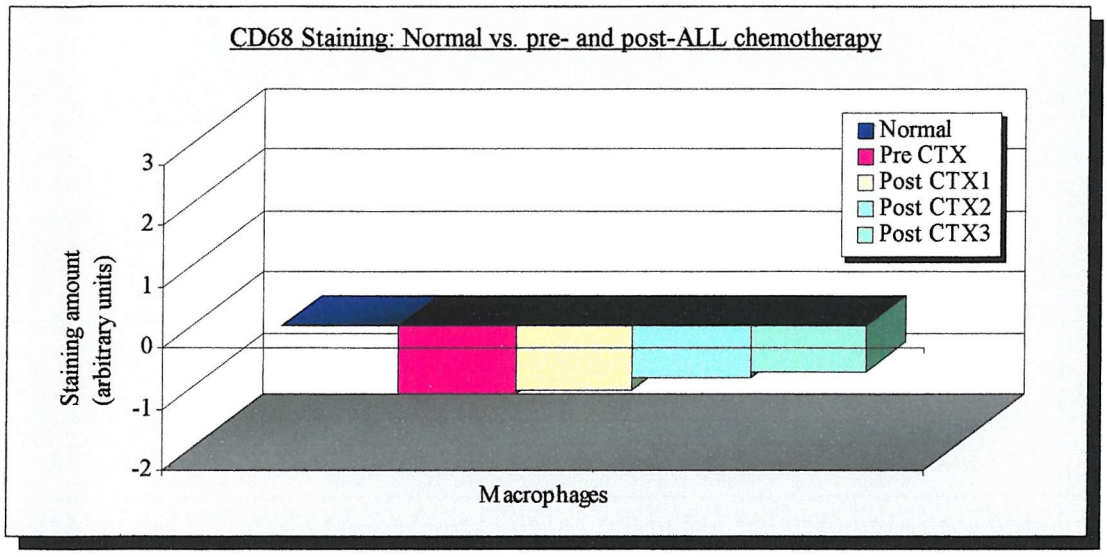


Figure 7.19. Number of CD68 positive stromal macrophages pre- and post-chemotherapy (CTX) for AML. The number of stromal macrophages following chemotherapy was either similar to normal or decreased depending on AML subtype. All values are plotted as a difference from normal values.

7.4.4 Expression of CD68

The number of stromal macrophages in biopsy sections from patients with ALL was similar to that observed in patients with AML. Prior to receiving chemotherapy, the number of CD68-positive stromal macrophages, identified using the PGM-1 antibody, was noticeably reduced compared with normal (Figure 7.19). These macrophages were characteristically small and elongated. After each course of chemotherapy, the number of macrophages increased, as did the size and the proportion of macrophages with round morphology. However, after the third course, the number of macrophages was still considerably lower than that observed in normal biopsy sections.

7.4.5 Reactivity with VS38C

After a single course of chemotherapy, the number of plasma cells identified using VS38C was increased in patients with ALL compared with biopsy sections from untreated patients. These plasma cells were scattered throughout the biopsy sections, similar to those observed in patients with AML. With each course of chemotherapy, the number of plasma cells increased gradually (Figure 7.20). Interestingly, as with chemotherapy for AML, each course of treatment resulted in an increased clustering of plasma cells around blood vessels and osteoblasts around trabecular margins.

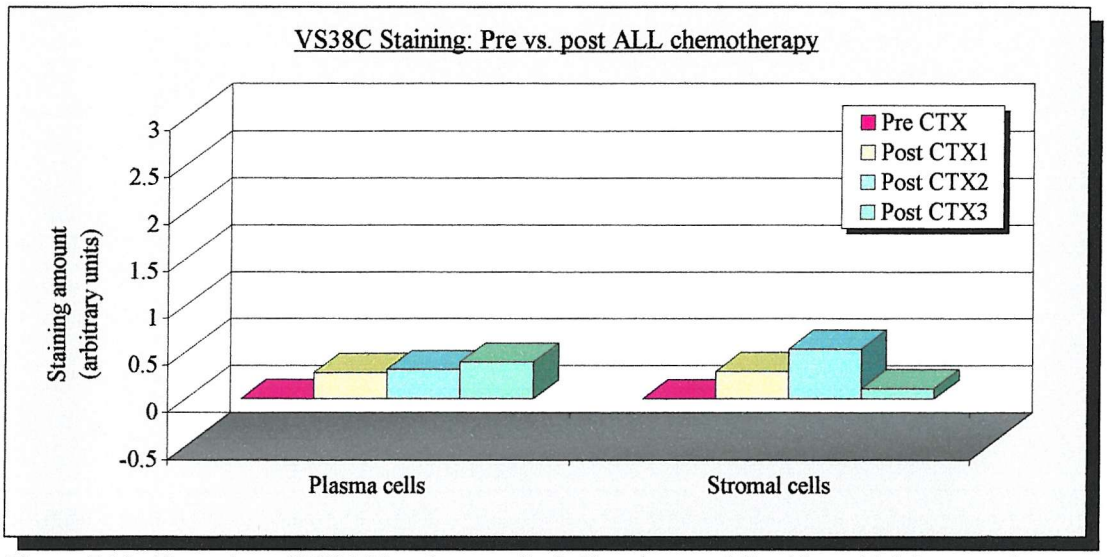


Figure 7.20. Expresion of VS38C by plasma cells (left) and stromal fibroblasts (right) pre- and post-chemotherapy (CTX) for AML. All values are plotted as a difference from pre-chemotherapy values.

Staining of stromal fibroblasts with VS38C antibodies was also increased in BMT sections from patients who had received treatment for ALL. After the first two courses of chemotherapy, a gradual increase in VS38C-positive stromal fibroblasts was observed. After the third course, however, the number of VS38C-positive stromal fibroblasts was reduced to about the same as that seen prior to treatment (Figure 7.20).

7.4.6 Expression of Ki67 antigen

Chemotherapy for ALL resulted in an initial decrease in the number of proliferating haemopoietic cells compared with pre-treatment ALL trephine bone marrow biopsy sections. Further courses of chemotherapy resulted in a gradual increase in the number of proliferating cells, towards that seen in untreated ALL (Figure 7.21). The same technical difficulties were encountered when assessing the number of proliferating stromal cells in BMT sections from patients with ALL as were encountered with AML sections described in section 7.3.6. Despite these difficulties, it was noticed that, after the first course of treatment, the number of proliferating stromal cells had increased compared with pre-treatment samples. After a second and third course of treatment, the number of proliferating stromal cells decreased to below that seen in biopsies obtained prior to treatment.

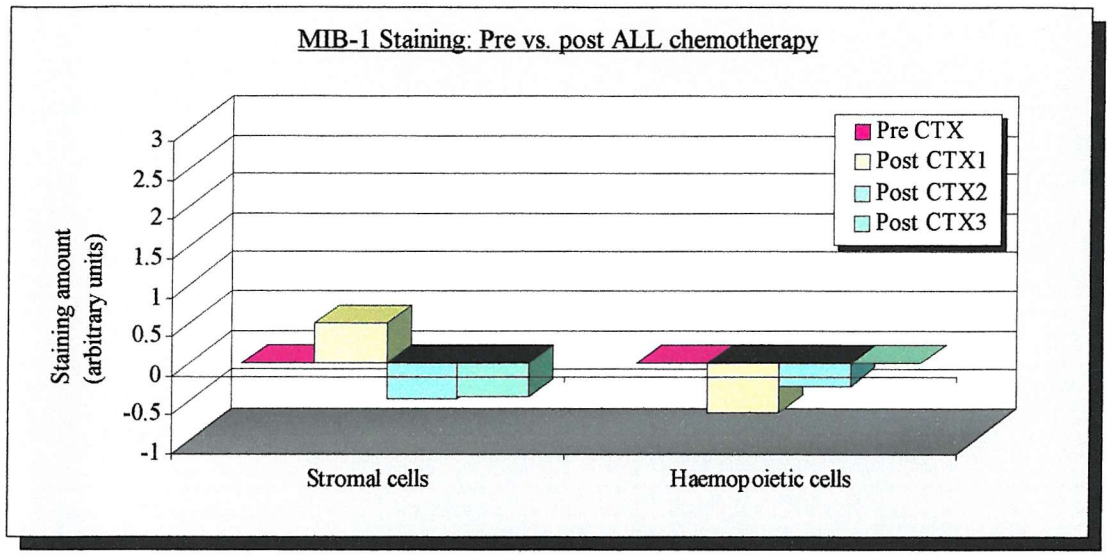


Figure 7.21. Relative numbers of proliferating stromal and haemopoietic cells pre- and post-chemotherapy (CTX) for AML. Proliferating cells were identified using MIB-1 antibodies. All values are plotted as a difference from pre-chemotherapy values.

7.5 Expression of CD34 in AML Subtypes

Initial observations while investigating the effects of leukaemia therapy on bone marrow stroma identified the presence of a possible correlation between CD34 expression by leukaemia blast cells and AML subtypes. For this reason, this was investigated further to quantify whether a statistically significant correlation exists. In a number of biopsies, small clusters of CD34 negative erythroid precursor cells were identified. These were also investigated to determine whether any correlation exists between the presence of these erythroid clusters and AML subtype. It was also investigated whether any correlation exists between CD34 positive endothelium and AML subtype.

7.5.1 Patient samples used to investigate CD34 expression in AML subtypes

Trephine biopsy specimens were obtained from 48 patients (including the 24 described in section 7.2.1) with AML to compare CD34 expression by haemopoietic and endothelial cells between AML subtypes. None of these patients had received prior chemotherapy and none represented previous disease relapse. The number of biopsies from each AML subtype, mean patient age and age range are shown in Table 7.1.

Table 7.1. Patient details in study of CD34 expression in AML subtypes

Morphologic Diagnosis		Patient Details		
		Number of Patients	Age Range (Years)	Mean Age (Years)
Undifferentiated	AUL	3	30-64	47.0
Myeloblastic	M0	5	12-75	57.8
	M1	10	16-79	54.9
	M2	10	15-54	51.7
Promyelocytic	M3	6	42-59	50.0
Myelomonocytic/ Monocytic	M4	9	32-78	58.8
	M5	5	64-67	65.5
TOTAL		48	12-79	55.1

7.5.2 Expression of CD34 leukaemic blast cells

Leukaemic blast cells positive for CD34 were present in all trephine biopsy specimens from patients with AUL and patients diagnosed with AML subtypes M0 to M3. Seven out of eight cases of AML-M4 and 3/5 cases of AML-M5 also contained a proportion of CD34 positive leukaemic blast cells. For statistical analyses, AML subtypes were grouped according to their morphologic diagnosis and categorised as either myeloblastic (subtypes M0, M1 and M2), promyelocytic (M3) or monocytic (M4 and M5). The proportions of CD34 positive leukaemic blast cells from each AML subtype are summarised in Table 7.2 and Figure 7.22. With the exception of AUL, the number of CD34 positive leukaemic blast cells decreased throughout the AML subtypes, from M0 to M5. The number of CD34 positive leukaemic blast cells from patients with AUL was similar to that observed from patients with AML-M2.

Table 7.2. Morphological comparison of CD34 expression in AML subtypes

Morphologic Diagnosis		Haemopoietic cells (0-3)*	Endothelial cells (0-3)*	No. of BMT containing erythroid clusters
Undifferentiated	AUL	2.0	2.0	2/3
Myeloblastic	M0	2.6	1.7	4/5
	M1	2.3	0.5	6/10
	M2	1.9	0.8	4/10
Promyelocytic	M3	1.2	1.5	4/6
Myelomonocytic/ Monocytic	M4	1.1	1.4	4/8
	M5	0.7	1.3	0/5

* The number of positive cells was estimated on a scoring system of 0-3 with increments of 0.5. Values shown represent the mean for each patient group.

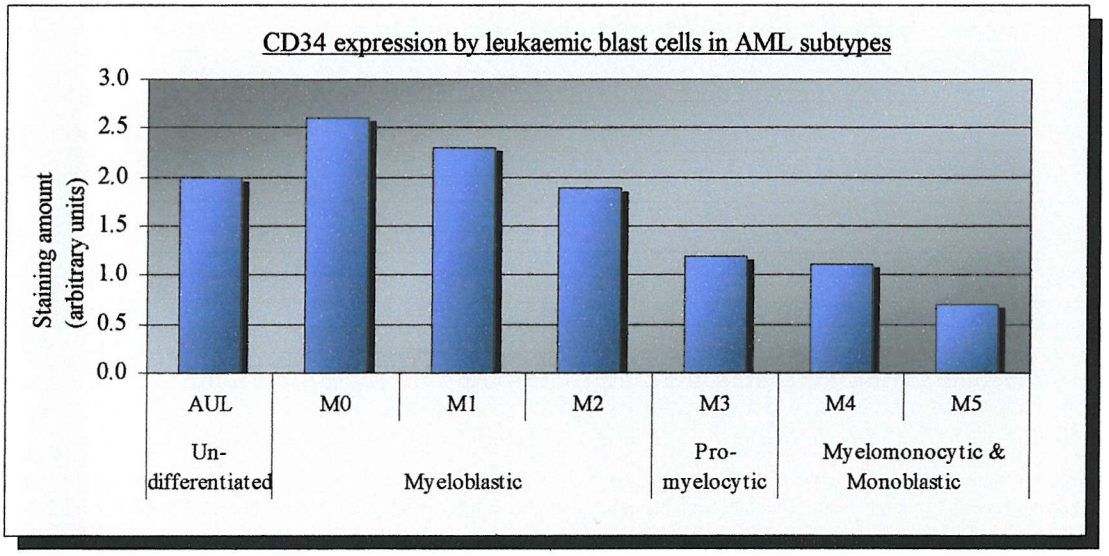


Figure 7.22. Expression of CD34 by leukaemic blast cells in bone marrow trephine biopsies among AML subtypes from untreated patients.

The average proportion of CD34 positive leukaemic blast cells in BMT sections from patients with myeloblastic AML was 76%. This was significantly different from BMT from patients with promyelocytic AML subtypes, which contained a mean of 40% (p=0.02). The difference in CD34 expression by leukaemic cells between myeloblastic and monoblastic AML subtypes (mean 30%) was highly significant (p=0.001). Photographs representative of CD34 expression among AML subtypes are shown in Figure 7.23.

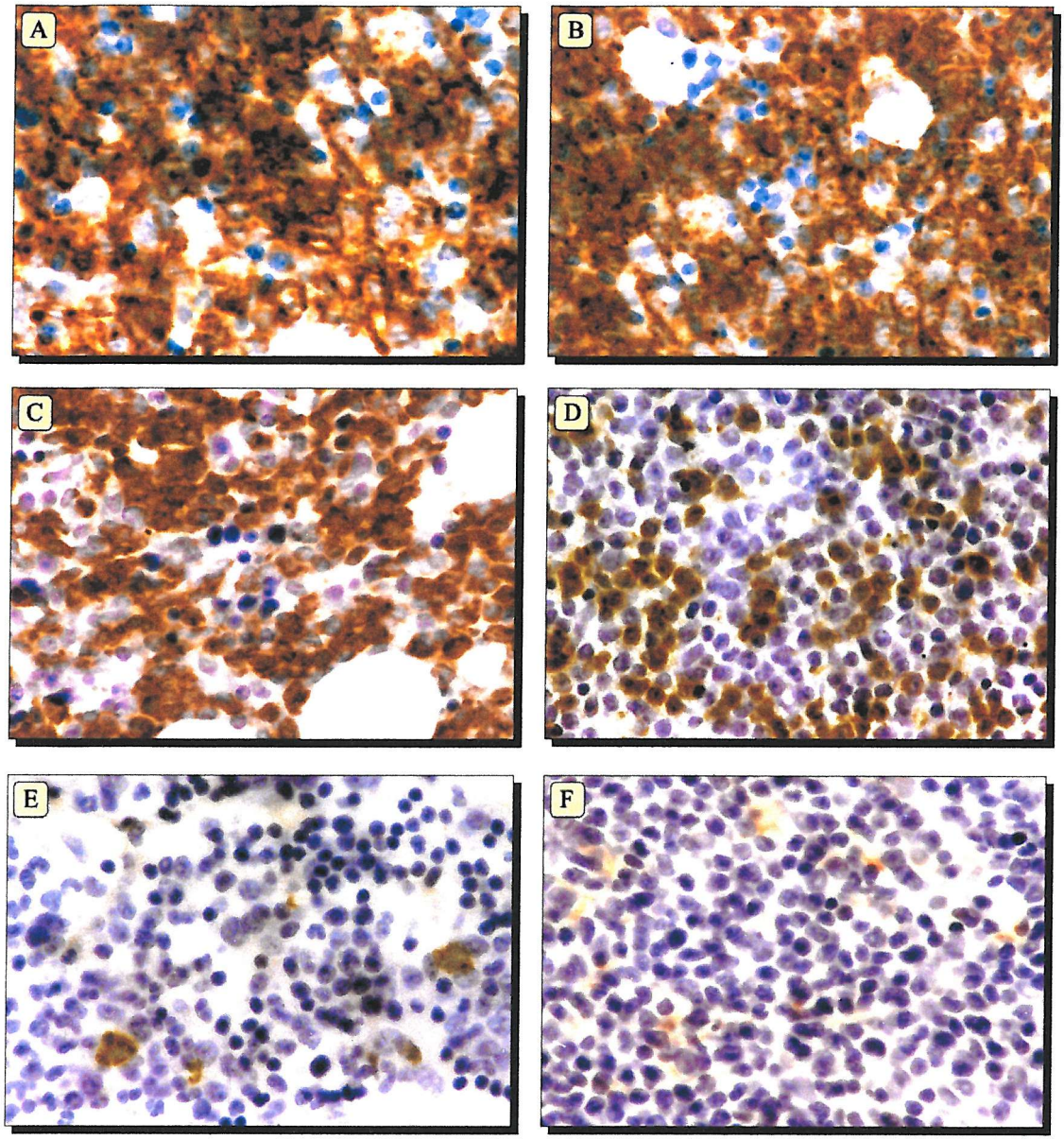


Figure 7.23. Expression of CD34 by leukaemic blast cells in acute myeloid leukaemia subtypes. A=AML-M0; B=AML-M1; C=AML-M2; D=AML-M3; E=AML-M4; F=AML-M5. Expression of CD34 by leukaemic blast cells in AML was found to decrease progressively from AML-M0 through to AML-M5.

7.5.3 Presence of erythroid clusters in AML subtypes

In a number of the biopsy specimens from patients with AML, small clusters of CD34 negative erythroid precursor cells were present among the haemopoietic cell population. Biopsies used to assess CD34 staining were re-assessed to investigate whether any correlation might exist between the presence or absence of erythroid precursors and AML subtype. The number of biopsies that contained erythroid clusters in each AML subtype was converted to a percentage and is shown in Table 7.2

(section 7.5.2) and Figure 7.24. The presence of erythroid clusters in AML subtype appears to parallel CD34 expression, with more biopsy specimens containing erythroid clusters in subtypes containing higher numbers of CD34 positive haemopoietic cells. As for haemopoietic cell CD34 expression, a trend was seen associating the relative immaturity of AML subtypes and the presence of erythroid clusters, as the proportion of biopsies containing erythroid clusters from subtypes AML-M0 to M3 and from AML-M4 to M5 decreased. It is interesting to note that erythroid clusters were not detected in any of the 5 biopsies obtained from patients with AML-M5.

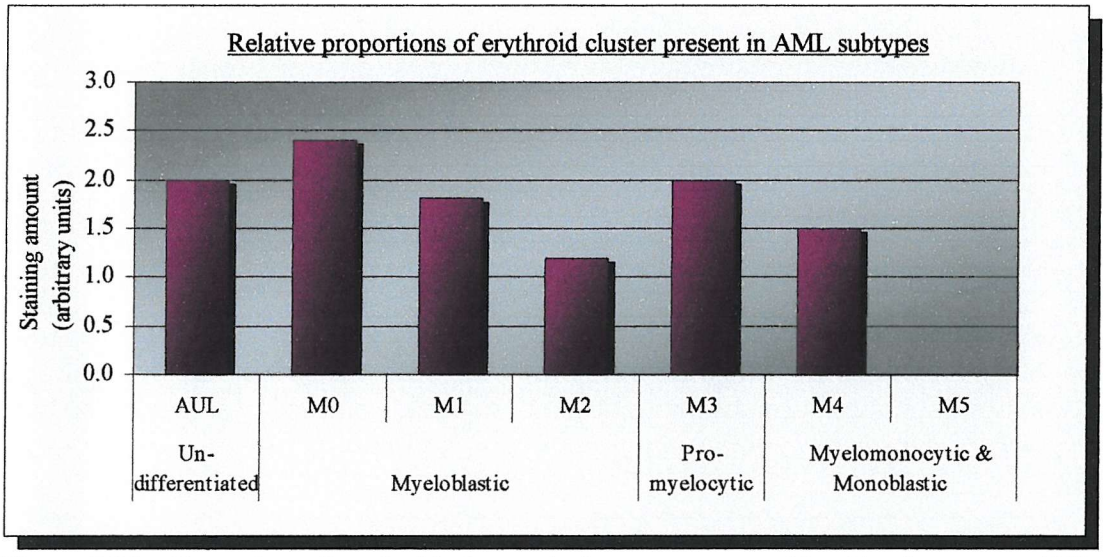


Figure 7.24. Proportion of erythroid clusters in bone marrow trephine biopsies among AML subtypes from untreated patients.

7.5.4 Expression of CD34 in endothelial cells

Antibodies reactive with CD34 were used to identify endothelial cells lining arterial blood vessels and venous sinusoids in AML subtypes. The aim was to determine whether any correlation exists between number of blood vessels and AML subtype (Table 7.2, section 7.5.2). In some biopsies, however, quantification of blood vessels stained with CD34 antibodies was difficult due to overpopulation of the marrow with CD34 positive leukaemic blast cells. Biopsies from patients with AUL and AML-M0 had relatively large numbers of blood vessels, the majority of which were thin-walled sinusoids (Figure 7.25). In contrast, the myeloblastic subtypes (AML-M1 and AML-M2) had a smaller number of blood vessels present (22%) compared with biopsies

from promyelocytic leukaemia marrow sections (67%; p=0.01) and sections representing AML-M4 and M5 (45%; p=0.03).

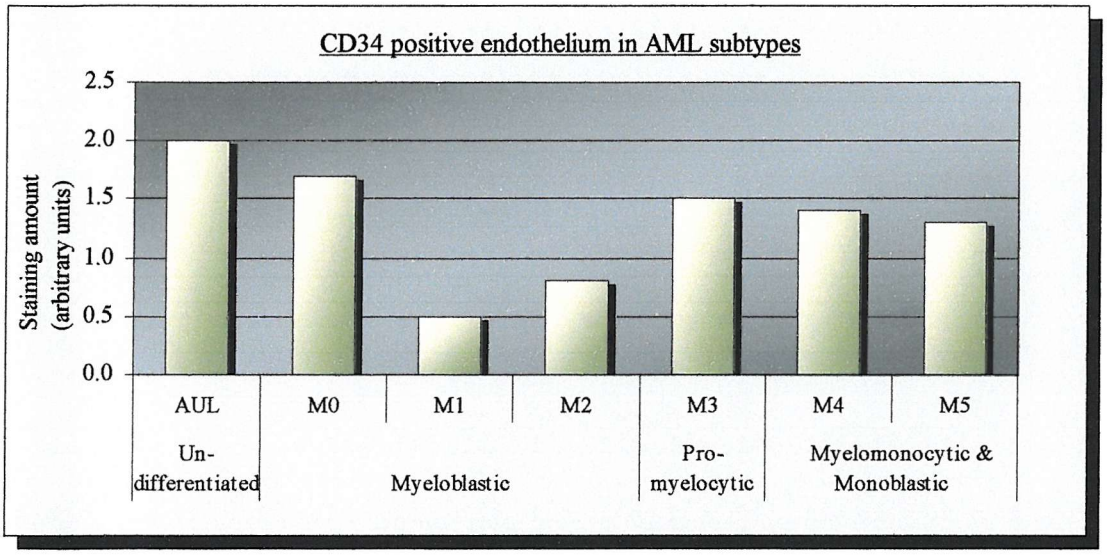


Figure 7.25. Amount of CD34 positive endothelium in bone marrow trephine biopsies among AML subtypes from untreated patients.

7.6 DISCUSSION OF RESULTS

Treatment of acute leukaemias with current chemotherapeutic regimes results in the depression of haemopoiesis and variations in haemopoietic regrowth between patients (Wilkins *et al.*, 1993). Bone marrow stroma is vital for the support and regulation of haemopoiesis (Eaves *et al.*, 1991b; Singer *et al.*, 1985), although the causes of variations in haemopoietic regrowth remain largely unknown. The aim of the analyses reported in this chapter was to investigate cellular changes occurring in the bone marrow stroma of patients with acute myeloid (AML) and acute lymphoid (ALL) leukaemia and to identify stromal cell abnormalities associated with the chemotherapeutic treatment of AML and ALL. Evaluation of the immunocytochemical staining of biopsies used in this chapter was carried out semi-quantitatively as described in section 2.14.6. Although this method of evaluation does not permit the identification of small changes in staining (i.e. <1.0 arbitrary unit) changes in staining greater than this can confidently be treated as true differences. This limitation was taken into account when drawing conclusions from these data.

The FAB classification for acute leukaemias, used in this study, subtypes AML according to the degree of differentiation along myeloid cell lineages and the stage of maturation within that lineage. Subtype AML-M0-M2 represent myeloblastic AML with increasing stages of granulocytic differentiation, AML-M3 represents promyelocytic differentiation and AML-M4 and M5 represent AML with increasing degree of monocytic differentiation.

The study of CD34 expression by leukaemic blast cells and its association with AML subtype revealed a significant correlation between AML subtypes and expression of CD34. This is as expected since CD34 has been shown to be highly expressed on primitive haemopoietic cells with a progressive decrease as the cells differentiate (Civin *et al.*, 1987). Patients with myeloblastic AML subtypes, AML-M0 to M2, characterised by the presence of primitive leukaemic blast cells with varying degrees of granulocytic differentiation, demonstrated very high numbers of CD34 positive haemopoietic cells. Highest numbers were observed in AML-M0 with a progressive decrease to AML-M2 due to the increase in maturity of blast cells from AML-M0 to M2. The number of CD34 positive leukaemic cells in myeloblastic forms of AML was significantly higher than in either promyelocytic (M3) and monoblastic/monocytic (M4 and M5) subtypes. The M5 subtype showed no increase in the proportion of CD34 positive haemopoietic cells relative to that which would be expected in normal bone marrow. After an initial course of chemotherapy, the decrease in number of CD34 positive cells present was greatest in AML-M0 and progressively declined through to M5. In all AML subtypes, the decrease in number of CD34 positive haemopoietic cells was disproportionate to the overall decrease in cellularity that occurred following initial chemotherapy.

The small number of BMT biopsies from patients with AML-M5 did not enable subtypes AML-M5a and M5b to be studied separately. From the FAB classification described in section 1.7 (Chapter 1), it would be appropriate to speculate that M5a would contain a higher number of CD34 positive cells than M5b, since M5a contains predominantly monoblasts and M5b contains predominantly promonocytes and monocytes. Of the four AML-M5 biopsies studied, the number of CD34 positive leukaemic cells was low in 3 patients (less than 1 on a scale of 0-3) and high in 1 patient (2.5 on a scale of 0-3). It could be considered that, of the M5 cases, 3 were

consistent with M5b and one was consistent with M5a. Unfortunately, this could not be confirmed since in Southampton University Hospitals NHS Trust, M5a and M5b are not routinely distinguished and attempts to trace aspirate films for retrospective sub-classification were unsuccessful.

An unexpected finding was the observation that, in parallel with a higher proportion of CD34 positive cells, earlier, more-primitive AML subtypes also showed a greater number of CD34 negative erythroid cell clusters. Like CD34 expression, as maturity of the blast cells increases through the AML subtypes, the number of erythroid clusters present decreased from M1 to M5; essentially no erythroid clusters were observed in monoblastic/monocytic AML. The reasons for the presence of erythroid clusters are not known. They may presumably represent small areas throughout the marrow stroma, which provide an environment in which non-neoplastic progenitor cells, committed to erythropoiesis, can survive and differentiate.

Investigation of CD34 positive endothelium throughout the biopsies of patients with AML and ALL was hindered by the overpopulation of the marrow with CD34 positive haemopoietic cells. Despite this, in untreated marrow biopsies, significant differences in vasculature were observed between myeloblastic forms of AML (M1 and M2) and promyelocytic and monoblastic forms of AML. However, the results obtained in biopsies obtained after chemotherapy were too varied to draw any conclusions.

Investigation of number and distribution of fibroblasts in the bone marrow stroma was carried out using antibodies reactive with L-NGFR and αSMA. In normal trephine biopsy sections, expression of L-NGFR is widespread throughout the bone marrow stroma. Fibroblastic "reticular" cells form a complex network of cells upon which haemopoietic cells lie (Eaves *et al.*, 1991a); it seems probable that L-NGFR positive cells form all or part of this population (Singer *et al.*, 1985). Expression of αSMA, however, has been shown in this study using bone marrow trephine biopsy samples (Chapter 6 and 7) and other studies using aspirated bone marrow biopsies (Wilkins & Jones, 1998) to be absent from stromal fibroblasts in normal bone marrow. The distribution of stromal fibroblasts in this study was investigated by assessment at three main locations: surrounding bony trabeculae, surrounding blood vessels and dispersed elsewhere in the bone marrow stroma.

Staining of stromal fibroblasts around trabeculae with both L-NGFR and αSMA antibodies was hindered by technical limitations. Bone marrow trephine biopsy samples are prepared with decalcification prior to paraffin embedding. This renders the tissue fragile and the heat-mediated (microwave) antigen retrieval method used resulted in lifting of trabeculae from the glass slides from many sections. It was observed that the majority of biopsies (15/23) in which trabeculae had become disrupted had been obtained after a second course of chemotherapy. This may be a direct result of the particular drugs taken prior to the second post-treatment biopsy or, perhaps more likely, may represent the cumulative result of both courses of chemotherapy in conjunction with the decreased cellularity of post-treatment biopsy sections when compared with normal marrow.

The most noticeable trend that occurred throughout the bone marrow stroma with treatment was that, in post-chemotherapy biopsies from both AML and ALL patients, expression of L-NGFR by stromal fibroblasts was either similar to normal or, in most subtypes, had markedly decreased. Parallel with this was the observation that, compared with normal, expression of αSMA by stromal fibroblasts had markedly increased after chemotherapy. A number of possible explanations could account for these observations. Firstly, stromal fibroblasts expressing αSMA may represent a separate population of cells than those expressing L-NGFR; chemotherapeutic agents may, in this scenario, be causing a replacement of L-NGFR positive cells with myofibroblasts that express αSMA. These αSMA positive cells may either lack, or have a decreased ability to produce, the necessary cytokines and stimuli to support haemopoietic development of particular cell lineages, influencing the rate of recovery of haemopoiesis after chemotherapy. Alternatively, the same population of cells may express L-NGFR and αSMA; under normal circumstances, these cells may express high levels of L-NGFR and after chemotherapy, as a response to injury, there may be down-regulation of L-NGFR and up-regulation of αSMA expression.

Many of the chemotherapeutic agents are cell cycle specific and are usually targeted towards rapidly proliferating cells rather than those with a slow turnover rate. Analysis of stromal fibroblasts for their expression of the proliferation-associated antigen Ki67 was carried out to identify the proportion of quiescent, or static stromal cells versus

proliferating stromal cells. However, no clear trends were observed since the number of proliferating stromal cells in relation to proliferating haemopoietic cells was low. This made it technically difficult to identify proliferating stromal cells among the haemopoietic cells and thus almost impossible to obtain an accurate estimate of the number of proliferating stromal cells. Analysis of proliferating haemopoietic cells in patients treated for AML also showed no clear trends. However, in patients with ALL, the number of proliferating haemopoietic cells decreased after the first course of chemotherapy and, with successive courses, gradually increased towards normal levels. This may be a reflection upon the overall reduction in cellularity post-chemotherapy or may represent a temporary impairment in regenerative capacity after successive courses of chemotherapy.

Patients with AML and ALL were found, pre-treatment, to have reduced numbers of stromal macrophages compared with normal controls. Initial treatment with chemotherapy in both patient groups had little effect. In some cases macrophage numbers reduced further. Since bone marrow macrophages are believed to be of haemopoietic origin, this may be a reflection upon the reduced number of haemopoietic cells after chemotherapy. However, macrophage numbers appeared to increase towards normal levels after the third course of treatment. In untreated leukaemic patients, these macrophages appeared small and had irregular morphology. With successive courses of chemotherapy, the size of the macrophages had noticeably increased with many becoming a more rounded shape. This is probably a result of increased phagocytic activity in the presence of cell death due to the effects of the chemotherapy. It cannot be excluded that it might represent a direct action of the chemotherapeutic drugs on the macrophages.

The VS38C antibody reacts with a rough endoplasmic reticulum-associated antigen, p63, known to be expressed by fibroblastic stromal cells and plasma cells in bone marrow (Turley *et al.*, 1994). The immunostaining results obtained, studying stromal cells with this antibody, were varied in AML and ALL. After an initial course of chemotherapy, there was clearly an increase in the number of p63-positive stromal fibroblasts but, after subsequent courses, no staining was observed. Staining of plasma cells with this antibody revealed no consistent changes pre- and post-chemotherapy. However, one striking finding of this study was the observation that p63-positive

osteoblasts became prominent in close proximity to trabecular margins (Figure 7.11B). This phenomenon was found in patients with both AML and ALL and was only seen after chemotherapy indicating new bone formation stimulated by chemotherapy. This could suggest either that stromal cell injury, caused by chemotherapy, is being replaced with new bone formation as part of a recovery mechanism or, alternatively or in addition, chemotherapy is causing stasis of normal bone turnover and is followed by "catch-up" remodelling at trabecular surfaces.

8. IMMUNOPHENOTYPIC CHARACTERISATION OF MOBILISED PERIPHERAL BLOOD STEM CELLS

8.1 INTRODUCTION

The work described in this chapter was a joint study undertaken by Manyee Cheung, a PhD student in the department of Pathology who was carrying out a related project, and myself. The majority of the work was performed by me and a manuscript is currently in preparation for publication. Below are details of the authorship of this manuscript:

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8.2 BACKGROUND

Haemopoietic stem cells can be mobilised from the bone marrow in large numbers following administration of low-dose chemotherapy regimes and growth factors such as granulocyte colony-stimulating factor (G-CSF) (Barr *et al.*, 1975). The mechanisms by which mobilisation occurs are poorly understood but are believed to involve disruption of cyto-adhesive interactions between stem cells and the bone marrow stroma (To *et al.*, 1997). Harvesting and transplantation of mobilised peripheral blood stem cells (PBSC) is now widely used, as an alternative to bone marrow transplantation, to restore haemopoietic function following bone marrow ablative therapy. Successful haemopoietic recovery following PBSC transplant is dependent upon complex interactions between haemopoietic stem cells and the bone marrow stroma (Quinones, 1993). This stroma is composed of extracellular matrix components and a variety of cell types, including fibroblasts, endothelial cells, adipocytes and macrophages (Allen & Dexter, 1984; Strobel *et al.*, 1986; Wilkins & Jones, 1995a).

The origin of bone marrow stroma remains poorly understood. Marrow-derived cells expressing CD34 include cell populations that can generate stroma as well as giving rise to haemopoietic cells (Simmons & Torok Storb, 1991), whereas PBSC usually require pre-formed stroma for long term growth in culture (Rice *et al.*, 1989). It remains unknown whether both stromal and haemopoietic components are derived from a common multi-potential stem cell or whether they originate from different stem cell populations. Current evidence favours the latter (Ojeda-Uribe *et al.*, 1993) with the exception of stroma-associated macrophages, which are presumed to be of haemopoietic origin (Thiele *et al.*, 1998). Mobilised PBSC used in transplantation are known to contain stem cells that can reconstitute haemopoiesis, partly differentiated cells such as CFU-GM and more-mature nucleated cells (Rice *et al.*, 1989) plus precursors for the generation of functional natural killer cells (Takenaka *et al.*, 1995). However, experimental evidence that PBSC harvests contain mobilised cells that are of stromal origin and have stroma-forming potential is lacking.

In this study, the question has been addressed as to whether mobilised PBSC populations contain cells capable of generating stromal layers under human long-term bone marrow culture (hLTBMC) conditions. We have also performed immunocytochemistry, using cytocentrifuge preparations of PBSC, to demonstrate expression of antigens shown previously to be expressed by bone marrow stromal cell populations (Wilkins & Jones, 1995a; Wilkins & Jones, 1998).

8.3 PATIENTS STUDIED

Fifteen samples from 12 patients with various types of lymphoma were used in this study (Table 8.1). Sequential PBSC samples were obtained from 2 of these patients (numbers 4 & 7 in Table 8.1). The samples obtained from 7 patients (patients 1-7 in Table 8.1) were pilot tubes containing approximately 1 ml of harvested cells in dimethyl sulphoxide-containing medium. These samples had been stored in liquid nitrogen for up to 5 years following controlled-rate freezing. Samples from a further five patients (patients 8-12 in Table 8.1) were obtained fresh, without prior freezing, to study differences between PBSC samples that had not been exposed to freezing or thawing and samples obtained after a period of frozen storage.

Table 8.1. Patient Characteristics

Patient No.	Patient Age	Diagnosis*	Prior Treatment**	Priming Regimen***
1	56	NHL	• CHOP • IM-VP16	• Cyclophosphamide 4g/m^2 • G-CSF[1] days 7-13
2	33	Angiocentric T- cell NHL	• Failed CHOP. Response to MACOP-B	• Cyclophosphamide 3g/m^2 • G-CSF[1] 300μg daily
3	46	NHL Mantle cell lymphoma, Relapsed to Large Bowel	• CHOP×6, • Surgery then CHOP×3 following relapse	• Cyclophosphamide 3g/m^2 • G-CSF[2] 263μg daily
4a	45	High grade B cell NHL	• MACOP-B	• G-CSF
4b	As Above	As Above	• As Above	• As Above
5	26	B cell sclerosing mediastinal lymphoma	• CHOP×6 • MACOP-B • IM-VP16×2	• Cyclophosphamide 3g/m^2 • G-CSF[2] 263μg daily
6	53	Gastric B cell lymphoma	• CHOP×5	• High dose CHOP • G-CSF[2] 263μg daily from day 5
7a	51	Low-grade NHL	• MACOP-B • DHAP	• G-CSF[2] 526μg daily
7b	As Above	As Above	• As Above	• As Above
7c	As Above	As Above	• As Above	• As Above
8	49	Follicular NHL	• Mitozantrone • Chlorambucil • Prednisole	• G-CSF[1] 960μg daily days 1-5
9	32	Hodgkin's Disease	• ABDV x 6	• Cyclophosphamide 3g/m^2 • G-CSF[1] 300μg daily
10	62	Diffuse large cell B-NHL	• CHOPx3 • Radiotherapy	• Cyclophosphamide 3g/m^2 • G-CSF[1] 300μg daily
11	56	Diffuse large cell B-NHL	• CHOPx3 • Radiotherapy	• G-CSF[1] 600μg daily days 1-5
12	55	Hodgkin's Disease	• CHOPx6 • P-MitCEBOM x8wk	• Cyclophosphamide 1.5g/m^2 • G-CSF[1] 300μg daily

* **Patient diagnosis:** NHL, non-Hodgkin's lymphoma

** **Prior treatment:** CHOP, cyclophosphamide, doxorubicin, vincristine and prednisolone; IM-VP16, Ifosfamide, methotrexate and etoposide; MACOP-B, methotrexate with leucovorin, doxorubicin, cyclophosphamide, vincristine, prednisolone and bleomycin; DHAP, dexamethasone, high-dose cytarabine and cisplatin; PMitCEBOM, Prednisolone, Mitozantrone, Cyclophosphamide, Etoposide, Bleomycin, Vincristine, Methotrexate.

*** **Priming Regimens:** G-CSF, granulocyte-colony stimulating factor

G-CSF[1]: Filgrastim (Neupogen® (Amgen))

G-CSF[2]: Lenograstim (Granocyte® (Chugai))

8.4 PBSC SAMPLES

Peripheral blood stem cells were harvested by leucopheresis (Baxter CS3000 Plus) 10-14 days post-priming with chemotherapy and growth factor (G-CSF), or 5-7 days post-priming with growth factor alone. Samples of PBSC were either frozen and stored, as described in Chapter 2 (section 2.12), or sent unfrozen to the laboratory for immediate study. Upon receipt in the laboratory, or immediately after thawing, cytocentrifuge preparations were made and cells were established in culture. The cytocentrifuge preparations were immunostained as described in Chapter 2 (section 2.15) using antibodies reactive with the stromal cell-associated antigens listed in Table 8.2. Remaining cells were cultured in a standard 4ml slide chamber under hLTBMC conditions, as described in Chapter 2 (section 2.10.1). After 4 weeks in culture, cytocentrifuge preparations were made from the non-adherent cells. These, plus any adherent cells on the growth surface, were stained for morphological evaluation.

Table 8.2. Antibody Details

Antibody	Antigen	Stromal Cell Type	Source
QBEnd10	CD34	Endothelial cells	Novocastra, UK
Anti-L-NGFR	Low affinity nerve growth factor receptor	Stellate cells	Dako, Denmark
Anti-VCAM-1	Vascular cell adhesion molecule 1	Most stromal cells	Dako, Denmark
Anti-collagen IV	Collagen IV	As above	Dako, Denmark
Anti-laminin	Laminin	As Above	Sigma, Poole, UK
BerMAC3	CD163 (110/130kDa macrophage-associated protein)	Stroma-adherent macrophages	Dako, Denmark

Samples of fresh PBSC received immediately after harvest contained an abundance of cells. In addition to cytocentrifuge preparations and cultures in 4ml slide flasks, cell cultures were also established in cell culture inserts as described in section 2.10 to assess further their ability to generate stroma in a culture system optimised for stromal

growth. To investigate the effects of freezing (and thawing) on PBSC, remaining cells from fresh PBSC samples were frozen and stored in liquid nitrogen for up to one week. These were thawed, cytocentrifuge preparations made and immunostained as described above, and cultures were established in both 4ml slide-flasks and cell culture inserts for 4 weeks.

8.5 RESULTS

8.5.1 PBSC cultures

A total of 14 PBSC samples from 11 patients were available for culture. One frozen sample could not be cultured due to a lack of viable cells upon thawing. Of the PBSC samples obtained frozen, only the growth surface of patient 7 showed any adherent cells after 4 weeks in culture. Three PBSC samples were studied from this patient and, when cultured, all resulted in the development of adherent cells. These cells were scattered over the growth surface but a confluent monolayer did not form in any of the 3 cultures. The appearance of these cells was identical to marrow-derived cells of the monocyte/macrophage lineage cultured in the absence of stromal fibroblastic cells (Wilkins & Jones, 1996) and was clearly different to the adherent fibroblasts in human long-term bone marrow cultures (Figure 8.1A and B).

Cultures established from fresh PBSC all contained a proportion of adherent cells after four weeks in culture. Cells cultured in cell culture inserts contained a greater proportion of adherent cells after four weeks than those cultured in 4ml slide flasks. These adherent cells were identical to those present in cultures from frozen PBSC and morphologically identifiable as cells of the monocyte/macrophage lineage. Subsequent immunocytochemistry of the growth surface of these cultures using antibodies reactive with L-NGFR and CD163, revealed that all adherent cells expressed the macrophage–associated antigen CD163 (identified using BerMAC3 antibodies) (Figure 8.1C). No L-NGFR positive cells were present in any of the cultures (Figure 8.1D).

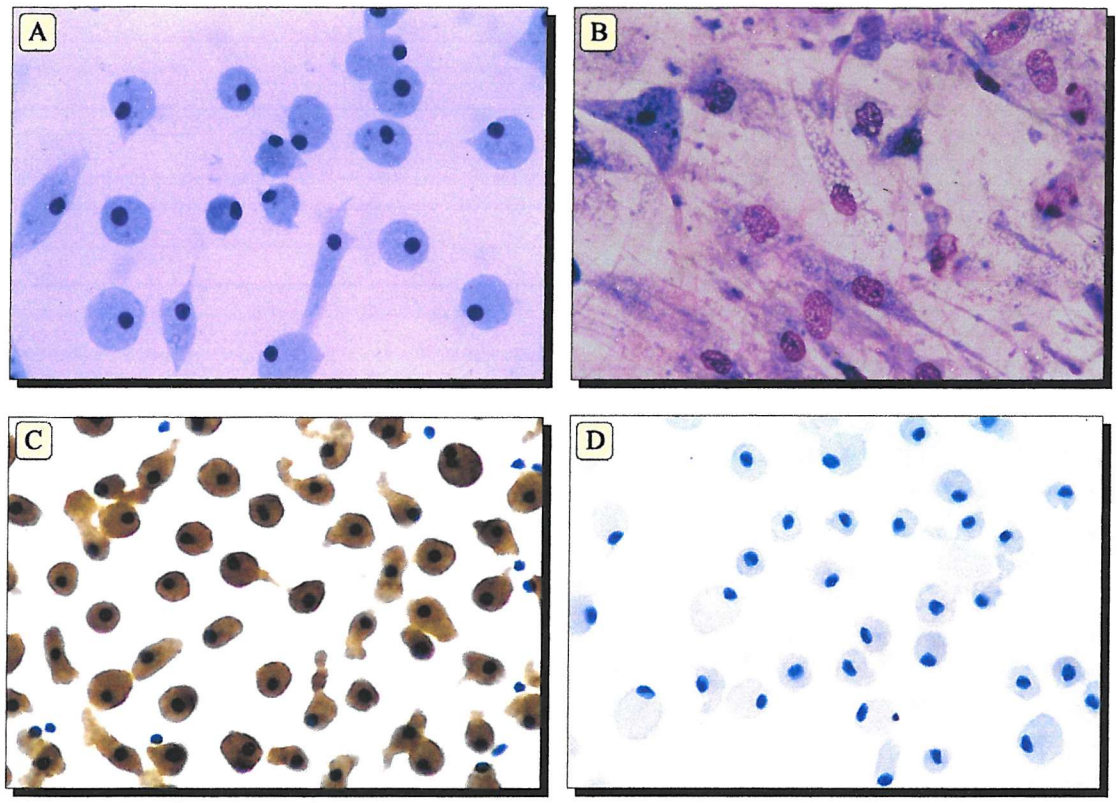


Figure 8.1. Adherent cells in cultures generated from mobilised peripheral blood stem cells (PBSC). The adherent cells in cultures generated from PBSC were morphologically identifiable as cells of the monocyte/macrophage lineage (A) and are clearly distinguishable from the layers of adherent cells present in human long-term bone marrow cultures (B). Immunostaining demonstrated that all adherent cells expressed the macrophage-associated antigen, CD163 (C), whereas no cells expressed the fibroblastic stromal cell-associated antigen, L-NGFR (D).

8.5.2 Immunocytochemical analysis of cytocentrifuge preparations

Quantitation of immunostain-positive cells among cytocentrifuge preparations of PBSC samples was performed by both Manyee Cheung and myself. The mean values for each group of PBSC were then calculated (Table 8.3). PBSC samples were grouped as either a) frozen, in which the samples had been stored for up to five years in liquid nitrogen; b) fresh (pre-freezing), representing the samples had been obtained fresh without freezing, or c) fresh (post-freezing), representing samples had been obtained fresh then frozen and stored for up to one week in liquid nitrogen prior to study.

Among all PBSC samples studied, the only positive immunostaining obtained was for CD34 and collagen IV. In contrast with previous studies of aspirated bone marrow cells (Wilkins & Jones, 1998), no cells expressing L-NGFR or VCAM-1 were found.

Cells expressing CD34 were present in the majority of PBSC samples and no significant differences in CD34 expression were observed between frozen and fresh PBSC samples. However, differences between frozen and fresh PBSC were observed with regard to collagen IV expression. This was greatest among PBSC that had been stored frozen prior to study (mean 11.1%; range 0.0-27%). In contrast, expression of collagen IV by PBSC obtained fresh was considerably lower (mean 0.3%; range 0.0-0.5%).

Table 8.3. Staining Quantitation

PBSC type	Mean percentage of cells stained					
	CD34	L-NGFR	VCAM-1	Collagen IV	Laminin	BerMac3
Frozen	0.3 (0.0-0.9%)	0	0	11.1 (0.0-27%)	0	0
Fresh (pre-freezing)	0.3 (0.0-0.7%)	0	0	0.2 (0.0-0.3%)	0	0
Fresh (post-freezing)	0.4 (0.0-1.0%)	0	0	0.3 (0.0-0.5%)	0	0

8.6 DISCUSSION

These results demonstrate that a small percentage of PBSC in most samples express the CD34 antigen and none express L-NGFR or VCAM-1. The mean percentage of CD34+ cells (0.3-0.4%) did not vary significantly between fresh PBSC and those stored frozen prior to study. These results are comparable to the results obtained by Bernder *et al*, 1994, who found the percentage of CD34+ cells among PBSC to be 0.15% (Bernder *et al.*, 1994), but are considerably lower than the percentage of CD34+ cells (1-4%) reported in normal adult bone marrow (Simmons & Torok Storb, 1991). When cultured under hLTBMC conditions, including optimisation to favour stromal growth, these PBSC samples did not give rise to adherent, fibroblastic, stromal cell layers.

Aspirated bone marrow contains a heterogeneous population of cells. In addition to haemopoietic and stromal progenitor cell populations that express CD34, there are cells present in aspirated marrow that express L-NGFR (Wilkins & Jones, 1998). When cultured under standard hLTBMC conditions, both CD34 positive and L-NGFR positive enriched cell populations from bone marrow aspirates give rise to adherent stromal layers (Kovacs *et al.*, 1999).

Each of the cultures from patient 7 showed a small number of adherent cells after four weeks growth, as did all cultures derived from fresh, unfrozen PBSC. These adherent cells were identified morphologically as macrophages and were shown by immunocytochemistry to express the macrophage-associated antigen, CD163. These cells were negative for L-NGFR. The growth of these macrophages may be due to the G-CSF priming regime (without additional cytotoxic therapy) used in this patient prior to harvesting. Similar results have been obtained by other investigators (Ojeda-Uribe *et al.*, 1993). However, this does not explain why sample 4a, from patient 4 in our study, obtained using an equivalent priming procedure, did not generate similar cells. From these results, it does not appear that the priming regime *per se* can account for the differences in cell populations identified from these 12 patients.

Immunocytochemical analysis of cytocentrifuge preparations of PBSC identified a high proportion of cells expressing collagen IV among PBSC that were stored frozen prior to study. Previous analysis of aspirated bone marrow cells has showed only very rare cells expressing this antigen (Wilkins & Jones, 1998; Wilkins & Jones, 1998). The proportion of cells expressing collagen IV was considerably lower among fresh PBSC obtained without prior freezing. Long-term storage of these cells in liquid nitrogen and subsequent thawing appears to produce this effect, but the possible mechanism is unknown.

The results of this study demonstrate that stem cells mobilised from the bone marrow to the peripheral blood do not include cells that have a stromal fibroblastic immunophenotype or stem cells capable of stromal reconstitution when cultured under these precise hLTBMC conditions. However, the presence of stem cells capable of stromal formation *in vitro* has been demonstrated in embryonic and umbilical cord blood (Erices *et al.*, 1999). Similarly, it has been shown that blood obtained from

patients with chronic myeloid leukaemia contains cells capable of generating endothelial components of marrow stroma (Gunsilius *et al.*, 2000). In these publications, however, differences in culture conditions and the addition of fibronectin in the experiments reported by Gunsilius and colleagues (2000) may have had a significant impact upon the cells they were able to generate. The findings of the current study suggest that PBSC mobilised for transplantation by routine methods may, however, contain only haemopoietic and not stromal stem cells in many, if not most, patients.

9. GENERAL CONCLUSIONS

9.1 INTRODUCTION

It has long been known that the cellular and non-cellular elements of human bone marrow stroma are together essential for the support and regulation of normal haemopoiesis (Eaves *et al.*, 1991a; Singer *et al.*, 1985). Many of these non-cellular, extracellular matrix components of bone marrow stroma are produced and secreted by bone marrow stromal cells (Zuckerman *et al.*, 1989). Despite previous studies investigating the importance of bone marrow stroma for the support and maintenance of haemopoiesis, the precise roles and significance of individual stromal cell types in this process remain unclear. Reliable techniques to isolate and culture these cells *in vitro* are hindered by their rarity in clinical samples of aspirated bone marrow (Wilkins & Jones, 1998). However, such techniques are critical to advance understanding of the functions of these cell types.

9.2 ISOLATION AND CULTURE OF HUMAN BONE MARROW STROMAL CELL POPULATIONS

In this study, magnetic activated cell sorting (MACS) was used to isolate stromal cells of interest (Miltenyi *et al.*, 1990) using antibodies shown previously to react with stromal cell-associated antigens (Wilkins & Jones, 1995a). These were cultured using Dexter-type long-term culture conditions (Dexter *et al.*, 1977). The use of MACS over the last decade has been advantageous over previous methods of magnetic cell sorting. The MACS microbeads are extremely small, internalised by the cells when cultured and do not interfere with the functional characteristics of the cells. Previous techniques, which used larger magnetic particles, required prior detachment of the magnetic beads before culture and could change the functional characteristics by cross-linking surface proteins. The MACS technique was used in this study for these reasons and because it is a relatively fast and simple technique requiring minimal cellular disruption. This was of particular importance since there was no control over the length of time it would take for the clinical samples of aspirated bone marrow to reach the laboratory. In general, samples that had taken longer than 24-48 hours to reach the laboratory were less likely to yield sufficient numbers of viable cells for culture.

Although MACS is an ideal technique for cell separation, a number of problems were encountered when using this technique. The degree of enrichment of positively selected cells was varied and unpredictable. A number of explanations could account for this. Firstly, enrichment will depend upon the proportion of cells of interest that are present among the starting cell population. The higher the proportion of cells of interest, the higher the purity of the enriched cell population. The clinical samples of bone marrow obtained were varied both in terms of the volume and quality of the samples. As a result, the number/proportion of available stromal cells would be varied in each sample rendering consistency between samples difficult. Secondly, one of the most important parameters with regard to magnetic labelling is temperature. While all stages of the procedure, where possible, were carried out at 4°C, as recommended by the manufacturers, the high laboratory temperature during summer months would almost certainly have influenced the outcome of the enrichment.

Since the number of isolated stromal cells from many of the aspirated bone marrow samples was small, a culture vessel with a small growth surface area was required. Porous polyethylene terephthalate (PET) membrane inserts were chosen as a suitable growth surface on which to culture the cells. These PET inserts have a small growth surface area and more closely resemble the *in vivo* environment than other, more traditional cell culture vessels, since they allow nutrient exchange both above and below the growth surface. To analyse the stroma-forming potential of each of a variety of stromal cell populations, multi-step sequential MACS separations were carried out. Immunocytochemical analysis required optimisation for use with bone marrow stromal cells cultured on PET membrane inserts. Fixation of cells was carried out using 4% paraformaldehyde in PBS for 5 minutes. Acetone cannot be used as it reacts with the material from which the membranes are produced, is a lipid extractant and results in loss of adipocyte cellular morphology in cultures. After haematoxylin counterstaining, the inserts are cut from their housing as described in Chapter 2 and mounted using an aqueous mountant. Mountants that require dehydration are not recommended, as dehydration was also found to result in poor preservation of adipocyte morphology.

9.3 Maximising Use of Clinical Samples: Double-Immunocytochemistry

The volume of clinical samples available from aspirated bone marrow was small and, following magnetic cell sorting for individual stromal components, a maximum of only two cultures could be established due to the small number of cells obtained. Owing to the limited number of available samples and the possibility of patient-to-patient variation, it was essential to obtain the maximum amount of information from each culture generated from these cells. Consequently, during the latter part of this study, double immunocytochemistry was optimised for use with bone marrow stromal cells cultured on PET cell culture inserts. Full details are provided in Chapter 4 but, in summary, the technique was carried out following similar procedures as those used for single immunocytochemistry but with an additional blocking step prior to addition of streptavidin-horseradish peroxidase complexes during the second round of staining. The active ingredients of the blocking solution comprised hydrogen peroxide, to saturate peroxidase binding sites, and sodium azide (a peroxidase inhibitor) to inhibit any residual peroxidase activity. This step was found to be critical to the success of the double immunocytochemical staining in which excessive endogenous peroxidase activity otherwise obscured the positive results. In addition, the concentration of bovine serum albumin, to prevent non-specific protein binding throughout the procedure, was increased from 1% to 10%.

9.4 Optimisation of RNA Methods

Optimisation of Northern blot hybridisation was carried out to enable detection of mRNA encoding L-NGFR after culture and was aided by the identification of the A875 human melanoma cell line, in which abundant expression of the L-NGFR receptor protein was demonstrated using immunocytochemistry. Using this cell line, amounts of RNA extracted from equivalent sources and cell numbers was superior with the QIAGEN RNeasy mini kit than with the Promega SV total RNA isolation system. Integrity gels gave optimal visualisation of signal if 2μg RNA was loaded into the gel with equal volume of load buffer and 1μl (1mg/ml) ethidium bromide. Northern blot hybridisation was successfully carried out to demonstrate L-NGFR

mRNA in both the A875 cell line and hLTBMC using a cocktail of oligonucleotide probes (Appendix 3) and a hybridisation temperature of 70°C.

9.5 Characterisation of Human Bone Marrow Stromal Cells

9.5.1 Stromal Fibroblasts

Stromal fibroblasts were isolated from aspirated bone marrow using anti-L-NGFR and STRO-1 antibodies. Expression of the low-affinity nerve growth factor receptor (L-NGFR) by bone marrow fibroblasts was first described in 1993 (Cattoretti *et al.*, 1993). Since this time, few studies have investigated expression of this antigen in bone marrow (Cattoretti *et al.*, 1993; Wilkins & Jones, 1995a) and, to date, there has been no literature published describing possible functions of this antigen in bone marrow stromal cells. Antibodies reactive with L-NGFR were chosen for isolation of stromal fibroblasts because firstly, few other specific markers for this cell type are known and secondly, an aim of the study was to investigate the growth characteristics of cells expressing this antigen in order to provide some insight into its possible functions. The monoclonal antibody STRO-1 was also used to isolate stromal fibroblasts. This antibody, the reactivity of which was first described in bone marrow in 1991, is believed to react with multipotential stromal cell precursors (Simmons & Torok Storb, 1991). The STRO-1 antibody was used in this study to investigate similarities and differences between the biological behaviour in long-term culture of L-NGFR positive and STRO-1-positive bone marrow cells.

Cell cultures formed from L-NGFR- or STRO-1-enriched cells had very similar growth characteristics. Both types of culture gave rise to an adherent stromal cell layer with fibroblast morphology, containing an abundance of adipocytes. However, adipogenesis appeared to occur more rapidly in cultures formed from STRO-1-positive cells than in cultures generated from L-NGFR positive cells. The ultimate origin of bone marrow stromal cells is still unknown and it is not known whether the differentiation from stem cell to mature stromal cell is a reversible process or whether only unidirectional terminal differentiation occurs along one of a series of alternative pathways. Of particular interest in this study is the differentiation from stem cells to

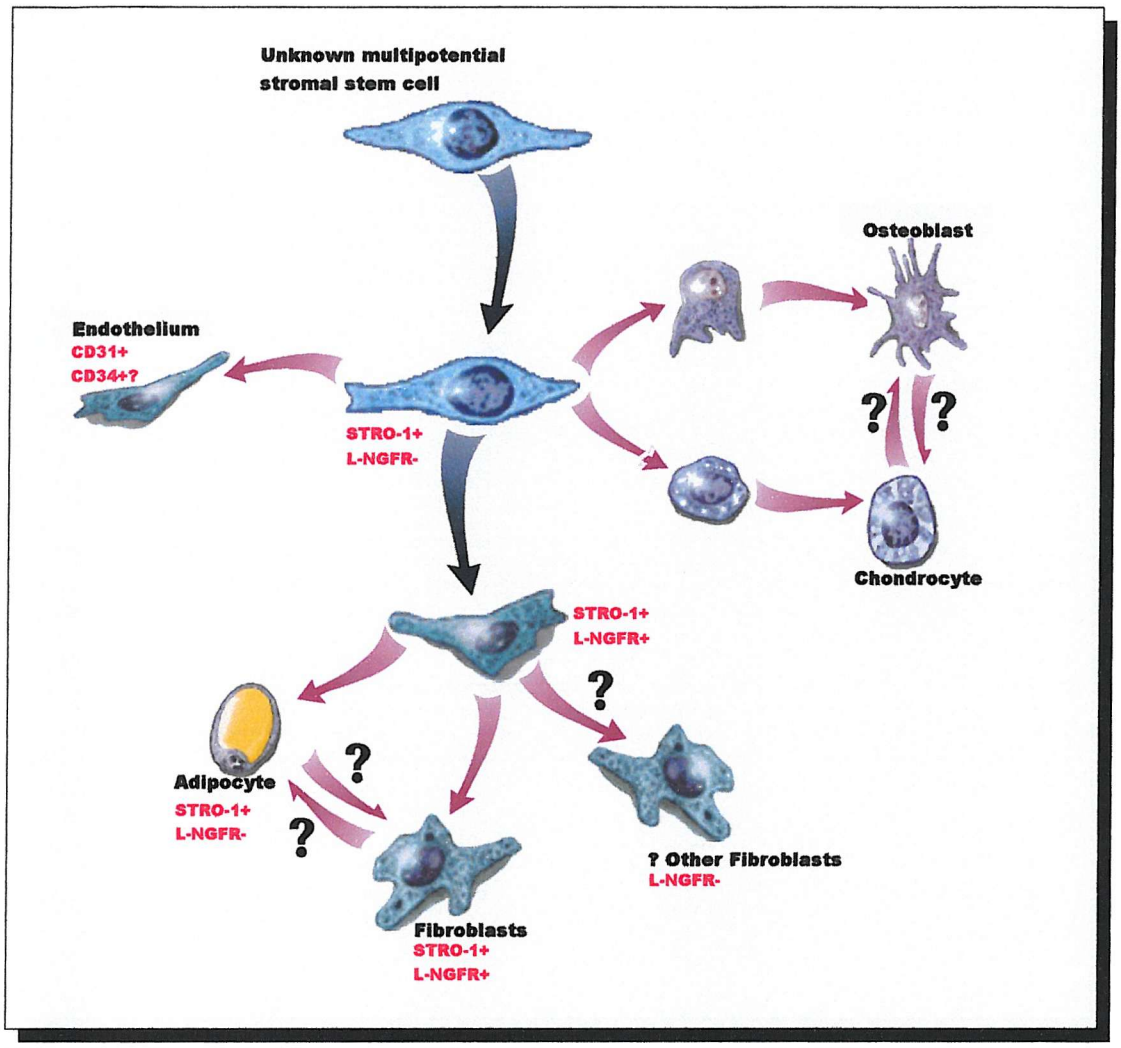


Figure 9.1. Hypothetical patterns of differentiation and expression of L-NGFR and STRO-1 from a stromal stem cell to mature fibroblasts and adipocytes. See section 9.5.1 for explanatory text. Cell images courtesy of Osiris Technologies Inc.

fibroblasts and adipocytes and the question of whether stromal fibroblasts have the capacity to differentiate into adipocytes under certain conditions. Possible hypotheses describing the differentiation of stem cell into mature stromal cells is shown in Figure 9.1.

Earlier onset of adipogenesis in cultures generated from STRO-1 enriched cells may be explained if the antigen to which STRO-1 binds is expressed further along the adipocyte differentiation pathway than that of L-NGFR. In this model, assuming fibroblasts and adipocytes both originate directly from a common stromal stem cell, populations of L-NGFR and STRO-1 enriched cells would both contain stromal stem cells with fibroblastic and adipogenic potential. However, those enriched for STRO-1 would contain more cells already committed to adipocyte differentiation and thus

would result in cultures able to undergo adipogenesis more rapidly than those derived from L-NGFR-enriched cells.

Immunostaining of cultures grown from L-NGFR-enriched cells identified the presence of lipid droplets in L-NGFR positive cells (Figure 3.4, Chapter 3). This, in association with the observation that only a small population of stromal fibroblasts continue to express L-NGFR after four weeks in culture, suggests that it is possible for adipocytes to be generated from a population of L-NGFR positive fibroblasts (Figure 9.1). It may also be possible for adipocytes to de-differentiate into fibroblasts. This is supported indirectly by observations in Chapter 6, investigating stromal alterations that occur in the development of idiopathic myelofibrosis (IMF). In this disease, it was observed that the increase in fibrous tissue within the marrow seen in IMF is associated with a decrease in the number of adipocytes throughout the bone marrow stroma, suggesting that in this disease, adipocytes may de-differentiate into stromal fibroblasts (see section 9.6). However, it may be possible that adipocytes do not de-differentiate into fibroblasts but instead undergo terminal maturation. In this hypothesis, the decreased in proportion of adipocytes and increase in proportion of fibroblasts in IMF could be due to increased adipocyte apoptosis and replacement with new fibroblasts originating from multipotential precursor cells.

The observation that only a small number of stromal fibroblasts continue to express L-NGFR after four weeks in culture may implicate a functional role of L-NGFR in stromal development. In developing neurons, L-NGFR is believed to act alongside TrkA in mediating neuronal interconnection (Miller, 1998), as described in Chapter 1. Investigation of other neurotrophin receptors in bone marrow (Labouyrie *et al.*, 1999) has shown recently that stromal fibroblasts also express the high affinity NGF receptor, tyrosine kinase A (TrkA). Further investigation is required in this area to elucidate the functions of these receptors in bone marrow stroma, but it is a distinct possibility that L-NGFR, together with TrkA, may act in a similar manner to that seen in developing neurons (Figure 1.4, Chapter 1). In this model, if stromal fibroblasts interconnect at the correct time and location, optimal levels of NGF would activate of both L-NGFR and TrkA. In a similar manner to neuronal survival, TrkA activation would override L-NGFR activation resulting in fibroblast survival. However, if the fibroblasts interconnect at either an inappropriate time or location, sub-optimal levels of NGF

would be present. Since L-NGFR, unlike TrkA, can be activated by other neurotrophins, L-NGFR activation would be sufficient to override TrkA activation and result in fibroblast apoptosis. Thus, L-NGFR and TrkA may mediate the connection of stromal fibroblasts in the bone marrow to form the network of interconnecting stromal fibroblastic cells found in normal adult bone marrow stroma. Immunocytochemical studies could be carried out using stromal cell cultures to investigate expression of L-NGFR versus TrkA and levels of various neurotrophins during stromal development. Studies such as this in normal bone marrow would give indications of whether this is a likely mechanism for stroma formation in bone marrow. If this is does prove likely, similar immunohistochemical studies could be carried out in diseases such as idiopathic myelofibrosis (IMF) to investigate whether failure of this mechanism could account for the inappropriate formation of fibrous tissue within the bone marrow.

9.5.2 CD34 positive stromal cells

Haemopoietic stem cells and endothelial components were separated from aspirated bone marrow using antibodies reactive with CD34. This antigen is expressed at high levels on primitive haemopoietic stem cells and expression has been shown to decrease progressively as these stem cells differentiate into mature blood cells (Civin *et al.*, 1987). Cultures generated from CD34-enriched cells differed from L-NGFR+ and STRO-1+ cultures by forming haemopoietic colonies during the 4-week culture period. It is currently unknown whether haemopoietic and stromal CD34+ stem cells are separate populations or whether stromal and haemopoietic components of bone marrow originate from a common precursor cell. From the results obtained in this study, stem cells expressing CD34 demonstrated the ability to generate both haemopoietic and stromal components of bone marrow. Two explanations could account for this observation. Firstly, stromal and haemopoietic components of marrow may originate separately (Figure 9.2, hypothesis A). In this hypothesis, both haemopoietic and stromal stem cells must express CD34 in order that CD34 positive cells may generate both haemopoietic and stromal components in culture. For this model to comply with the observations seen in L-NGFR+ and STRO-1+ cultures, stromal stem cells expressing CD34 would presumably co-express L-NGFR and STRO-1. However, this does not explain adequately the observation that cultures from

CD34 positive cells took up to a week longer to generate an adherent fibroblastic layer than cultures formed from L-NGFR+ or STRO-1+ cells.

A second explanation could be that stromal and haemopoietic stem cells are formed from a common progenitor cell (Figure 9.2, hypothesis B). If this progenitor cell, with the capacity to become either a haemopoietic or stromal stem cell, were CD34 positive, it would explain why CD34 cultures took longer to generate stroma, as they would first have to differentiate into stromal stem cells. It would also explain the ability of CD34 positive cells to give rise both to stromal and haemopoietic components of marrow. To comply with the observation that only stromal components of bone marrow are present in cultures formed from L-NGFR or STRO-1-positive cells, the common progenitor cells having both haemopoietic and stromal differentiation capacity would be negative for L-NGFR and STRO-1 and positive for CD34. The stromal progenitor cells, however, would be positive for both L-NGFR and STRO-1 (Figure 9.2, hypothesis B).

Multi-step sequential MACS separations were carried out in which cultures were established from enriched populations of L-NGFR-/CD34+ or CD34-/L-NGFR+ cells. Removal of L-NGFR positive cells prior to enrichment for CD34 did not affect the ability of these cells to generate stroma. This could be explained by hypothesis B, since removal of L-NGFR positive cells would remove the stromal stem cells but leave the common progenitor cell that could then differentiate to provide stromal stem cells. In addition, prior removal of CD34 positive cells did not affect the ability of L-NGFR positive cells to generate stroma. This could also comply with hypothesis B, since removal of CD34 positive cells would remove both the common progenitor cell, capable of stromal and haemopoietic cell formation, and also remove the haemopoietic stem cells, leaving only the stromal stem cells, positive for both STRO-1 and L-NGFR. Hypothesis B would, however, suggest that only common progenitor cells and haemopoietic stem cells are CD34 positive and that stroma-committed stem cells are CD34 negative.

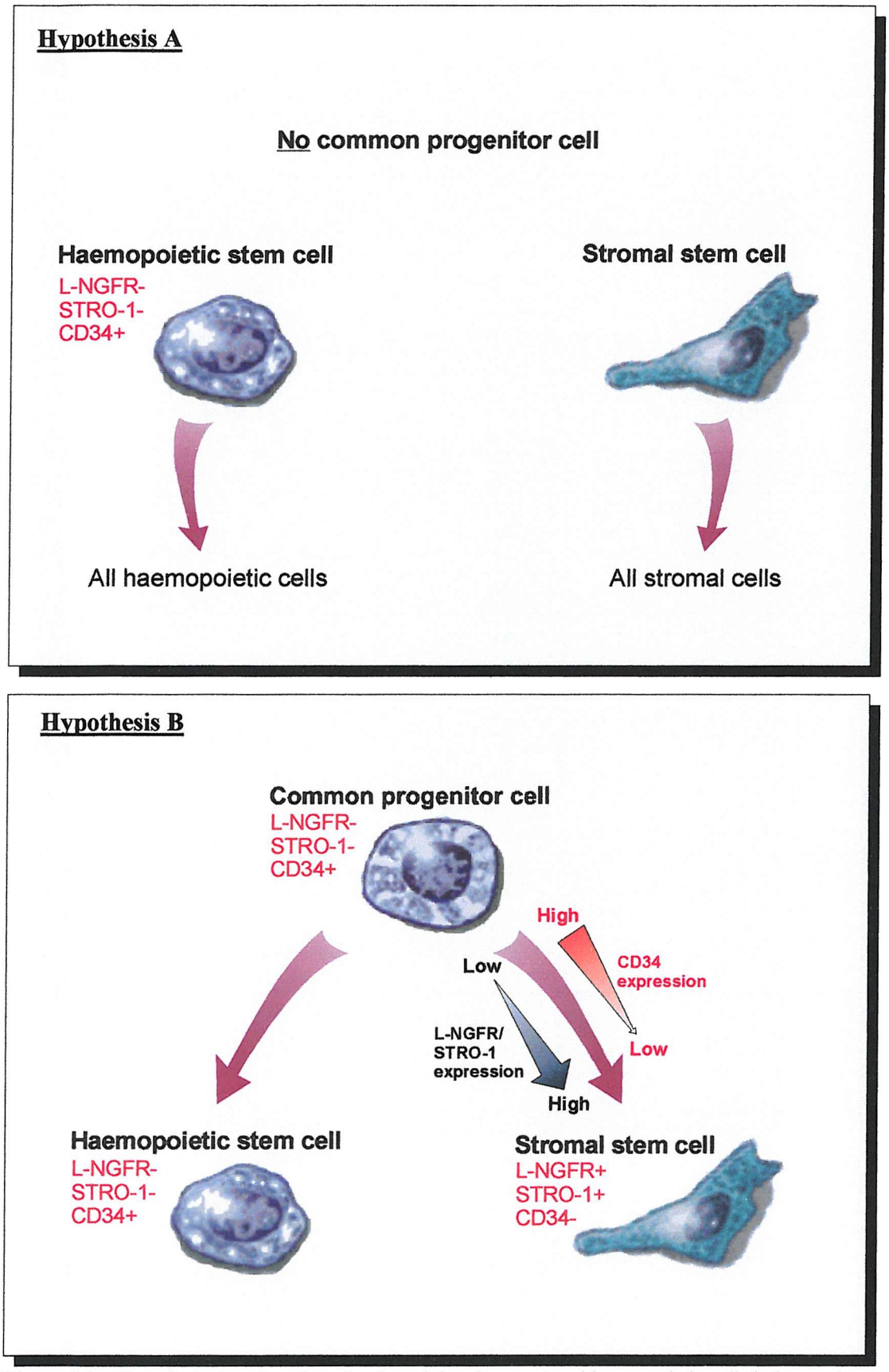


Figure 9.2. Hypothetical explanations for the origin of stromal and haemopoietic cells in human bone marrow. See section 9.2.2 for explanatory text. Cell images courtesy of Osiris Technologies Inc.

Further evidence for hypothesis B was found in the studies described in Chapter 8. These results showed that peripheral blood stem cells (PBSC) mobilised into the peripheral blood from bone marrow did not include cells with stroma-forming potential when cultured in hLTBMC conditions *in vitro*. These PBSC contained a proportion of cells that express CD34, but none expressing L-NGFR. The ability of these cells to undergo functional haemopoiesis after transplantation and the inability to generate stroma in culture are in keeping with the existence of CD34+/L-NGFR- haemopoietic stem cells. These results also suggest that the CD34 positive stem cells used in PBSC transplantation represent only haemopoietic stem cells and do not contain stromal stem cells. In addition, there must be other underlying differences between haemopoietic and stromal stem cells to account for the observation that stromal stem cells are not mobilised into the peripheral blood when treated with low-dose chemotherapy and growth factors.

Experiments investigating expression of STRO-1, L-NGFR and CD34 in cytocentrifuge preparations from aspirated bone marrow showed that, after enrichment for STRO-1, there were cells present in the STRO-1-positive fraction that expressed L-NGFR and cells present that expressed CD34. These were morphologically identical to STRO-1 positive cells. This would suggest that there are cells present in aspirated bone marrow with STRO-1+/L-NGFR+ and STRO-1+/CD34+ phenotypes and that there is a degree of overlap between expression of these antigens. The presence of cells with STRO-1+/L-NGFR+ phenotype, in hypothesis B would represent stromal stem cells. However, a proportion of the cells with a STRO-1+/CD34+ phenotype are likely to represent the erythroid precursors, which have been shown to express STRO-1 (Simmons & Torok Storb, 1991). It is also possible that during the maturation process from common progenitor cell to stromal stem cell, as expression of CD34 decreases, expression of STRO-1 increases, indicating that during maturation there is a degree of overlap between STRO-1 and CD34 expression (Figure 9.2, hypothesis B).

9.5.3 Stromal macrophages

Macrophages were isolated from aspirated bone marrow using antibodies reactive with CD14 and CD31, the latter of which is also expressed on endothelial components of marrow. Macrophages within the bone marrow stroma, unlike other stromal cells are

believed to be of haemopoietic rather than stromal origin, originating from monocytes and their precursors rather than the stromal precursor cells. Cultures generated from both CD31 and CD14 did, however, give rise to adherent stromal layers with an abundance of macrophages. It is unclear why adherent stromal layers with fibroblast morphology were generated from both CD31 and CD14-enriched cells. A subset of CD34 positive cells has been shown previously to co-express CD31 (Watt *et al.*, 1993), which may explain the formation of stroma from CD31 positive cells. However, the reasons why CD14-enriched cells gave rise to stroma remain unclear. It is important to note that MACS is an enrichment technique only and does not provide 100% pure populations of positively-selected cells.

Immunophenotypic characterisation of stromal macrophages isolated and cultured using antibodies reactive with CD31 and CD14 revealed the presence of two macrophage variants (Figure 9.3). There were large macrophages, which stained weakly for BerMac3 and smaller macrophages, which stained strongly for BerMac3. Cultures generated from CD31-enriched cells contained a heterogeneous population of large and small macrophages, whereas those formed from CD31-depleted cells contained only the large macrophage variant. It is unknown at present whether these two macrophage variants represent different functional variants or whether they may represent changes in antigen expression during maturation. The observation that cultures formed from CD31-depleted cells contained only the large macrophage variant may provide a means for the separation of large and small macrophages in order to further investigate the functions of these two macrophage variants.

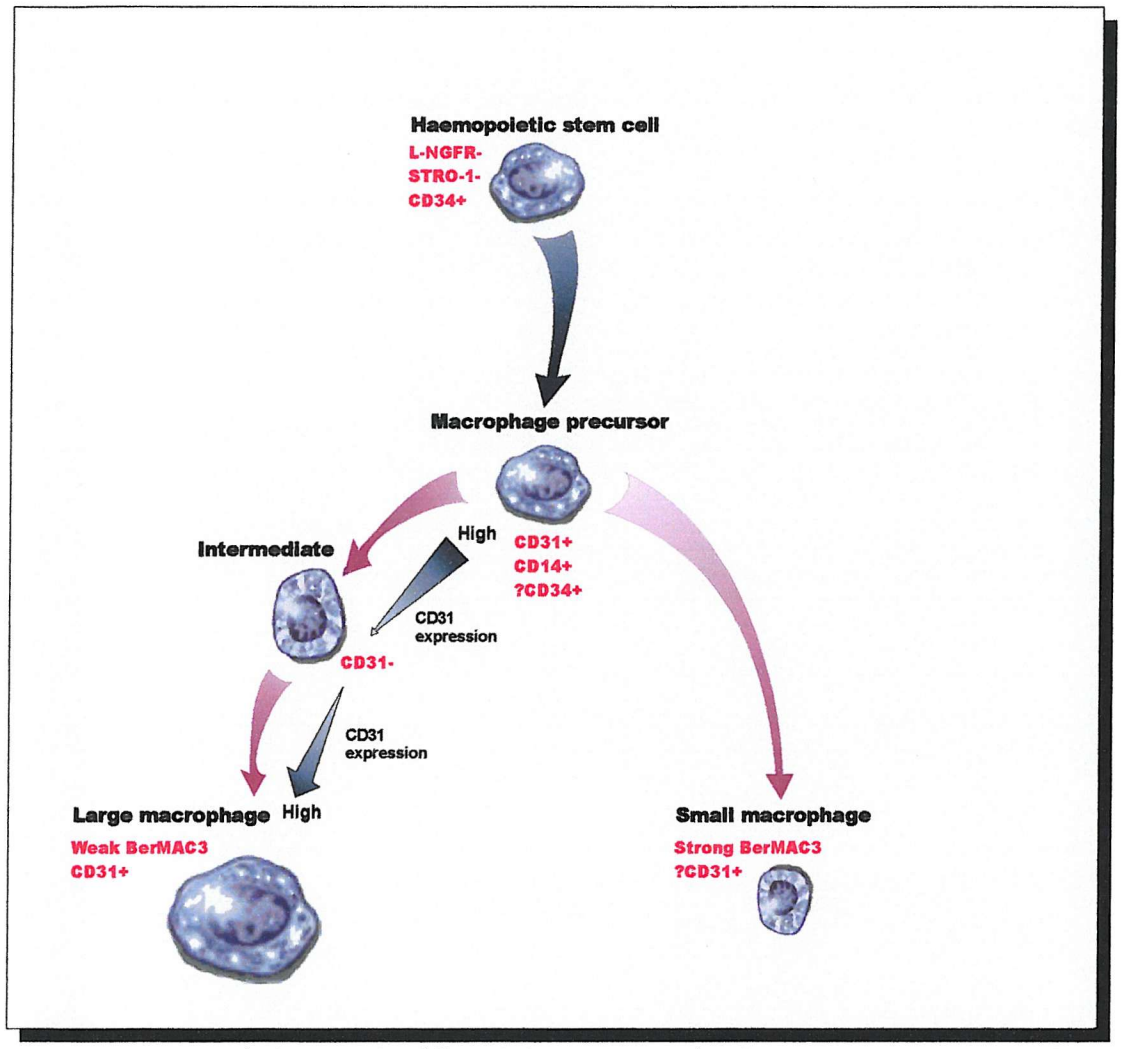


Figure 9.3. Hypothetical pattern for differentiation and expression CD31, CD14 and BerMAC3 from haemopoietic stem cells to bone marrow marophages. See section 9.5.3 for explanatory text. Cell images courtesy of Osiris Technologies Inc.

9.6 Stromal Alterations in the Development of Idiopathic Myelofibrosis

Idiopathic myelofibrosis (IMF) is a disorder characterised by an increase in stromal fibroblasts and endothelium in the bone marrow (Thiele *et al.*, 1997), believed to result from an underlying haemopoietic stem cell defect. In this study, abnormalities in the proportion and distribution of stromal fibroblasts, sinusoids, blood vessels and macrophages were investigated in bone marrow trephine biopsy sections from patients with IMF, using a panel of antibodies reactive with these stromal components of bone marrow.

Investigation of the bone marrow stroma of patients with IMF identified a number of differences when compared with sections obtained from essentially normal bone marrow. On the basis of L-NGFR expression by stromal fibroblasts, IMF patients could be divided into two groups. There were the majority (approximately 75%) whose bone marrow contained a high number of L-NGFR positive stromal fibroblasts, comparable to normal bone marrow biopsies, and there were others (approximately 25%) whose marrow contained very few. However, whether these two groups represent clinically distinct subcategories remains to be seen. It would be interesting to investigate whether any correlation exists between expression of L-NGFR and disease prognosis/outcome.

In normal bone marrow trephine biopsy sections, it was observed that there was no αSMA expression by stromal fibroblasts, consistent with the finding that there are no cells present in normal aspirated bone marrow that express αSMA (Wilkins & Jones, 1998). However, in almost all bone marrow trephine biopsies from IMF patients there were a high number of αSMA positive stromal fibroblasts. It has been discussed in section 9.5 that there may be the potential for adipocytes to originate, or differentiate from stromal fibroblasts. If this is possible, it is also possible that the reverse may happen. It was observed that, in the bone marrow of patients with IMF, there was a reduction in the number of adipocytes in conjunction with an increase in fibrous components. An explanation for this may be that, in IMF, the conditions within the bone marrow stroma are appropriate to cause the de-differentiation of adipocytes to stromal fibroblasts, or rather myofibroblasts, as identified by their expression of αSMA. However, an alternative explanation could be that the adipocytes in IMF undergo apoptosis and subsequent replacement by myofibroblasts. These theories are enhanced by the observation that, in IMF, stromal macrophages contained evidence of lipid debris within their cytoplasm. It may be that, as the adipocytes undergo a transition to stromal fibroblasts, they release excess lipid debris into the stroma, which is subsequently removed by the macrophages or alternatively, as the adipocytes undergo apoptosis, the fat is ingested by the macrophages.

It was also observed that, in the bone marrow of patients with IMF, there was an increase in the proportion of tissue occupied by bony trabeculae, compared with

normal. Since human trabecular bone cells have been shown to be capable of expressing both osteoblastic and adipocyte phenotype (Nuttall *et al.*, 1998), it is interesting to speculate that under certain conditions stromal fibroblasts, adipocytes and osteocytes may have the potential to interchange their phenotypes. The increased vascularity in IMF involved an increase both in size and number of capillaries and sinusoids. This increase in vascularity in IMF has been associated previously with intravascular haemopoiesis (Thiele *et al.*, 1997). A role for megakaryocytes has been proposed in causing both increased vascularity and fibrosis, since megakaryocytes release a variety of growth factors, including endothelial cell growth factor (ECGF) epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and transforming growth factor β_1 (TGFβ_1). This would fit the observation that in the marrow of patients with IMF (data not shown) an increase in megakaryocyte numbers was observed. Co-culture experiments could be carried out to investigate this further by culturing megakaryocytes from normal and IMF marrow in the presence of an established fibroblastic stromal cell layer. This would determine whether megakaryocytes isolated from IMF patients result in the formation of fibrosis.

9.7 Bone Marrow Stroma in Patients With Acute Leukaemia, Before and After Chemotherapy

It is well documented that chemotherapeutic agents used in the treatment of many malignancies, including the acute leukaemias, result in suppression of normal haemopoiesis. After treatment, haemopoietic regeneration is unpredictably varied between patients (Wilkins *et al.*, 1993). The mechanisms accounting for this variation in haemopoietic regrowth are unknown at present, although the importance of bone marrow stroma in support and regulation of normal haemopoiesis is well documented (Singer *et al.*, 1985). In this part of my study, the cellular composition of bone marrow stroma was investigated before and after treatment for acute leukaemia, in order to identify underlying cellular and immunophenotypic changes that may account for the post-treatment haemopoietic variation.

Analysis of stromal fibroblasts throughout the marrow before and after chemotherapy for both AML and ALL showed that, in post-treatment biopsy sections, there is in

many cases a marked decrease in number of L-NGFR positive stromal fibroblasts compared with normal. Parallel to this, it was observed that the number of stromal fibroblasts expressing αSMA greatly increased following chemotherapy for both AML and ALL, compared with normal. This suggests that, in the acute leukaemias, following treatment with these chemotherapeutic agents, there is an induced change in the fibroblastic component of bone marrow stroma from essentially normal, L-NGFR positive stromal fibroblasts to pathological, αSMA positive myofibroblasts. Whether these are the same cells changing phenotype or whether they represent two different populations remains to be seen. The results obtained with sequential post-chemotherapy biopsy sections clearly show that as haemopoietic activity recovers, expression of αSMA gradually decreases and expression of L-NGFR gradually increases. This was most noticeable with the results obtained from patients with ALL.

The significance of changes in αSMA and L-NGFR remains to be seen, but it would be in keeping with a "wound healing" type of response following stromal injury, as αSMA positive myofibroblasts are essential for such responses at other body sites. It would be interesting to determine whether these αSMA positive myofibroblastic marrow stromal cells have a normal or reduced ability to support haemopoiesis. It may be likely that they have a reduced ability to support haemopoiesis since myofibroblasts can be found in fetal marrow, which is essentially non-haemopoietic as the majority of haemopoietic activity occurs in the liver until post-natal life. Studies could be carried out to investigate the ability of these cells to support haemopoiesis by obtaining aspirated bone marrow post-chemotherapy, isolating either αSMA or L-NGFR positive cells and establishing a stromal cell layer. Co-culture experiments could then be carried out in the presence of CD34+ haemopoietic cells to determine the ability of the stromal layer to support functional haemopoiesis. However, these experiments would be hindered by technical limitations relating to the quality of the samples. During the period of this study a small number of aspirated bone marrow biopsies were obtained post-chemotherapy but a confluent stromal layer did not form in any of the samples. This may be due to insufficient numbers of viable cells or may reflect a reduced ability of these cells to grow in culture.

A previous study investigating the effects of chemotherapy on the bone marrow suggests that cytotoxic agents, many of which target proliferating cells, also exert their effects on bone marrow stroma by impairing the ability of stroma to support the proliferation of haemopoietic cells (Uhlman *et al.*, 1991). Our results also show that, although the stroma may appear physically unaffected by chemotherapy, immunophenotypic changes can be found that could reflect fundamental functional changes that, from clinical observations, may contribute to their diminished ability to support haemopoiesis. If it could be identified that these cells cannot adequately support functional haemopoiesis, this could provide an insight into the time course of haemopoietic regeneration following therapy for acute leukaemia. It may be possible to identify a relationship between reduction in L-NGFR expression and/or increase in αSMA expression with the time taken for haemopoietic recovery.

In addition to changes of the fibroblastic component of bone marrow stroma, a reduction in stromal macrophage number was identified in patients with both AML and ALL prior to treatment. The number of macrophages subsequently increased following chemotherapy, returning to normal levels in most patients after the third course. However, after treatment, the macrophages were noticeably larger and more rounded, possibly as a direct action of the chemotherapy on the macrophages or, more probably, in response to increased phagocytic activity in the presence of leukaemic cell death due to the chemotherapy. Analysis with VS38C antibodies, reactive with the rough endoplasmic reticulum-associated antigen p63 identified that, after chemotherapy, a large number of osteoblasts were present surrounding trabecular margins and a large number of plasma cells surrounded blood vessels and sinusoids. These features were not seen prior to treatment in sections from any of the biopsies but were common in post-treatment biopsy sections. The presence of osteoblasts reflects new bone formation, probably stimulated by cessation of chemotherapy. Such bone regeneration suggests that chemotherapy is causing stromal cell injury that includes temporary disruption of normal trabecular bone turnover. After chemotherapy, a period of increased bone remodelling then occurs at trabecular surfaces to make up for reduced osteogenesis during the period of treatment.

9.8 SUMMARY

In summary, the methods used to isolate, culture and characterise human bone marrow stromal cells from clinical samples of aspirated bone marrow were optimised to enable investigation of their immunophenotypic characteristics and some aspects of their function. Stromal components of bone marrow were isolated by MACS using antibodies reactive with L-NGFR, CD34, CD31 and CD14. The use of multi-step sequential MACS enabled prior depletion of one stromal cell type before enrichment of another to characterise these cells further and investigate possible origins of some of the stromal components of bone marrow shown in previous diagrams (Figures 9.1-9.3). These diagrams have been collated and a summary diagram is shown in Figure 9.4. A miniaturised cell culture system was developed to enable the small numbers of stromal cells isolated from each sample to be cultured successfully. This cell culture system uses porous polyethylene terephthalate (PET) membranes on which the cells are cultured to enable diffusion of nutrients and growth factors from the culture medium to both sides of the growth surface. Since only a limited amount of material is available from the clinical bone marrow samples, and therefore only a small number of cultures can be generated from each, double immunocytochemistry was optimised towards the latter part of the study. This will enable more phenotypic information to be obtained from each bone marrow sample in future experiments.

In situ hybridisation (ISH) was investigated to identify the presence of mRNA encoding L-NGFR before and after MACS and pre-and post-culture. This was, however, largely unsuccessful and will require further experimentation to determine optimal hybridisation conditions. Northern blot analysis was optimised as an alternative to ISH for the detection of L-NGFR mRNA following culture of stromal cells. A non-isotopic chemiluminescent detection system was chosen for these experiments, for both safety reasons and the high degree of sensitivity that can be achieved. The optimisation of Northern blot analysis was successful but application was limited because of the very limited number of clinical samples at this stage of the study. The major disadvantage of Northern blot analysis is the requirement for prior RNA extraction. As a result, the spatial distribution of stromal cells that synthesise L-NGFR mRNA could not be investigated. Further experiments to optimise ISH would be advantageous to overcome this problem.

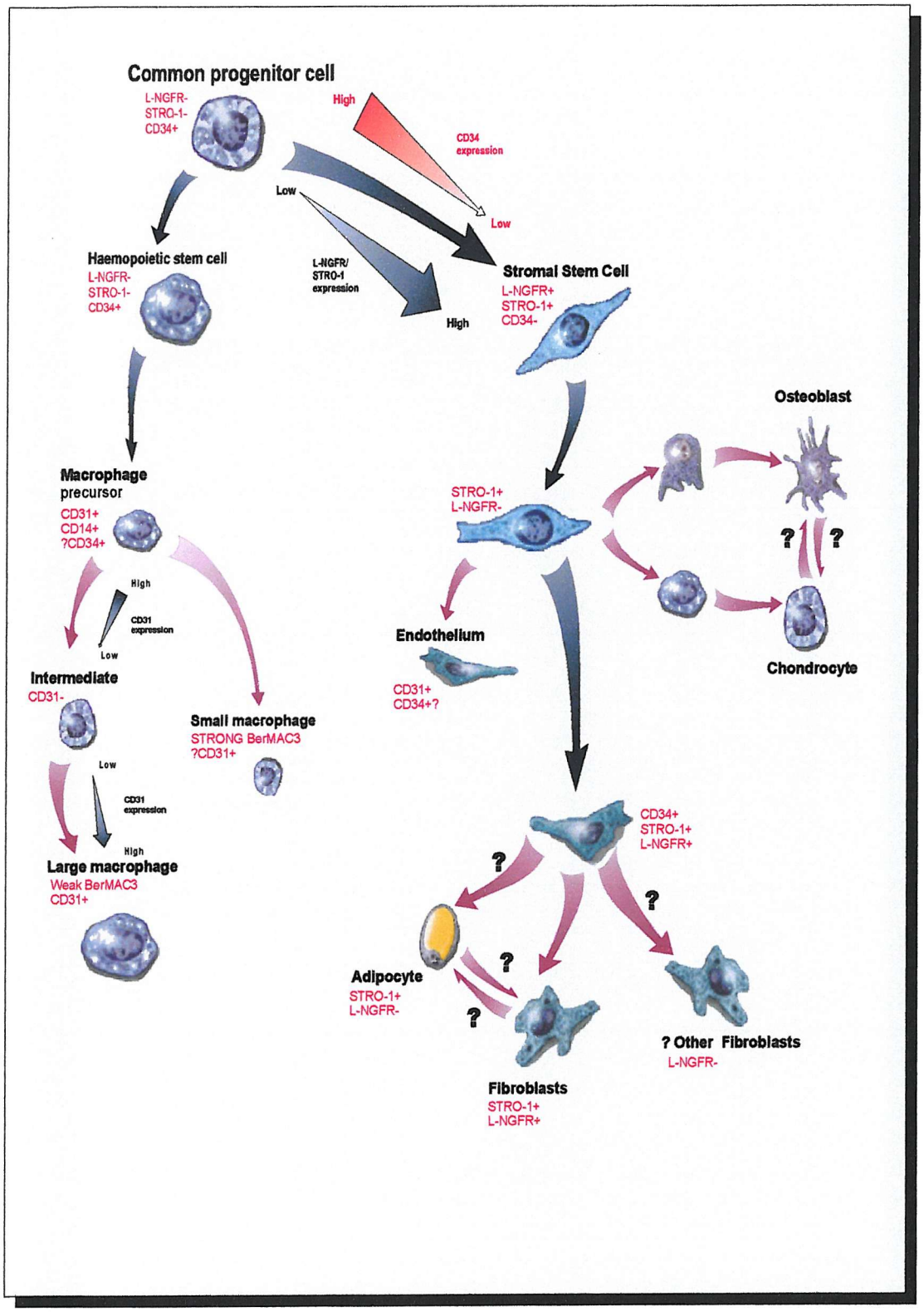


Figure 9.4. Summary diagram illustrating hypothetical patterns for differentiation and expression of stromal cell-associated antigens, from undifferentiated multipotential stem cells to bone marrow stromal cells. Cell images courtesy of Osiris Technologies Inc.

A major limiting factor throughout this study was the small number of aspirated bone marrow samples available. For this reason, the A875 human melanoma cell line was identified and obtained for use in all optimisation experiments requiring cells with abundant L-NGFR expression. In terms of functional analyses, had more samples been available, the requirements of individual stromal cell populations to support haemopoiesis would have been investigated using recombination experiments. In these experiments, varying combinations of MACS-separated stromal cells would be isolated and cultured together over a fixed period of time. Haemopoietic progenitor cells isolated on the basis of CD34 expression and stored frozen prior to culture, would be added to the pre-formed stromal layer and the formation of haemopoietic colonies assessed. The aim of this would be to determine which of the main stromal elements are essential for successful haemopoiesis *in vitro*.

Another limiting factor was the variability of MACS enrichment. The precise reasons for this are not clear although there may be a number of contributory factors which, together, may account for this variability. Firstly, the volume and quality of each clinical sample is different, as is the proportion of stromal cells. Secondly, enrichment is optimal at 4°C. While all incubations were carried out at this temperature, the laboratory temperature was invariably higher than this and may account for some variation in enrichment. An alternative technique would have been Fluorescence-activated cell sorting (FACS). While this is a sensitive method for the isolation of small cell populations, it is a very time consuming technique and facilities for sterile FACS sorting were not readily available for use in this study. In addition, cell sorting using FACS can result in reduction in the number of viable cells after sorting. Since the sizes of the clinical samples used were already small, this may have significantly reduced the number of viable cells remaining after sorting. However, had the facilities been available, it would have been interesting to address possible differences between MACS and FACS techniques.

Immunohistochemical analyses of bone marrow stroma have identified a number of differences in the composition of stroma in diseases such as IMF, AML and ALL compared with normal. The results of these studies, in combination with stromal cell characterisation in cell culture experiments, have provided insight into possible mechanisms to account for such changes. One of the main findings was the

phenotypic change that occurs in bone marrow fibroblasts after chemotherapy for acute leukaemia. With each course of chemotherapy, a progressive decline in L-NGFR expression by stromal fibroblasts was observed, paralleled by a progressive increase in expression of αSMA. This indicates a replacement of L-NGFR positive stromal fibroblasts with αSMA positive myofibroblasts. However, it was not clear whether the same cell type expressed both L-NGFR and αSMA or whether they represented two separate populations. If it could be shown that these αSMA positive myofibroblasts have an impaired ability to support haemopoiesis, this may provide a key explanation for the impaired haemopoiesis that occurs after chemotherapy.

One way in which this could be studied *in vitro* would be to investigate stromal cell cultures and find ways to favour growth of L-NGFR+ stromal fibroblasts over αSMA positive fibroblasts. Stromal cell cultures investigated in this study contained a proportion of fibroblastic stromal cells that expressed αSMA. This was accompanied by the observation that in some cultures, cells from granulocytic or monocytic cell lineages were present but erythroid cells or megakaryocytes were never observed. Since cells expressing αSMA are not present in normal marrow (Wilkins & Jones, 1998), it would be interesting to see whether cultures generated from L-NGFR+/αSMA- stromal fibroblasts favour growth of erythroid cells and megakaryocytes and perhaps support the growth/differentiation of a wider array of haemopoietic cell lineages.

In conclusion, the major findings resulting from this study are outlined below:

- Stromal components were separated from aspirated bone marrow using MACS/ multi-step sequential MACS and antibodies reactive with L-NGFR, STRO-1, CD14, CD31 and CD34. They were cultured using a miniaturised cell culture system comprising porous membrane inserts. Stromal cells were characterised using single and double immunocytochemistry and Northern blot analysis.

- Morphological and immunophenotypic characterisation of bone marrow stromal cells has provided some insight into the possible functions/ origin of these cells.

- Stem cells mobilised from bone marrow to peripheral blood do not include cells that have a stromal fibroblastic immunophenotype or that are capable of stromal reconstitution under hLTBMC conditions *in vitro*.

- Bone marrow stroma from patients with IMF contained a reduced number of adipocytes and increased number of αSMA+ myofibroblasts. In the majority of biopsy sections, expression of L-NGFR by stromal fibroblasts was high. In a small proportion, L-NGFR expression was low. It is unknown whether expression of L-NGFR may correlate with disease prognosis.

- A significant correlation was found between CD34 expression by leukaemic blast cells and AML subtype. Expression of CD34 was highest in more primitive AML subtypes (AML-M0/M1) and progressively decreased towards more mature AML subtypes (AML-M5).

- After chemotherapy for AML/ALL, the bone marrow stroma contained an increase in macrophage size and number, accumulation of osteoblasts around trabecular margins and plasma cells around blood vessels.

- As the number of courses of chemotherapy increased, expression of L-NGFR by stromal fibroblasts decreased. This was paralleled by an increase in expression of αSMA by these cells. If αSMA positive stromal cells can be shown to have a reduced capacity to support haemopoiesis, this may provide an explanation for the impaired haemopoiesis that occurs after chemotherapy.

APPENDIX ONE: DETAILED METHODS

A1.1 BIOTIN LABELLING OF OLIGONUCLEOTIDE PROBES

AIM

Oligonucleotides dT and dA are used for *in situ hybridisation* as positive and negative controls respectively. They are supplied unlabelled and must be labelled prior to use. Biotin labelled probes were detected using a three-stage streptavidin/biotinylated alkaline phosphatase technique.

METHODS

Biotin Labelling

Label 10 sterile (autoclaved) microcentrifuge tubes, five for dT and five for dA. This amount utilises the full amount of TdT and one aliquot of biotin-16-dUTP supplied with the kit.

1. Add the following into the autoclaved microcentrifuge tubes **in the order given**. This will yield approximately 100µl of labelled product. Spin the microcentrifuge tubes after each addition. Do four of the dT and four of the dA before doing the fifth to allow for any evaporation.
   i) 5µl biotin-16-dUTP (Boehringer: 1mM solution)
   ii) 5µl oligo dT, dA or specific probe (1µg/5µl)
   iii) 2.5µl of 1M tris-HCl (pH 6.8)
   iv) 3.3µl TdT (Gibco: as supplied)
   v) 16.7µl TdT buffer (Gibco: as supplied)
   vi) 47.5µl sterile DEPC-H_2O
2. Mix the contents of each tube thoroughly and incubate for 2-3 hours in a waterbath at 37°C. Stand the tubes carefully in a holder to prevent water reaching the neck of the microcentrifuge tubes.
3. Terminate the labelling by adding 3µl of 0.25M EDTA (prepared two days before in a sterile 25ml universal container).

Sephadex column separation of biotinylated probes

This must be carried out to separate biotin labelled oligonucleotides from unlabelled probe fragments and unattached biotin-dUTP molecules. Ten columns are required. Prepare each column as described below.

4. Take a sterile 1ml syringe and remove the plunger.
5. Roll a small amount of sterile quartz wool into a ball and insert into the barrel of the syringe using the plunger.
6. Attach the syringe nozzle to a length of well fitting tubing such as pharmaceutical quill and attach the other end to a 50ml syringe. Use silicone wax if necessary to ensure an airtight fit.
7. Place the open end of the 1ml syringe into the sephadex beads and pull hard on the 50ml syringe to draw the sephadex into the 1ml syringe until it is packed full with sephadex.
8. Place the filled 1ml syringe into a 15ml round-bottomed scintillation tube for centrifugation.

When ten columns have been prepared:

10. Pack the sephadex columns by centrifuging at 2970rpm at room temperature. 2 min
11. Equilibrate the columns with 1xTE by adding 100µl 1xTE buffer and centrifuging at 2970rpm. 4 min
12. Repeat step 11 four times to have completed five centrifugations throughout steps 10 and 11.
13. For the sixth centrifugation, place a sterile 1.5ml microcentrifuge tube in each round-bottomed tube beneath the sephadex column before adding 100µl 1xTE buffer and centrifuging at 2970rpm. This is done to check the 100µl elutes. 4 min
14. Discard the microcentrifuge tubes and replace with fresh empty ones, marked clearly with the probe that is to be separated.

15. Place a 100µl aliquot of the labelled probe, prepared as above, onto the top of each column and centrifuge at 2970rpm, collecting the eluent into the microcentrifuge tube. 4 min

16. Divide into 10µl aliquots and store frozen at -20°C. One aliquot contains sufficient amount of labelled probe for one section or patch of cultured cells.

A1.2 DOT BLOT HYBRIDISATION TO TEST PROBE LABELLING EFFICIENCY

AIM

The labelling efficiency of TdT as described in oligonucleotide probe labelling varies. A dot blot hybridisation can be carried out to ensure that the oligonucleotide probes are labelled to a sufficient extent to enable detection with streptavidin/biotinylated alkaline phosphatase when used for *in situ* hybridisation or Northern blot analysis.

METHODS

1. Label 6 sterile microcentrifuge tubes 1 to 6, for each probe used.
2. Into each tube, except number 3, place 36µl of 50mM Tris, 0.3M NaOH buffer (pH7.5) containing 1.5mg/ml sheared salmon sperm DNA prepared as described in Appendix 2.
3. Into tube 3, place 38µl of the above buffer.
4. Add 4µl probe to tube 1 at the concentration that would normally be used. Mix well and transfer 4µl to tube 2. Mix the contents of tube 2 and transfer 4µl to tube 4, not tube 3. Mix the contents of tube 4 and transfer 4µl to tube 5. Tube six is used as a negative control and has no probe added.
5. To tube 3, add 2µl from tube one and mix.
6. The dilutions produced are as described below:

Tube	1	2	3	4	5	6
Dilution	1/10	1/100	1/200	1/1 000	1/10 000	----------

7. Cut a square of nitro-cellulose membrane to fit into a black staining box.
8. On the nitro-cellulose membrane, mark in pencil the name of each probe at the top and down the side mark 1-6 for each dilution.
9. Put 1µl from each microcentrifuge tube into the appropriate place on the nitro-cellulose membrane.

10.	Place the membrane between sheets of blotting paper and between paper towels and incubate at 80°C. Use a flat weight to prevent the membrane curling in the oven.	2 hours
11.	Turn on the water-bath for step 11 and prepare dot-blot blocking solution by dissolving 2.9g BSA in 100ml TBS (pH 7.6) and adding 50µl Tween 20.	
12.	Remove the membrane from the oven and place in a black staining box, add 20ml of the above blocking solution and incubate at 50°C in a water-bath.	20 min
13.	Bake again as in step 9.	20 min
14.	Prepare streptavidin/biotinylated alkaline phosphatase complex using 50µl streptavidin and 50µl alkaline phosphatase in 10ml 0.05M tris-HCl (pH7.6).	
15.	Remove the membrane from the oven and re-hydrate with DEPC-H_2O. Pour away and replace with 20ml blocking solution at room temperature.	10 min
16.	Pour away and replace with 10ml streptavidin/biotinylated alkaline phosphatase solution. Agitate slowly on a shaker at room temperature.	1 hour
17.	Wash with 20ml blocking solution.	3x5 min
18.	Pour away the blocking solution and replace with veronal acetate buffer (pH 9.2).	5-10 min
19.	Prepare fast red substrate solution as described in Appendix 2.	
20.	Pour away the veronal acetate buffer and replace with fast red substrate solution. Agitate at room temperature until as many dots as possible have turned pink without excessive background staining.	15-30 min
21.	Rinse thoroughly in tap water.	
22.	Bake at 80°C between blotting paper until dry. The membrane is now fixed.	

Interpretation of results

The presence of a coloured signal beyond row 1 indicates a level of labelling that should be adequate for *in situ* hybridisation. Labelling beyond row 3 indicates the likelihood of a very strong signal by *in situ* hybridisation.

A1.3 IN SITU HYBRIDISATION

AIM

In situ hybridisation is used to locate, using biotin labelled oligonucleotide probes, specific nucleic acid sequences within intact tissue sections. The biotin-labelled probes are hybridised to the nucleic acid under stringent conditions such that only complementary nucleotide sequences will hybridise to form a stable double helix. Unbound probe is washed from the tissue sections leaving only bound probe hybridised to the nucleic acid of interest. The biotin-labelled probe is enhanced using a three-stage streptavidin/biotinylated alkaline phosphatase complex, which is detected using fast red, a substrate for alkaline phosphatase. Fast red is converted to a red product in the presence of alkaline phosphatase. All stages of the protocol are carried out at room temperature unless otherwise stated.

GLASS/PLASTIC-WARE AND SOLUTIONS

All solutions should be prepared as described in the solutions Appendix using, where applicable, DEPC treated water and ISH washed glass and plastic-ware. All solutions, glass and plastic-ware must remain sterile and gloves should be worn throughout.

METHODS – DAY ONE

Pre-treatments for cytocentrifuge preparations and hLTBMC

For paraffin embedded tissue sections proceed to step 7.

1.	Allow preparations to air-dry and fix in 4% paraformaldehyde in PBS.	10 min
2.	Rinse in 1x PBS	3 min
3.	Rinse in DECP-H_2O	2 x 1 min
4.	Dehydrate through graded alcohol's (70% x 1, 100% x 2)	2 min each
5.	Allow to dry at 37°C	15 min
6.	Mark as required using a hydrophobic pen, allowing 10 min to dry before proceeding with pre-hybridisation stage (Steps 20 onwards).	

Pre-treatments for paraffin embedded tissue sections

7.	Place slides in metal staining rack and de-wax in xylene	2 x 5 min
8.	Re-hydrate through graded alcohol's (100% x 2, 70% x 1)	2 min each
9.	Transfer slides to black plastic slide box filled with DEPC-H_2O to rinse	
10.	Pour away the DEPC-H_2O and replace with 0.2N HCl	20 min
11.	Replace with 0.3% triton-x-100 in PBS	15 min
12.	Rinse in 1x PBS	1 min
13.	Rinse in 0.05M tris-HCl, pH 7.6	1 min
14.	Lay slides in staining tray, hydrated with TBS. Cover each section with 0.5ml proteinase K (2.5µg/ml in 0.05M tris-HCl, pH 7.6). Incubate at 37°C.	1 hour
15.	Return slides to staining rack and rinse in 0.2% glycine in PBS	1 min
16.	Rinse in 1x PBS	2 x 3 min
17.	Post-fix in 0.4% paraformaldehyde in PBS pre-cooled to 4°C	20 min
18.	Rinse in 1x PBS	3 min
19.	Rinse in DEPC-H_2O	2 x 1min

Slides are now ready for pre-hybridisation

Pre-hybridisation/hybridisation

20.	While slides are in paraformaldehyde, prepare sufficient ISH-hybridisation buffer to allow for both pre-hybridisation (200µl/slide) and hybridisation (50µl/slide).	
21.	Lay slides out in staining tray and cover each section with 200µl ISH-hybridisation buffer to pre-hybridise. Cover and incubate at the hybridisation temperature for the probe used.	1 hour
22.	Thaw aliquots of probe and dilute to the desired concentration in hybridisation buffer. Mix well by pipetting followed by pulse centrifugation at 13 000rpm.	
23.	After pre-hybridisation, remove excess hybridisation buffer and replace with 50µl probe solution prepared in step 22. Use 50µl hybridisation buffer as a no probe control.	

24. Cover each section with a coverslip replace the lid of the staining tray and incubate at the hybridisation temperature. Overnight

With hLTBMC, use 100µl probe solution/patch of cells and do not coverslip. This will allow for any evaporation.

METHODS – DAY TWO

Stringency Washes

25. From stock 20x SSC prepare sufficient 2x SSC and 0.2x SSC.
26. Pre-warm to room temperature/42°C as necessary.
27. Remove the slides from the overnight incubation and remove the coverslips.
28. Rinse slides in 2x SSC and place in a staining rack.
29. Immerse in 2x SSC 2 x 10 min
30. A. For oligo dT and oligo dA:
    - i. 0.2x SSC at 42°C 20 min
    - ii. 0.2x SSC at room temperature 2 x 20 min

30. B. For other oligos:
    - i. 0.2x SSC at 42°C 2 x 20 min
    - ii. 0.2x SSC at room temperature 20 min

Detection of Oligonucleotide probes

31. Lay slides in staining tray and cover each with 1ml 0.1% triton-x-100 in TBS. 15 min
32. Drain and cover with 0.5ml streptavidin solution diluted 1:1000 in TBS 30 min
33. Rinse in TBS 3 x 5 min
34. Drain and cover with 0.5ml biotinylated alkaline phosphatase solution diluted 1:1000 in TBS 30 min
35. Rinse in TBS 3 x 5 min
36. Repeat steps 32-35 for increased detection
37. Drain TBS and replace with 0.5ml veronal acetate buffer, pH 9.2 10 min

38.	Drain and replace with 1ml fast red substrate solution prepared immediately before use	15 – 20 min
39.	Rinse in DEPC-H_2O and place slides in slide rack. Rinse in running tap water	3 – 5 min
40.	Counter-stain with Mayer`s haematoxylin	1 min
41.	Rinse in tap water to blue	5 min
42.	Lay on slide tray and cover with 1 – 2 drops crystal mount. Allow to dry	Overnight
43.	Double mount in DPX and coverslip.	

A1.4 ISOLATION AND QUANTITATION OF TOTAL RNA FROM ANIMAL CELLS USING THE QIAGEN RNEASY MINI KIT

AIM

To allow the isolation of total RNA from animal cells particularly where the amount of starting material is small. This method was carried out using the RNeasy mini kit and QIAshredders from QIAGEN and is described in the handbook supplied with the kit. This kit supplied all buffers used by this method. It is of utmost importance that all appropriate measures are undertaken to avoid contamination with RNases.

METHODS

Harvesting cells

Cells grown in suspension should be centrifuged at 1100rpm. for 5 minutes to pellet the cells. A maximum of 1 x 10^7 cells can be used for each RNeasy preparation. Totally remove and discard supernatant and proceed to cell lysis. For cells grown as a monolayer, complete removal of the supernatant must be carried out before proceeding to cell lysis.

Cell lysis

Ensure buffer RLT has been supplemented with 10% β-mercaptoethanol (β-ME) before use. Once β-ME has been added to buffer RLT, the solution is stable for one month. Add buffer RLT to either the pelleted cells or the monolayer using 350µl for up to 5 x 10^6 cells or 600µl for up to 1 x 10^7 cells. Vortex or pipette to mix, until no cell clumps are visible.

Homogenisation and RNA extraction

1.	Pipette lysate directly onto a QIAshredder column sitting in a 2ml-collection tube and centrifuge at maximum speed.	2 min
2.	Add 1 volume of 70% ethanol to the homogenised lysate and mix well by pipetting. Do not centrifuge.	
3.	Apply up to 700µl of sample, including any precipitate that may have formed, to an RNeasy mini spin column sitting in a 2ml-collection tube. Centrifuge at 10,000rpm.	15 sec
4.	If the sample volume in step 3 exceeds 700µl, successively load aliquots onto the RNeasy column and centrifuge as above. Re-use the collection tube but discard the flow through after each step.	
5.	Pipette 700µl buffer RW1 onto the RNeasy column and centrifuge at 10,000rpm. to wash.	15 sec
6.	Discard flow through and collection tube. Transfer RNeasy column into a new 2ml-collection tube.	
7.	Pipette 500µl buffer RPE (diluted 1:4 with 100% ethanol) onto the RNeasy column. Centrifuge at 10,000rpm. to wash.	15 sec
8.	Pipette 500µl buffer RPE diluted as above onto the RNeasy column. Centrifuge at maximum speed to dry the RNeasy membrane.	2 min
9.	Carefully transfer the RNeasy column into a new 1.5ml collection tube. Ensure the column does not contact the flow through as ethanol will be carried over as a result and may interfere with subsequent reactions.	
10.	Pipette 30-50µl RNase-free water directly onto the RNeasy membrane. Centrifuge at 10,000rpm to elute.	1 min
11.	Repeat step 10 if the expected RNA yield is >30µg.	

RNA Quantitation and Integrity

Following RNA isolation, the concentration and purity of the RNA should be determined by measuring the absorbance at 260nm and 280nm in a spectrophotometer.

The concentration of the RNA extracted can be used to determine the amount of RNA required to load onto a formaldehyde-agarose gel for electrophoresis in order to determine the integrity of the RNA extracted and to use for Northern blot analysis. Formaldehyde-agarose gel electrophoresis is described in further protocols.

Determination of RNA yield using spectrophotometry

12	Take 2µl RNA sample from step 10/11 into a sterile microcentrifuge tube and make up to 500µl with DEPC-H_2O	
13	As a control, put 500µl DEPC-H_2O into another sterile microcentrifuge tube	
14	Vortex all and pulse centrifuge at 13 000rpm	10 sec
15	Put onto a heat block pre-heated to 65°C	10 min
16	As a zero reference for the spectrophotometer use the DEPC-H_2O control.	
17	Measure the absorbance at 260nm and at 280nm to determine the RNA yield and purity. The absorbance at 230nm may also be measured if low RNA yields are observed.	

Interpretation of spectrophotometer results

The absorbance at 260nm is used to determine the concentration of RNA in the sample. The absorbance at 280nm is used to determine the concentration of protein contamination in the sample. The 260/280 ratio demonstrates the purity of the RNA in the samples versus the amount of protein contamination. A 260/280nm ratio of 1.7-2.1 is normal; of 2.0 is considered pure RNA. Readings outside this range are indicative of excessive protein contamination.

Low RNA yields may be due to GITC contamination. This can be determined by measuring the 260/230nm ratio. Normal ratios are in the region of 1.8-2.2. Readings outside this range indicate GITC contamination and appropriate measures during the early stages of the protocol should be looked at to eliminate this contamination.

A1.5 FORMALDEHYDE-AGAROSE GEL ELECTROPHORESIS

AIM

Formaldehyde-agarose (FA) gel electrophoresis is used to separate nucleic acid molecules on the basis of their size. Nucleic acid molecules have a net negative charge and migrate throughout the agarose gel toward the anode when an electrical current is switched on. The pores within the gel retain larger nucleic acid molecules relative to smaller ones and thus smaller nucleic acid molecules travel further down the gel. FA gel electrophoresis can be used to determine the integrity of RNA following RNA extraction from cells and tissues and is also used where the RNA is to be transferred onto a nylon membrane for Northern blot analysis.

METHODS

Glassware and Gel tank preparation

All glassware must be prepared before use to ensure that it is RNase free. It must be washed with a detergent at a high temperature and rinsed. It must then be oven baked at ≥160°C for 3 hours or more. Equipment such as the gel tank that cannot be oven baked must be soaked overnight in 0.5M NaOH followed by several rinses with DEPC-H_2O.

Gel Preparation

Soak the gel equipment and treat glassware as described above. Put autoclave tape along both ends of the gel boat to hold the gel while it sets. Turn on the heat block for the preparation of RNA samples. Put the gel boat into the gel tank and the comb into the gel boat, ensuring that only about 2mm remain between the bottom of the comb and the base of the gel boat.

1. Prepare a 1% agarose gel as follows:
    i. Weigh 0.5g agarose into a conical flask (0.5g/50ml gel solution
    ii. Add 45ml DEPC-H_2O
    iii. Heat until dissolved
    iv. Cool slightly and add 5ml, 10x MOPS
    v. Cool further and add 2.5ml formaldehyde in a fume hood due to toxicity
2. Pour gel solution immediately into the gel tank and leave to set in the fume hood for 30-40 minutes. Remove any air bubbles using a pipette.
3. Prepare 350ml of 1x MOPS from 10x MOPS stock solution for use as running buffer.

RNA preparation

4. Into a sterile microcentrifuge tube add Xµl RNA sample. For an integrity gel, X= the volume of RNA sample containing 2µg RNA. For Northern blot analysis, X= the volume of RNA sample containing 10µg RNA. Repeat for each RNA sample.
5. Into a separate sterile microcentrifuge tube add the appropriate volume of RNA markers containing 2µg RNA both for integrity gels and for Northern blot analysis.
6. To each microcentrifuge tube add Xµl (an equal volume) load buffer prepared in a fume hood as described below:
    i. 0.75ml formamide
    ii. 0.15ml 10x MOPS
    iii. 0.24ml 37% formaldehyde
    iv. 0.1ml DEPC-H_2O
    v. 0.1ml glycerol
    vi. 0.08ml 10% w/v bromophenol blue
7. Vortex all and pulse centrifuge for 10 seconds.
8. Heat on a heat block for 15 minutes at 60°C.
9. If the gel is to be used to determine RNA integrity following RNA extraction, add 1µl, ethidium bromide (1mg/ml). If the gel is to be used for Northern transfer onto nylon or nitrocellulose membrane omit the addition of ethidium bromide as this can hinder the transfer of nucleic acids from the gel.

10. Carefully remove the comb from the gel and add the running buffer prepared in step 7 until the level of running buffer just exceeds the height of the gel.
11. Load the samples into the gel and run the gel for 60 minutes at 100V, or until the samples are approximately three-quarters the way down the gel.
12. Once the gel has run sufficiently, gels prepared as above integrity gels may be viewed and photographed using an ultra-violet light source, or if the gel is to be used for Northern blot analysis this protocol may now be followed

Determination of RNA integrity

The integrity of total RNA extracted from tissues and cells can be determined using the FA gel described above. Upon viewing with an ultra-violet light source, the relevant ribosomal RNA species will appear as sharp bands on the gel. The upper 28s ribosomal RNA band (human/mouse RNA) should be present in approximately twice the amount of 18s (lower) ribosomal RNA band. If the bands appear as a smear towards smaller sized RNA species, it is likely that the RNA has been degraded throughout preparation. Likewise, if the bands appear reversed, i.e. the 18s RNA band appears in approximately twice the amount of the 28s RNA band, this is indicative of degraded RNA since the 28s ribosomal RNA degrades into and 18s-like ribosomal RNA species.

A1.6 NORTHERN BLOT ANALYSIS

AIM

Northern blot analysis is a technique whereby RNA molecules are transferred from a gel, following gel electrophoresis onto a piece of nitrocellulose or nylon membrane. This membrane is hybridised with labelled probe specific for the RNA sequence of interest. The labelled probe is detected using an appropriate detection method depending on the label used. Probes used in this protocol are labelled with biotin since they can be detected using a chemiluminescent detection system such as CDP-star. Northern blot analysis is not used for the location of specific RNA within tissues but instead offers a more sensitive method by which RNA sequences in specific cell types can be identified.

METHODS – DAY ONE

Northern Transfer

Prior to starting, the RNA must be extracted, the integrity determined and then size fractionated by gel electrophoresis. Details can be found in other protocols. It is vital throughout this protocol, as with other RNA methods, to ensure that all glassware is washed well with a detergent, rinsed and baked at ≥160°C for at least three hours. All plastic-ware as well as the vacuum blotting equipment must be soaked overnight with 0.5M NaOH (20g/1000ml) and rinsed well with DEPC-H2O.

1.	Soak the gel equipment and treat glassware as described above.	
2.	Once the gel has run sufficiently following gel electrophoresis, remove the gel from the gel tank and carefully remove one corner for orientation using an RNase free scalpel.	
3.	Wash the gel in an RNase free container with 200ml DEPC-H_2O	10 min
4.	Drain and wash the gel with 100ml 50mM NaOH	20 min
5.	Drain and wash the gel with 200ml DEPC-H_2O	10 min
6.	Drain and wash the gel with 100ml 10x SSC	2 x 10 min
7.	Soak the Nylon membrane in 20x SSC	5 min

8. Prepare the vacuum blotting equipment and rinse the porous membrane support in DEPC-H_2O.
9. Place the porous membrane support onto the vacuum blotter. Lay the nylon membrane onto this followed by the plastic mask. Ensure the plastic mask overlaps with the membrane. Place the gel onto the plastic mask ensuring that this overlaps with the mask. This is important to ensure a vacuum is created.
10. Remove any air bubbles beneath the gel before sealing the edges and filling in the wells with a 1% agarose solution.
11. Switch on the vacuum blotter pump and adjust to 50mbar pressure.
12. Cover only the gel with 0.25M HCl — 7 min
13. Blot off and replace with denaturation solution — 7 min
14. Blot off and replace with neutralising solution — 7 min
15. Immerse gel in transfer solution (20x SSC) — 1 hour
16. Switch off the pump and pour away the 20x SSC and remove the blot. Place the blot in 20x SSC to rinse.
17. Blot dry between several sheets of filter paper. Repeat.
18. Fix the membrane in an oven pre-heated to 80°C — 30 min
19. The blot may be stored at 4°C between sheets of blotting paper in a sealed bag.

METHODS- DAY TWO

Hybridisation

20. Wet the membrane in 0.25M disodium phosphate, pH 7.2
21. Pre-hybridise in 20ml northern blot hybridisation buffer at the hybridisation temperature (70°C for L-NGFR oligonucleotide probes). — 1 hour
22. Drain and replace with hybridisation buffer containing biotinylated probe (40pmole/20ml). Incubate at the hybridisation temperature. — 2 hours

Stringency washes

23.	Drain the hybridisation buffer and wash the membrane at room temperature in 2x SSC, 1.0%SDS. Use 1ml/cm^2	2 x 5 min
24.	Drain and wash at the hybridisation temperature in 1x SSC, 1.0%SDS. Use 1ml/cm^2	2 x 15 min
25.	Drain and wash at room temperature in 1x SSC. Use 1ml/cm^2	2 x 5 min

Membrane blocking and chemiluminescent detection

26.	Wash the membrane in blocking buffer. Use 0.5ml/cm^2	2 x 5 min
27.	Replace and incubate in blocking buffer. Use 1ml/cm^2	10 min
28.	Dilute Avidx-AP conjugate, supplied with the CDP-Star kit, 1:5000 in blocking buffer using 0.1ml/cm^2. Incubate with constant agitation.	20 min
29.	Drain and wash the membrane in blocking buffer using 0.5ml/cm^2	5 min
30.	Wash the membrane in wash buffer (1ml/cm^2)	3 x 5 min
31.	Wash in 1x assay buffer (0.25ml/cm^2). Prepare from 10x assay buffer supplied with the CDP-star kit.	2 x 2 min
32.	Drain the blot by touching a corner on a paper towel and place on saran wrap on a flat surface. Do not let the blots dry.	
33.	Pipette a thin layer of CDP-star onto the blot (3ml/100cm^2) and incubate	5 min
34.	Drain excess CDP-star and wrap in saran wrap.	

Exposure

Place the wrapped membrane in direct contact with standard X-ray film in a development folder. Exposure times may vary from between 1-30 minutes depending on the intensity of the chemiluminescence. This will vary depending on the amount of specific RNA present.

A1.7 LYMPHOPREP SEPARATION OF LEUKOCYTES FROM BONE MARROW ASPIRATE SAMPLES

AIM

Bone marrow aspirate samples obtained from donors contain a mixture of erythrocytes, leukocytes and platelets of which only the leukocytes are of any use for the establishment of cell cultures. These may be separated from the sample by lymphoprep separation as described here. All stages of the protocol should be carried out in a class II hood where samples from patients with haematological and other malignancies are being used.

METHODS

1. Add 5ml lymphoprep to a 20ml sterile universal container.
2. Using a pipette, carefully layer the aspirated bone marrow down the side of the 20ml universal container onto the lymphoprep ensuring the marrow sample does not mix with the lymphoprep.
3. Centrifuge at 1970rpm. for 30 minutes to produce a erythrocyte cell pellet at the bottom of the tube, a clear layer of lymphoprep, an opaque interface layer containing the leukocytes and a layer on top containing plasma and medium.
4. Use a sterile pasteur pipette to harvest the leukocytes from the interface layer between the lymphoprep and the plasma. Collect into a new 20ml sterile universal container.
5. Fill the universal container with medium and centrifuge at 11 000rpm. for 5 minutes.
6. Remove the supernatant and re-suspend the pelleted leukocytes as required.

A1.8 ESTABLISHMENT AND MAINTENANCE OF HUMAN LONG-TERM BONE MARROW CULTURES

AIM

To establish and maintain human long-term bone marrow cultures (hLTBMC). All cell cultures of human bone marrow cells used in this study were cultured under hLTBMC conditions as described here. All stages of the method described here are carried out in a class II hood due to the use of clinical bone marrow samples from patients with a variety of haematological malignancies.

METHODS

Iscove`s modified Dulbecco`s medium (Iscove`s medium; IM) is used for the culture of human bone marrow cells. This is first treated with antibiotics to prevent infections.

Treatment of IM with antibiotics

1. To 500ml IM, add the following:
   i. 1ml benzylpenicillin (300mg/500ml)
   ii. 1ml Streptomycin (50mg/500ml)

Preparation of culture medium

Prepare culture medium as described below. For 10ml:

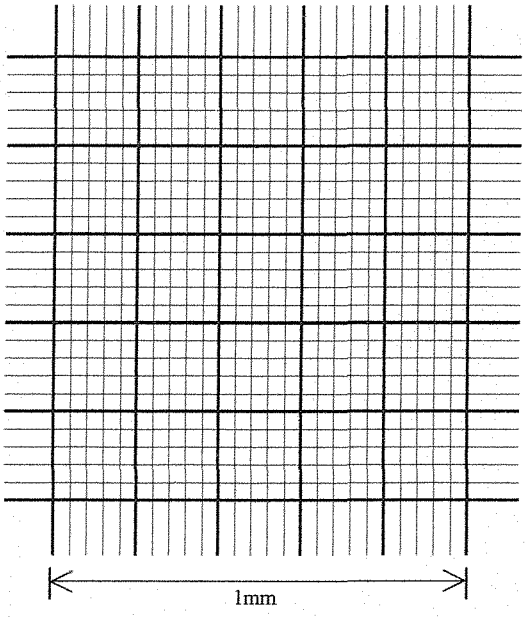
i.	Iscove`s medium	7.9ml
ii.	Horse Serum (10%)	1.0ml
iii.	Foetal calf serum (10%)	1.0ml
iv.	Hydrocortisone Solution* (1%)	100µl

*Hydrocortisone as sodium hemisuccinate (5×10^{-5}M) in sterile distilled water stored frozen at -20°C in 500µl aliquots.

2. Dilute the required number of cells in the appropriate volume of cell culture medium. For the initial establishment of a hLTBMC, an initial cell concentration of $1 - 2 \times 10^6$ cells/ml of cell culture medium is required.

Cell counting

The number of viable cells in a given sample can be determined with the use of a standard haemocytometer. Pipette 50µl cell suspension into a bijou tube and add 50µl trypan blue. Prepare haemocytometer by applying a drop of water to each side of the chambers and applying a coverslip. Pipette a small amount of the cell/trypan blue mixture under the coverslip to fill both of the chambers. Count the number of viable cells in both grids by light microscopy. Non-viable cells are denoted by the presence of trypan blue staining within their cytoplasm. Calculate the number of viable cells per millilitre cell suspension as described below.



Calculation of Number of cells/ml:

THEORY
*Area of smallest square = $1x10^{-4}mm^2$
*Area of one grid = $1mm^2$
*Depth between grid and coverslip = 0.1mm
*Volume of solution between grid and coverslip = 0.1mm x $1mm^2$ = $0.1mm^3$

To account for dilution actor multiply No. cells in grid by 10 000 = $0.1mm^3$ x 10 000 = $1000mm^3$ = No. cells in 1ml

To account for equal dilution with trypan blue solution, multiply No. cells in one grid by 20 000 or No. cells in two grids by 10 000

PRACTICE
1. Count No. cells in both grids (Or equivalent)
2. Multiply No. cells in both grids by 10 000

Volumes of cell culture medium required

The volume of cell culture medium required for the establishment of cell cultures is dependent upon the container in which the cells are to be cultured. Following lymphoprep separation, the cell pellet should be re-suspended in the following volumes.

4ml Slide flask

i. 4ml cell culture medium

8-well slide flask

i. 500µl/well cell culture medium

ii. Hydrate flask by filling remaining wells with 500µl IM

96-well plate

i. 250µl/well cell culture medium

ii. Hydrate plate by adding 250µl IM to immediately surrounding wells

24-well cell culture inserts

i. Prepare 350µl/insert cell culture medium

ii. Add 900µl cell culture medium to well

iii. Add insert to well using sterile forceps. Insert at an angle to prevent the introduction of air bubbles under the insert.

iv. Pipette the 350µl cell suspension into the insert.

Culture conditions

All cell cultures are initially incubated at 37°C, 5% CO_2 overnight with flask lids applied loosely to allow the entry of the CO_2 into the flask. The lids of culture flasks are tightened and transferred to 33°C incubator. Culture flasks/plates with loosely fitting lids are left at 37°C in 5% CO_2 for incubation during culture.

Feeding of cells

Cells are initially left for two weeks without feeding to prevent disruption while cells adhere. After two weeks feed cells by replacing half the initial volume of cell culture medium with freshly prepared cell culture medium.

A1.9 MAGNETIC ACTIVATED CELL SORTING (MACS)

AIM

Immunomagnetic cell separation techniques such as MACS may be used to isolate cell types on the basis of the antigens they express. This is of particular use for the investigation of growth patterns and functional characteristics of particular cell types. This protocol was been adapted from that supplied with the MACS kit (Miltenyi Biotec Ltd. Surrey, UK) for use with the isolation of stromal cells from bone marrow aspirate samples. As with all cell culture work involving clinical samples, all stages of this protocol were carried out in a class II hood.

METHODS

Antibody incubations

1. Harvest leukocytes from bone marrow aspirate sample by method shown in Appendix A1.7
2. Re-suspend cell pellet in 4ml IM and transfer to a 5ml sterile collection tube.
3. Wash by centrifugation at 11000rpm for 5 minutes at 4°C.
4. Remove supernatant and apply 300µl primary antibody diluted as appropriate in PBS supplemented with 0.5% w/v BSA (PBS/BSA).
5. Incubate at 4°C in primary antibody for 20 minutes with gentle agitation after 10 minutes to re-suspend cells.
6. Add 4ml cold PBS/BSA and wash as in step 3.
7. Remove supernatant and add MACS Ig-G conjugated micro-beads diluted 1:7 with PBS/BSA.
8. Incubate for 15 minutes at 4°C.
9. Add 4ml cold PBS/BSA and wash as in step 3.

MACS separation

10. Pass 500µl PBS/BSA through the MACS column to remove hydrophobic coating.
11. Remove supernatant from cells and re-suspend in 500µl cold PBS/BSA.
12. Add to the column and collect eluent as negative fraction.

13. Once this has passed through the column, pass 3 x 500µl PBS/BSA through the column to ensure any unlabelled cells are eluted. Ensure that each 500µl has passed through the column before the next is added. Collect with negative fraction.
14. Remove the column from the magnet and place in another 5ml sterile collection tube.
15. Add 1ml PBS/BSA to the column and using the plunger provided, plunge firmly to elute the labelled cells.
16. Wash both positive and negative fractions as in step 3.
17. Remove the supernatant and re-suspend in freshly prepared culture medium if cells are to be cultured. If sequential separations are to be carried out, re-suspend cells in primary antibody and follow the protocol through from step 4.

A1.10 LIQUID NITROGEN STORAGE OF VIABLE CELLS

AIM

Liquid nitrogen storage can be used to maintain cells in a viable state for many years. Cells may be frozen upon harvesting or grown in culture, trypsinised from their growth surface and then frozen. If cells are cultured prior to liquid nitrogen storage, it should be noted that upon their thawing and re-culture, their life span would be shortened due to prior culture. This is important when considering bone marrow cells cultured under hLTBMC conditions, some of which only have a life span of between one and two months.

METHODS

Trypsinisation to remove adherent cells from their growth surface

1. Liquid nitrogen storage of adherent cells requires prior removal of non-adherent cells and trypsinisation to break the bonds holding these cells to their growth surface.
2. Gently pipette supernatant up and down and remove to dislodge non-adherent cells. Discard the supernatant if not required
3. If cell adherence is expected to be a problem, add an appropriate volume of 1x Hanks buffered salt solution (HBSS) to the cells for 10 minutes at room temperature. HBSS is deficient in calcium and magnesium and will cause a net diffusion of calcium and magnesium into solution. Since these are both required for the adherence of cells to their growth surface, removal of these will weaken the adherence of the cells.
4. Discard and replace with a small volume of trypsin, just large enough to cover the growth surface. Incubate at 37°C for a maximum of 5 minutes or until the cells have dislodged.
5. Pipette the cells into a sterile 20ml universal container and add 10% foetal calf serum to inactivate the trypsin activity.
6. Fill the universal container with IM and centrifuge at 11000rpm for 5 minutes to wash.

Liquid nitrogen storage

7. Prepare freeze medium as described below allowing up to 10^7 cells/ml. Cell numbers less than this should be frozen in 1ml freeze medium.

   For 10ml freeze medium:

   i. 7.5ml RPMI

   ii. 1.5ml FCS. Invert gently.

   iii. 1ml DMSO added drop-wise while mixing with a sterile pasteur pipette.

   If added too quickly, the DMSO will cause the FCS to precipitate. If this happens discard and start again.

8. Divide into 1ml aliquots in cryotubes and wrap well in tissue paper.
9. Place in polystyrene container, filled with tissue paper and place overnight in a freezer set at -70°C. Wrapping well in tissue paper and polystyrene container will ensure that the cells are frozen slowly – approximately 1°C/minute.
10. Transfer to liquid nitrogen using thick gloves and eye protection to protect from the cold temperature of the liquid nitrogen.

Cell thawing

11. Add 3ml IM to a 5ml sterile collection tube and keep at 4°C until required.
12. Remove one aliquot of cells from liquid nitrogen and place in a water-bath pre-heated to 40°C to thaw. This should take between 1 and 2 minutes.
13. Transfer immediately to the IM prepared in step 10. Wash remaining cells by pipetting between the two containers.
14. Wash by centrifugation at 11000rpm for 5 minutes.
15. Re-suspend cell pellet as required for re-culture or other procedure.

A1.11 IMMUNOCYTOCHEMICAL STAINING OF CELL CULTURE INSERTS

AIM

To allow the detection of antigens from cells grown in cell culture inserts. This method enables detection of antigens without disruption of cells from their growth surface. Antigens are detected using an indirect immunoperoxidase technique comprising monoclonal primary antibodies, biotinylated sheep anti-mouse secondary antibodies, streptavidin and biotinylated horseradish peroxidase. The peroxidase is demonstrated using the chromogen diaminobenzidine. This method enables antigen detection without disruption of cells from their growth surface.

METHODS

1.	Fix cells by adding 100µl of cold 4% paraformaldehyde in PBS, pH 7.2.	5 min
2.	Add 100µl avidin solution to block endogenous biotin activity.	20 min
3.	Replace with 100µl biotin solution to block endogenous avidin activity.	20 min
4.	Replace with 100µl 1% BSA in TBS.	20 min
5.	Rinse with 100µl TBS.	
6.	Apply 100µl primary antibody diluted as appropriate in TBS.	60 min
7.	Rinse with 100µl TBS.	3 x 2 min
8.	Apply 100µl biotinylated sheep, anti-mouse immunoglobulin for monoclonal primary antibodies diluted 1:200 in TBS.	30 min
9.	Prepare streptavidin-biotinylated peroxidase complex for step 11 by adding the equivalent of 2µl streptavidin solution, 2µl biotinylated horse-radish peroxidase solution to 396µl TBS to produce a 1:200 dilution. Leave to complex	30 min
10.	Rinse with 100µl TBS.	3 x 2 min
11.	Apply 100µl pre-prepared streptavidin-biotinylated peroxidase complex (step 9).	30 min
12.	Rinse with 100µl TBS.	3 x 2 min

13. Prepare and apply DAB chromogen (wearing gloves due to carcinogenicity). 10 min

Preparation of DAB substrate solution

Prepare DAB substrate solution (wearing gloves due to carcinogenicity) by adding 0.25ml of 10x substrate to 2.25ml reverse osmosis water. Add 2 drops of DAB chromogen and mix. Add 1 drop of hydrogen peroxide substrate solution and mix. Add 50µl 15% sodium azide to inhibit endogenous peroxidase. This is inhibited now as oppose to earlier since hydrogen peroxide degrades unfixed, fresh tissue.

Counterstaining

14. Rinse once in 100µl TBS followed by a rinse in tap water.
15. Counter-stain with Harris haematoxylin. 1 min
16. Rinse in tap water. 1 min
17. Differentiate in 1% acid alcohol. 5 sec
18. Blue in running tap water. 5 min

Mounting

Using a scalpel, carefully remove the cell membrane from its plastic housing. Remove the membrane from the housing with tweezers taking note of the orientation of the growth surface. Apply one drop of aqueous gel mount to an APES coated, glass mounting slide. Carefully lay the membrane onto the gel mount, growth surface uppermost. Apply another drop of gel mount directly onto the growth surface and coverslip. Invert and press firmly onto filter paper to both soak excess mountant and ensure the membrane is mounted uniformly to prevent loss of optical clarity.

A1.12 IMMUNOHISTOCHEMICAL STAINING OF TREPHINE BONE MARROW BIOPSIES

AIM

To allow detection of antigens using monoclonal antibodies. These are detected using an indirect immunoperoxidase technique comprising biotinylated sheep anti-mouse secondary antibodies, streptavidin and biotinylated horseradish peroxidase. The peroxidase is demonstrated using the chromogen diaminobenzidine (DAB).

METHODS

1.	De-paraffinise sections in xylene.	2 x 5 min
2.	Re-hydrate through graded alcohols (100% x 2, 70%).	1 min each
3.	Prepare endogenous peroxidase inhibitor (0.5% hydrogen peroxide in methanol) by adding 0.2ml 30% hydrogen peroxide to 11.8ml 100% methanol. Apply 500µl/slide.	2 x 15 min
4.	Wash well in TBS and proceed with antigen retrieval below.	

Antigen Retrieval

The antigen retrieval methods described below are dependent on the primary antibody used. Antibody data sheets provide information on the optimal method of antigen retrieval for that antibody. If antigen retrieval is not required, proceed to step 5.

i. Pronase Pre-treatment

Prepare 0.05% pronase solution by adding 0.1ml stock pronase (10mg/ml) and adding 1.9ml TBS. This solution is stable for 5 days at 2-8°C. Apply 0.05% pronase solution (200µl/slide) and incubate at room temperature for 25 minutes.

ii. Microwave pre-treatment

Fill a plastic slide rack with 24 slides and place in polythene box. To maintain a constant load, 3 polythene boxes must each be loaded with a slide rack filled with 24 slides. Fill each box with 330ml 0.01M citrate buffer, prepared by adding 2.1g citric acid crystals to 1 litre reverse osmosis water and adjusting the pH to 6.0 by adding approximately 25ml 1M NaOH. Place the lid on each box and place in the microwave oven at equal distances around the microwave plate. Run the

microwave at medium power for 25 minutes (time and setting optimal for Panasonic 800watt oven). Remove boxes from the microwave, wearing thick gloves and eye protection to prevent injuries from the hot steam and fill quickly with running water. Leave in running water for 3 minutes to cool.

Antibody Incubations

5.	Place slides back in staining tray and wash in TBS.	2 x 5 min
6.	Apply primary antibody diluted as appropriate in TBS (200µl/slide). Incubate at 4°C.	Overnight
7.	Remove slides from cold and allow them to warm to room temperature.	15 min
8.	Wash slides in TBS	3 x 5 min
9.	Apply biotinylated sheep anti-mouse antibody for monoclonal primary antibodies diluted 1:200 in TBS (200µl/slide). Incubate at room temperature.	30 min
10.	Prepare streptavidin-biotinylated peroxidase complex (1:200) by adding 2µl streptavidin solution and 2µl biotinylated horseradish peroxidase solution to 396µl TBS. Leave to complex.	30 min
11.	Wash in TBS.	3 x 5 min
12.	Apply pre-prepared streptavidin-biotinylated peroxidase complex from step 10 (200µl/slide). Incubate at room temperature.	30 min
13.	Wash in TBS.	3 x 5 min

Prepare DAB substrate solution (wearing gloves due to carcinogenicity) by adding 0.25ml of 10x substrate to 2.25ml reverse osmosis water. Add 2 drops of DAB chromogen and mix. Add 1 drop of hydrogen peroxide substrate solution and mix.

14.	Apply DAB substrate solution (200µl/slide). Incubate until colour has developed adequately.	3-10 min
15.	Rinse in TBS.	
16.	Wash in running tap water.	2 min
17.	Counterstain with Harris haematoxylin.	30 sec

18.	Rinse in tap water.	1 min
19.	Differentiate in 1% acid alcohol.	5 sec
20.	Blue in running tap water.	5 min
21.	Dehydrate through graded alcohols (70%, 100% x 2) and xylene (x 2).	1 min each
22.	Mount in DPX and coverslip.	

A1.13 SINGLE-ENZYME DOUBLE IMMUNOCYTOCHEMICAL STAINING OF CELL CULTURE INSERTS

AIM

Clinical bone marrow aspirate samples contain only limited numbers of viable cells of interest available for culture. Each clinical sample is different, being either from different patients or from patients with different haematological disorders. In order to increase the amount of information available from each sample, double immunostaining allows the demonstration of two antigens from each cell culture. Where multiple cultures are established from one aspirate sample, much information can be obtained from the one bone marrow aspirate sample. This protocol allows consecutive demonstration of antigens using two detection systems. Primary antibodies may be detected using streptavidin-biotinylated HRP and different enzyme substrates. This protocol demonstrates the use of single-enzyme, double immunocytochemistry using the HRP detection systems DAB (brown) and Vector VIP (purple; Vector Laboratories Ltd., Peterborough, UK). Note that for best optical clarity, DAB should always be used as the first substrate and should be used to detect the cell type anticipated to be most abundant. All steps are carried out at room temperature.

METHODS

1.	Fix cells by adding 100µl of cold 4% paraformaldehyde in PBS, pH 7.2. This must be prepared in a fume hood due to the toxicity of the fumes.	5 min
2.	Add 100µl avidin solution to block endogenous biotin activity.	20 min
3.	Replace with 100µl biotin solution to block endogenous avidin activity.	20 min
4.	Replace with 100µl 10% BSA in TBS.	20 min
5.	Rinse with 100µl TBS.	
6.	Apply 100µl mouse, anti-human primary antibody diluted as appropriate in TBS.	30 min
7.	Rinse with 100µl TBS.	3 x 2 min

8.	Apply 100µl biotinylated sheep, anti-mouse immunoglobulin for monoclonal primary antibodies diluted 1:200 in TBS.	30 min
9.	Prepare streptavidin-biotinylated peroxidase complex for step 11 by adding the equivalent of 2µl streptavidin solution, 2µl biotinylated horseradish peroxidase solution to 396µl TBS to produce a 1:200 dilution. Leave to complex.	30 min
10.	Rinse with 100µl TBS.	3 x 2 min
11.	Apply 100µl pre-prepared streptavidin-biotinylated peroxidase complex (step 9).	30 min
12.	Rinse with 100µl TBS.	3 x 2 min
13.	Prepare and apply DAB chromogen (wearing gloves due to carcinogenicity).	10 min

Preparation of DAB substrate solution

Prepare DAB substrate solution (wearing gloves due to carcinogenicity) by adding 0.25ml of 10x substrate to 2.25ml reverse osmosis water. Add 2 drops of DAB chromogen and mix. Add 1 drop of hydrogen peroxide substrate solution and mix. Add 50µl 15% sodium azide to inhibit endogenous peroxidase. This is inhibited now as oppose to earlier since hydrogen peroxide degrades unfixed, fresh tissue.

14.	Wash in TBS.	3 x 5 min
15.	Add 100µl avidin solution to block endogenous biotin activity.	20 min
16.	Replace with 100µl biotin solution to block endogenous avidin activity.	20 min
17.	Replace with 100µl 10% BSA in TBS.	20 min
18.	Rinse with 100µl TBS.	
19.	Apply second mouse, anti-human primary antibody diluted as appropriate in TBS	30 min
20.	Wash in TBS	3 x 2 min
21.	Apply biotinylated sheep, anti-mouse biotinylated secondary antibody for monoclonal primary antibodies, diluted as before.	30 min

22.	Prepare streptavidin-biotinylated horseradish peroxidase by adding the equivalent of 2µl streptavidin solution and 2µl biotinylated horseradish peroxidase solution to 396µl TBS to produce a 1:200 dilution	
23.	Wash cells in TBS	3 x 2 min
24.	Prepare and apply peroxidase inhibitor: 15ml PBS + 150µl sodium azide (15%) and 500µl hydrogen peroxide (30%). Note this step is essential to prevent cross-reactivity between detection systems	15 min
25.	Wash cells in TBS	3 x 2 min
26.	Apply streptavidin-biotinylated horseradish peroxidase prepared in step 18.	30 min
27.	Wash in TBS	3 x 2 min
28.	Prepare and apply Vector VIP substrate solution	15 min

Preparation of Vector VIP substrate solution

To 5ml phosphate buffered saline, in ascending order, add 1 drop of solutions 1-4, mixing well after the addition of each solution.

29.	Rinse in TBS	
30.	Wash in running tap water	5 min
23.	Counterstain with Harris haematoxylin.	30 sec
24.	Rinse in tap water.	1 min
25.	Differentiate in 1% acid alcohol.	5 sec
26.	Blue in running tap water.	5 min
27.	Mount and coverslip	

Mounting

Using a scalpel, carefully remove the cell membrane from its plastic housing. Remove membrane from housing with tweezers taking note of the orientation of the growth surface. Apply one drop of aqueous gel mount to an APES coated, glass mounting slide. Carefully lay the membrane onto the gel mount, growth surface uppermost. Apply another drop of gel mount directly onto the growth surface and coverslip. Invert and press firmly onto filter paper to both soak excess mountant and ensure the membrane is mounted uniformly to prevent loss of optical clarity.

APPENDIX TWO: PREPARATION OF SOLUTIONS

A2.1 DENATURATION SOLUTION

Sodium chloride (MW 58.44)..... 1.5M (17.52g/200ml)

Sodium hydroxide (MW 40)..... 0.5M (4g/200ml)

Dissolve in 200ml DEPC-H_2O and autoclave. Store at 4°C.

A2.2 DEPC-TREATED ULTRA-PURE WATER

Ultra-pure water obtained by reverse-osmosis..... 2000ml

Diethyl pyrocarbonate (DEPC)..... 2ml

In a fume hood add the DEPC to the reverse-osmosis water. DEPC is toxic and potentially oncogenic. Take care not to contaminate the DEPC stock solution with water since it is explosive under such circumstances.

A2.3 EDTA (0.25M)

EDTA (MW 372.24)..... 0.25M (0.93g/10ml)

Dissolve EDTA in 10ml DEPC-H_2O and store at 4°C.

A2.4 DISODIUM PHOSPHATE 7-HYDRATE

Disodium phosphate..... 0.5M (13.4g/100ml)

85% Orthophosphoric acid..... (0.4ml/100ml)

A2.5 FAST RED SUBSTRATE SOLUTION FOR ALKALINE PHOSPHATASE

Veronal acetate, pH 9.2..... 20ml

Fast Red TR salt..... 10mg

Levamisole..... 5mg

Napthol AS-B1 phosphate..... 10mg

Dimethyl formamide..... 30µl

Dissolve Fast Red salt and levamisole in veronal acetate buffer. Dissolve Napthol AS-B1 phosphate in dimethyl formamide and add to the aqueous salt solution. Mix and filter prior to immediate use.

A2.6 Hybridisation Buffer for In Situ Hybridisation (5ml)

Formamide..... 2.5ml

Heat-denatured salmon sperm DNA..... 100µl (10mg/ml)

Sodium chloride (MW 58.44)..... 600µl, 5M (2.92g/10ml)

10x PE..... 500µl

50% Polyethylene glycol or 50% dextran sulphate..... 500µl

DEPC-H_2O..... 800µl

Add the above in the order given and mix well.

A2.7 Hybridisation Buffer for Northern Blot Analysis

EDTA..... 1mM (0.25ml of 0.2M EDTA)

Sodium dodecyl sulphate..... 7% (17.5ml of 20% SDS)

Disodium phosphate 7-hydrate..... 25ml of 0.25M

Make up to 50ml using DEPC-H_2O and mix well. Make up fresh each time.

A2.8 10x 3-(N-Morpholino)Propanesulphonic Acid (MOPS)

MOPS..... 200mM (20.93g/500ml)

Sodium acetate..... 50mM (2.05g/500ml)

EDTA..... 10mM (1.86g/500ml)

Dissolve the above in 450ml DEPC-H_2O, adjust pH to 7.0 using 1M NaOH and autoclave. Store in the dark (wrapped in foil) at room temperature.

A2.9 NEUTRALISING SOLUTION, pH 7.5

Tris (MW 121.14)..... 1M (24.22g/200ml)
Sodium Chloride (MW 58.44)..... 1.5M (17.52g/200ml)
Dissolve in 190ml DEPC-H_2O and adjust pH to 7.5. Correct volume and autoclave prior to storage at 4°C.

A2.10 PARAFORMALDEHYDE (0.4%) IN PBS

Paraformaldehyde..... 0.8g/200ml
This solution should be prepared fresh for immediate use. In a fume hood, add 0.8g paraformaldehyde to 200ml PBS, pH 7.6, into a screw-topped heat-proof bottle. Add a magnetic stirrer. Unscrew the lid slightly and while in the fume hood heat while stirring until the solution becomes clear. This will happen shortly before boiling but do not allow to boil. Cool the solution to 4°C prior to use. Store for a maximum of 48 hours.

A2.11 10X PHOSPHATE BUFFERED SALINE (PBS), pH 7.6

Sodium chloride (MW 58.44)..... 1.3M (75.97g/l)
Sodium dihydrogen orthophosphate (MW 156.01)..... 70mM (10.92g/l)
Di-sodium hydrogen orthophosphate dihydrate (MW 177.99)..... 30mM (5.34g/l)
Dissolve in 900ml DEPC-H_2O, adjust pH to 7.6 using sterile 1M NaOH and adjust volume to 1000ml. Autoclave prior to storage at room temperature.

A2.11 PEROXIDASE INHIBITOR FOR DOUBLE IMMUNOSTAINING

Phosphate Buffered Saline.....15ml
Sodium Azide (15%)..... 150µl
Hydrogen Peroxide (30%).....500µl
Mix well and use immediately

A2.12 50% Polyethylene Glycol and 50% Dextran Sulphate

Dissolve 5g polyethylene glycol or 5g dextran sulphate in 5-6ml DEPC-H_2O. For dextran sulphate solution, use a roller-mixer to agitate the viscous solution. Adjust volume to 10ml and store at 4°C.

A2.13 10x PVP-EDTA (PE)

0.5M Tris-HCl, pH 7.5.....500ml
Sodium pyrophosphate..... 1% (5g/500ml)
Polyvinylpyrolidone (MW 40 000)..... 2% (10g/500ml)
Ficoll (MW 400 000)..... 2% (10g/500ml)
EDTA (MW 372.24)..... 50mM (9.31g/500ml)

Dissolve the above with heating at 65°C. Maintain at this temperature for 15 minutes. Store at 4°C. This solution has a pale yellow colour which becomes slightly darker with time.

A2.14 Sheared Salmon Sperm DNA

Using a sterile scalpel blade, cut 100g salmon sperm DNA from the "skein" in which it is purchased. Dissolve in 10ml DEPC-H_2O overnight using a roller-mixer. Shear by sonication (MSE 150 sonicator), using 20-30 cycles of 30 seconds each, cooling the solution on ice between cycles. Store at -20°C in 100µl aliquots.

A2.15 20x Standard Saline Citrate (SSC), pH 7.0

Sodium chloride (MW 58.44)..... 3M (175.32g/l)
Tri-sodium citrate (MW 294.10)..... 0.3M (88.23g/l)

Dissolve in 900ml DEPC-H_2O, adjust pH to 7.0 using sterile 1M NaOH and adjust volume to 1000ml. Autoclave prior to storage at 4°C.

A2.16 Tris Buffered Saline (TBS), pH 7.6

Tris (MW 121.14)..... 0.05M (6.06g/l)
Sodium chloride (MW 58.44)..... 0.15M (8.77g/l)
Magnesium chloride 6-hydrate (MW 203.30)..... 2mM (0.4g/l)
Bovine serum albumin (BSA)..... 0.1% (1g/l)

Dissolve salts in 900ml DEPC-H_2O, adjust pH to 7.6 and correct volume to 1000ml. Autoclave prior to storage at 4°C. Add BSA immediately prior to use and do not store thereafter. Note, for immunohistochemistry, magnesium chloride 6-hydrate and BSA are not required and water does not need to treated with DEPC. Water purified by reverse-osmosis may be used.

A2.17 10x Tris-EDTA (TE), pH 8.0

Tris (MW 121.14)..... 0.1M (6.06g/500ml)
EDTA (MW 372.24)..... 0.01M (1.86g/500ml)

Dissolve tris in 450ml DEPC-H_2O, then add EDTA. Adjust pH to 8.0, make up to 500ml and autoclave. Store at 4°C.

A2.18 Tris-HCL Buffers

0.05M Tris-HCl buffer, pH 7.6..... 0.05M (1.21g Tris/200ml)
0.5M Tris-HCl buffer, pH 7.5.....0.5M (30.29g Tris/500ml)
1M Tris-HCl buffer, pH 6.8..... 1M (12.11g Tris/100ml)

Dissolve Tris in DEPC-H_2O and adjust pH using 1N HCl. Correct volume and autoclave. Store at 4°C for no more than 2 weeks.

A2.19 Tris (50mM), NaOH Buffer (0.3M), pH 7.5

Tris (MW 121.14)..... 50mM (0.606g/100ml)
Sodium hydroxide (MW 40)..... 0.3M (1.2g/100ml)

Dissolve tris and NaOH in 90ml DEPC-H_2O and adjust pH to 7.5. Correct volume to 100ml prior to storage.

A2.20 VERONAL ACETATE BUFFER, PH 9.2

Sodium acetate trihydrate (MW 136.08)..... 30mM (0.82g/200ml)
Sodium barbitone (MW 206.18)..... 30mM (1.24g/200ml)
Sodium chloride (MW 58.44)..... 0.1M (1.17g/200ml)
Magnesium chloride 6-hydrate (MW 203.30)..... 50mM (2.03g/200ml)

Dissolve all of the above in 190ml DEPC-H_2O, adjust pH to 9.2 with HCl and correct volume. A cloudy precipitate will form if the solution becomes too acidic and requires NaOH to correct. This buffer cannot be autoclaved and must be stored at 4°C for no longer than 2 weeks. Discard if a precipitate forms during storage.

APPENDIX THREE: OLIGONUCLEOTIDE PROBES

This appendix contains the Genbank report for the L-NGFR mRNA obtained from the National Centre for Biotechnology Information (NCBI) database via the internet (www address shown below). A cocktail containing four oligonucleotide probes specific for L-NGFR mRNA was used in this study. The location of each probe within the L-NGFR mRNA sequence is highlighted in red.

NATIONAL CENTRE FOR BIOTECHNOLOGY INFORMATION GENBANK REPORT FOR L-NGFR mRNA

http://www.ncbi.nlm.nih.gov/htbin-post/Entrez/query?uid=4505392&form=6&db=n&Dopt=g

```
LOCUS       NGFR         3386 bp    mRNA            PRI       30-SEP-1999
DEFINITION  Homo sapiens nerve growth factor receptor (TNFR superfamily,
            member
            16) (NGFR) mRNA.
ACCESSION   NM_002507
NID         g4505392
VERSION     NM_002507.1  GI:4505392
KEYWORDS    .
SOURCE      human.
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Mammalia;
            Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 3386)
  AUTHORS   Grob PM, Ross AH, Koprowski H and Bothwell M.
  TITLE     Characterization of the human melanoma nerve growth factor
            receptor
  JOURNAL   J. Biol. Chem. 260 (13), 8044-8049 (1985)
  MEDLINE   85234501
REFERENCE   2  (bases 1 to 3386)
  AUTHORS   Huebner,K., Isobe,M., Chao,M., Bothwell,M., Ross,A.H., Finan,J.,
            Hoxie,J.A., Sehgal,A., Buck,C.R. and Lanahan,A.
  TITLE     The nerve growth factor receptor gene is at human chromosome
            region
            17q12-17q22, distal to the chromosome 17 breakpoint in acute
            leukemias
  JOURNAL   Proc. Natl. Acad. Sci. U.S.A. 83 (5), 1403-1407 (1986)
  MEDLINE   86149312
REFERENCE   3  (bases 1 to 3386)
  AUTHORS   Chao MV, Bothwell MA, Ross AH, Koprowski H, Lanahan AA, Buck CR
            and
            Sehgal A.
  TITLE     Gene transfer and molecular cloning of the human NGF receptor
  JOURNAL   Science 232 (4749), 518-521 (1986)
  MEDLINE   86179876
REFERENCE   4  (bases 1 to 3386)
  AUTHORS   Rettig,W.J., Thomson,T.M., Spengler,B.A., Biedler,J.L. and
            Old,L.J.
  TITLE     Assignment of human nerve growth factor receptor gene to
            chromosome
            17 and regulation of receptor expression in somatic cell hybrids
  JOURNAL   Somat. Cell Mol. Genet. 12 (5), 441-447 (1986)
  MEDLINE   87019189
REFERENCE   5  (bases 1 to 3386)
  AUTHORS   Johnson,D., Lanahan,A., Buck,C.R., Sehgal,A., Morgan,C.,
            Mercer,E.,
            Bothwell,M. and Chao,M.
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  TITLE     Expression and structure of the human NGF receptor
  JOURNAL   Cell 47 (4), 545-554 (1986)
  MEDLINE   87051725
 REFERENCE  6  (bases 1 to 3386)
  AUTHORS   Welcher,A.A., Bitler,C.M., Radeke,M.J. and Shooter,E.M.
  TITLE     Nerve growth factor binding domain of the nerve growth factor
            receptor
  JOURNAL   Proc. Natl. Acad. Sci. U.S.A. 88 (1), 159-163 (1991)
  MEDLINE   91095418
 COMMENT    REFSEQ: This reference sequence was derived from M14764.1.
            Summary: Nerve growth factor receptor contains an extracellular
            domain containing four 40-amino acid repeats with 6 cysteine
            residues at conserved positions followed by a serine/threonine-
            rich
            region, a single transmembrane domain and a 155-amino acid
            cytoplasmic domain. The cysteine-rich region contains the nerve
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