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An investigation into the effects of short-chain fatty acids on primary and transformed urothelial cells in relation to their potential as an intravesical agent in the neobladder.

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Abstract

Colocystoplasty has an important role in bladder reconstructive surgery. However it can be affected by chronic inflammation and excessive mucous production which may lead to urinary tract infection, stone formation and occasionally malignant transformation. We postulate that changes in the colonic segment within the augmentation are affected by a condition known as diversion colitis, which is found in bowel segments diverted away from the faecal stream. The aetiology of this condition is a luminal deficiency of short chain fatty acids (SCFAs) which are produced by bacterial fermentation of dietary fibre and include butyrate, propionate and acetate. Studies have shown that they are the colonocytes' preferred energy substrate and have an important role in colonic mucosal health and prevention of malignant transformation.

The purpose of this study was to investigate the effect of SCFAs on the bladder which is an important consideration when contemplating intravesical therapy in colocystoplasty.

Using monolayer cell cultures of both primary urothelial cells and urothelial cancer cell lines the effects of SCFAs were investigated. The MTT (3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) cytotoxicity assay was employed to study cell growth. Fluorescence microscopy with acridine orange and flow cytometry were used to study apoptosis and the cell cycle. It was found that all three SCFAs inhibit cell growth, induce apoptosis and induce cell cycle arrest. Butyrate had the most potent effect *in vitro* followed by propionate and then acetate. To assess the significance of these results *in vivo*, intravesical instillation of SCFAs was performed in a rodent model which demonstrated no significant adverse effects both histologically and on urothelial cell turnover.

Contents **Page**

List of figures

List of illustrations

List of tables

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Abbreviations

ATP	Adenosine triphosphate
Bax	Bcl2 associated X-protein
Bcl2	B-cell leukaemia/ lymphoma 2
BUF	British Urological Foundation
CO_2	Carbon dioxide
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EPIC	European prospective investigation into cancer and nutrition
FBS	Foetal bovine serum
HBSS	Hanks balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HCl	Hydrochloric acid
HCO_3	Bicarbonate
HDI	Histone deacetylase inhibitor
HLA	Human leukocyte antigen
G0	Gap 0 or quiescent phase of cell cycle
G1	Gap 1 phase of cell cycle
G2	Gap 2 phase of cell cycle
IC50	Fifty percent inhibitory concentration
KSFM	Keratinocyte serum free medium
M	Mitosis

mRNA	Messenger ribonucleic acid
MTT	3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue
PBS	Phosphate buffered saline
RCS	Royal College of Surgeons
SCFAs	Short chain fatty acids
αSMA	α Smooth muscle actin
TCC	Transitional cell carcinoma
TGF-β1	Transforming growth factor beta 1
UC	Ulcerative colitis

Chapter 1

Introduction

History of bladder reconstruction

Over the last 20 years an array of bladder preserving and total substitution techniques have developed using nearly every segment of the gastrointestinal tract, each with their own advantages and disadvantages. Whether the indication for urinary diversion is following cystectomy for cancer, bladder dysfunction or malformation the choice between incontinent or continent urinary diversion, bladder augmentation or orthotopic reconstruction using ileum, caecum, colon or stomach has to be made. The underlying aim in all cases is to maintain good renal function and provide continence.

The use of bowel in the urinary tract has been described for over a century (Morris H 1903). Initially urine was diverted into the existing lower gastointestinal tract by formation of ureterosigmoidostomy (Coffey 1911; Coffey 1919; Leadbetter & Clarke 1955; Nesbit & Bohne 1949; Simon 1852) or rectal bladder (Bergenham 1896). This was followed by the non continent urinary diversion using a loop of ileum (Bricker 1950) or colon with tunnelled non refluxing ureterocolic anastomosis (Leadbetter and Clarke 1955). The ileocaecal segment was then used as both a continent catheterisable pouch or conduit (Bricker 1950; Gil-Vernet, Jr. 1965; Gilchrist et al. 1950). Augmentation ileocystoplasty was first described in a dog by Tizzoni and Foggi in 1888 (Tizzoni & Foggi 1888). Augmentation cystoplasty in humans was first described by Mikulicz in 1899 (Smith, III & Swierzewski, III 1997) but it did not become widespread until the 1950's when Couvelaire utilized the technique for increasing the capacity of small contracted tuberculous bladders (Couvelaire 1950).

In addition to ureterocolic diversion one of the key developments of reconstructive urology was clean intermittent self catheterisation which many patients with reconstructed bladders have come to rely on to drain their neobladders (Lapides et al. 1976). Both advances in

anaesthetic techniques and intensive care have made the risk of often long and complicated procedures acceptable, especially in the paediatric population.

Augmentation cystoplasty

Augmentation cystoplasty has become the most widely used procedure to create a suitable low pressure urinary reservoir that preserves upper tract function and facilitates continence. After initial development it was not often used until the 1950's when it became popular for the treatment of the small contracted tuberculous bladder (Couvelaire 1950). Since then the technique has been used in interstitial cystitis, bladder cancer, radiation cystitis, chemotherapy cystitis and small contracted bladders secondary to previous surgery. More recently augmentation cystoplasty has been used in the treatment of the detrusor overactivity and neurogenic bladder dysfunction refractory to pharmacotherapy, especially in patients with spinal cord injury, multiple sclerosis and myelodysplasia (Greenwell et al. 2001). In the paediatric population it has revolutionized the management of bladder exstrophy and other congenital malformations of the urogenital tract. The indications for augmentation are listed in table 1.1.

The clam type augmentation cystoplasty (Bramble 1982; Mundy & Stephenson 1985) is accepted as the best technique, but there is no clear scientific evidence to support the use of one type of bowel segment over another (Smith, III & Swierzewski, III 1997). In the paediatric population both colon and ileum are used equally (Bertschy et al. 2000; Blyth et al. 1992), and gastric segments are occasionally used in specific circumstances (Sumfest & Mitchell 1994).

Indication	
Neuropathic	Spinal cord injury Multiple sclerosis Spinal dysraphism
Non neuropathic	Detrusor overactivity Chronic cystitis Interstitial cystitis Radiation cystitis
Congenital abnormality	Bladder exstrophy Cloacal malformations Posterior urethral valves

Table 1.1 Indications for augmentation cystoplasty

Complications of augmentation cystoplasty

Although the gastrointestinal mucosa is currently the best material available for bladder augmentation in most patients, a significant number are compromised by complications including excess mucus production, urinary tract infection, stone formation, metabolic imbalance and an increased risk of malignancy.

Histologically there is villous atrophy giving a flattened endoscopic appearance, and progressive chronic inflammation that manifests clinically as excess mucopus production. The mucosa is often fragile and friable giving rise to contact bleeding (Solomon et al. 1998). Inflammatory infiltrates include lymphocytes, plasma cells and especially eosinophils. Other features include goblet cell depletion with increased nuclear: cytoplasmic ratio characteristic of inflamed intestine and increased mitotic activity and collagen deposition indicating regeneration and fibrosis. The appearances are similar to mild ulcerative colitis, but lack crypt abscesses (Moorcraft et al. 1983). It has been proposed that these changes are biphasic, initially an inflammatory response with loss of microvilli and infiltration of the lamina propria followed by regression including villous and crypt atrophy, and reduced goblet cells

with a corresponding reduction in mucus secretion (Aragona et al. 1998; Moorcraft et al. 1983). However not all studies have demonstrated a reduction in mucous secretion (N'dow et al. 2004). Histochemical studies have shown alterations in mucin expression in transposed ileum. At the urothelial-intestinal anastomosis, the pattern of mucin expression is similar to that found adjacent to colonic tumours which may be regarded as early pre-malignant change (Buisine et al. 1996; Filipe & Branfoot 1976; N'dow et al. 2000; Nurse & Mundy 1989a). There remains much speculation as to the aetiology of these histological changes, whether it is an adaptive response of the intestinal mucosa from an absorptive to a protective role, a direct toxic effect of urine or lack of contact with intestinal luminal effluent, which normally provides the colonocyte with its preferred energy substrate.

Urinary tract infection affects about one-third of patients. The most common offending organisms are *Escherichia coli, Klebsiella aerogenes, Proteus species and Citrobacter freundii* (Wagstaff et al. 1991). Unfortunately this is a consequence of providing urinary mucin, an excellent carbohydrate energy substrate and optimizing urinary pH for bacterial growth (George et al. 1991). Combined with the use of clean intermittent self catheterization (CISC) and incomplete emptying in these patients the risk of urinary tract infection and subsequent stone formation is high (Blyth et al. 1992). Infection may also lead to long term renal scarring in the presence of vesicoureteric reflux (Kass et al. 1981). Vitamin C lowers urinary pH and is recommended to reduce the risk of urinary tract infection. Ranitidine has been shown to reduce mucus production and may help prevent subsequent mucus precipitation which leads to outflow obstruction, chronic retention and infections (George et al. 1992).

The incidence of stone formation is between 20 and 30 % and the majority are triple phosphate implying that bacteriuria and subsequent urease production is the main aetiological factor (Blyth et al. 1992; Mast et al. 1995).

The metabolic complications of bladder augmentation can be divided broadly into two groups. There are those complications mainly due to increased permeability of gastrointestinal mucosa compared with native urothelium that occur as a result of placing gastrointestinal tissue within the urinary tract. Then there are a further group of complications that originate from the loss of a segment of gastrointestinal tract leading to both nutritional and metabolic abnormalities.

In contrast to the relatively impermeable urothelium, the gastrointestinal mucosa allows exchange of water and solute between the urine within the augmented bladder and the capillary bed. This leads to a number of metabolic complications depending on the segment of intestine used.

As part of normal function solutes are exchanged across the intestinal mucosa by both active and passive mechanisms via the transcellular and paracellular routes to maintain an electrochemical gradient across the basolateral membrane. Water movement is determined by the osmotic gradient and permeability of intercellular tight junctions. The main solutes involved include sodium, potassium, chloride, bicarbonate and ammonium (Benos 1982). Table 1.2 lists the main metabolic complications arising from the use of specific intestinal segments in the urinary tract.

Intestinal Segment	Complication	Treatment
Gastric	Hypochloraemic alkalosis Hypokalaemia	H-pump inhibitor
Jejunum	Hypochloraemic acidosis Hyponatraemia Hyperkalaemia Uraemia	Sodium chloride
Ileum and colon	Hyperchloraemic acidosis Hypokalaemia	Na and K citrate, Bicarbonate Chlorpromazine Nicotinic acid

Table 1.2 Treatment of metabolic complications of intestinal urinary diversion.

Jejunum is the least desirable segment to use as it is the most permeable to water and electrolytes resulting in dehydration, hyponatraemia, hyperkalaemia, hypochloraemia, uraemia, and metabolic acidosis. The metabolic disturbance seen with jejunum is more severe and affects more patients (25% to 40%) than ileum or colon (Klein et al. 1986). Jejunum is no longer used for these reasons.

Ileal and colonic segments in the bladder cause hyperchloraemic metabolic acidosis due to absorption of urinary chloride and ammonium (Stampfer et al. 1997). Hypokalaemia is more common with colonic than ileal segments (Koch et al. 1990). Although acidosis affects more patients (20-100%) it is less severe than in those with jejunal bladder segments. Hyperchloraemia is more common with colon (50%) than ileum (26%) (Mast et al. 1995; Nurse & Mundy 1989b). These abnormalities are rarely symptomatic but if problematic may be treated simply with bicarbonate supplements orally.

Gastrocystoplasty was developed in an attempt to overcome some of the problems with acidosis and mucus production seen with ileum and colon, however gastric mucosa in the urinary tract has its own set of complications (Sinaiko 1956). Hypochloraemic alkalosis is caused by systemic bicarbonate accumulation during the production of hydrochloric acid (HCL). In mild renal impairment this is exacerbated by reduced bicarbonate excretion, but in severe renal impairment offers advantages over ileum and colon by compensating renal acidosis (Piser et al. 1987). Secretion of acid into the urinary tract causes haematuria-dysuria syndrome in 25-36% of patients (Dykes & Ransley 1992; Murray et al. 1987; Nguyen et al. 1993)

The long term consequences of metabolic acidosis are skeletal demineralisation (Osteomalacia or ricketts) and impaired growth and development in the young (Lee et al. 1977; McDougal 1992; McDougal & Koch 1991; Mundy & Nurse 1992). Children with

colocystoplasties appear to be more at risk than those with ileocystoplasty because ileum is more efficient at reabsorbing excreted calcium from the urine.

Hepatic metabolism responds to the increased ammonium load preventing a rise in serum levels. Only in severe liver disease or when infection with urea splitting bacteria occurs does serum ammonia rise which can result in encephalopathy. Other electrolyte imbalance particularly magnesium deficiency, and altered drug metabolism may lead to neuromuscular and personality changes.

Nutritional consequences of loss of a segment of intestine from the gastrointestinal tract

The complications resulting from removal of a segment of bowel will depend on the role of that segment in the gastrointestinal tract. When ileum is removed vitamin B12, folate and bile salt absorption may be impaired resulting in macrocytic anaemia, secretory diarrhoea, steatorrhea, cholelithiasis, hyperoxaluria and hypercalciuria with urolithiasis (Akerlund et al. 1989; Canning et al. 1989; Stampfer et al 1997; Wagstaff et al. 1991). When the ileocaecal valve is lost accelerated transit time causes osmotic diarrhoea, and the resulting bacterial reflux into the small bowel and overgrowth leads to malabsorption (Steiner & Morton 1991). Use of gastric segments may result in reduced levels of intrinsic factor and hydrochloric acid secretion with subsequent Vitamin B12 deficiency, incomplete breakdown of protein and malabsorption leading to iron deficiency anaemia and hypocalcaemia. Combined ileo-caecal resection or a large resection of colon may result in significant diarrhoea, loss of bicarbonate and electrolytes leading to dehydration, hypokalaemia, hypomagnesaemia and acidosis (McDougal 1992; Steiner & Morton 1991).

Nutritional complication	Treatment
Decreased transit time	Anticholinergics and antispasmodics
	Opiates (codeine)
Hypocalcaemia, Hypomagnesaemia	Mineral supplements
Bile salt malabsorption	Cholestyramine
Steatorrhea	
Hyperammonaemia-encephalopathy	Protein restriction
(Liver function impairment)	Relieve urinary outflow obstruction
	Neomycin
	Lactulose
Intrinsic factor deficiency, Vitamin B12 deficiency	B12 supplements
Rickets, Osteomalacia, Metabolic bone disease	Alkalinising agents, Vitamin C, Vitamin D3, Calcium,

Table 1.3 Treatment of nutritional complications of ileo-colonic urinary diversion

Malignancy risk

The incidence of carcinoma in ureterosigmoidostomy is between 6% and 29% and potentially pre-malignant polyps occur in 40% of cases (McDougal 1992; Smeulders & Woodhouse 2001). It took over 40 years to recognize this risk which led to a fall in the popularity of ureterosigmoidostomy, although it is still performed in certain cultures where a stoma is unacceptable (Shaw & Lewis 1999). Augmentation cystoplasty is a much younger operation only performed in significant numbers from the 1980's. However malignancy has become recognized as a significant complication in both ileocystoplasty and colocystoplasty, despite early assumptions that separation of the urinary and faecal streams would alleviate this problem (Chiang et al. 1982; Egbert et al. 1980; Filmer & Spencer 1990; Golomb et al. 1989; Kirby & Lloyd-Davies 1985; Leedham & England 1973; Smith & Hardy 1971; Takasaki et al. 1983). More recently adenocarcinoma has been reported in colonic pouch urinary diversions (Gazzaniga et al. 2000; L'Esperance et al. 2001; Lisle et al. 2000). Currently the incidence is 1-2% (Shaw & Lewis 1999) and the latency period to tumour formation is 10-20 years, less with increasing age (McDougal 1992). A worrying observation is the similarity

between tumours found in ureterosigmoidostomy and augmentation cystoplasty. In addition ileum which normally has a very low risk of malignancy, 2% that of colon, is almost as vulnerable to malignant change as colon when part of a urinary diversion (Filmer & Spencer 1990). Eighty-five percent of tumours are adenocarcinoma, 10% are transitional cell carcinoma and the remaining 5% are other histological types (McDougal 1992). Tumours tend to occur at the anastomosis between urothelium and bowel mucosa. One explanation for this apparent preferential site is that there is increased cell proliferation and healing in this area which is more susceptible to mutagenic agents in the urine, such as nitrosamines and their activation products. The presence of suture material has been another suggestion. Although there is still some debate about the actual tissue of origin, Barrington et al suggested that adenocarcinomas originate from urothelium which has undergone metaplasia, dysplasia and intestinalisation, however others using specific staining techniques suggest that tumours arise from bowel mucosa (Barrington et al. 1997; Gitlin et al. 1999; McDougal 1992).

There are a number of theories regarding the aetiology of these tumours. The most carcinogenic environment arises in ureterosigmoidostomy where the anastomosis is exposed to a mixture of faeces and urine, resulting in formation of high levels of carcinogenic nitrosamines. The nitrosamine theory, as a result of chronic infection certainly has a place in the development of tumours in augmentation cystoplasty, but is not the whole story (Stewart et al. 1981). In an animal model inactivation with ascorbic acid did not prevent tumour formation, (Stribling et al. 1989) and in another animal study proliferative abnormalities occurred equally in the presence and absence of infection (Spencer et al. 1993). In a longer term animal study in rats (up to 27 months), all cases developed hyperplasia and metaplasia at the anastomosis, and 5 out of 32 cases developed non invasive tumours noted to be at or adjacent to the anastomosis. A state of unregulated growth factor interactions from adjacent

dissimilar tissues with induction of a different phenotype was proposed as a possible aetiological mechanism, similar to that occurring in embryonic tissues (Little, Jr. et al. 1994). The histological changes seen in intestinal mucosa (Moorcraft et al. 1983; Nurse & Mundy 1989a) include chronic inflammation, metaplasia, increased mitotic figures, nuclear-cytoplasmic ratio and inflammatory cell infiltrate could be regarded as pre-malignant changes. In other conditions chronic inflammation is well recognized as a cause of squamous malignancy. Inflammatory cells may promote carcinogenesis in a number of ways including production of free radicals and growth factors or by catalyzing secondary amine conversion (Filmer & Spencer 1990). Ornithine decarboxylase, involved in polyamine synthesis and normal cellular proliferation is increased in colon carcinomas, and its inhibition prevents carcinogen induced neoplasia. In an animal model of ureterosigmoidostomy elevated levels of ornithine decarboxylase were found prior to tumour development at the anastomosis (Treiger & Marshall 1991). Colonic mucin expression in augmentation cystoplasty has revealed patterns of expression that may represent early carcinogenesis at the anastomotic junction (Nurse & Mundy 1989a). The sulfomucins, dominant in the normal colon are replaced by sialomucins throughout the length of the crypt in cases of colon cancer, colon conduits, continent reservoirs and augmentation cystoplasty (Mansson & Willen 1988; Treiger & Marshall 1991). MUC5AC, a mucin gene expressed in cases of rectosigmoid villous adenoma and postulated to be an early event in the adenoma carcinoma sequence was found at the anastomosis in augmentation cystoplasty (Buisine et al. 1996; N'dow et al. 2000). The mechanism of carcinogenesis in augmentation cystoplasty is not completely understood, but mucin production, infection, inflammation, ornithine decarboxylase and nitrosamines may all contribute. However in light of increasing evidence that the control of mucosal proliferation by luminal effluent has a significant impact on the development of

colorectal cancer, transposed gastrointestinal segments may be at increased risk as a result of exclusion from the faecal stream, in addition to the effects of urinary factors.

Alternative materials in augmentation surgery

Despite the success seen with intestinal mucosa in the urinary tract the risk of significant complications has led to the search for a better material for bladder augmentation. Gastric mucosa became a widely used alternative popularized by the publication of several successful series in animals and humans in the1970's (Leong & Ong 1975; Piser et al. 1987). Features such as lack of absorption of hydrogen ions, bactericidal effect of acid and reduced mucus secretion made gastrocystoplasty a promising alternative. Unfortunately as the numbers have increased so to have the complications and it is no longer recommended unless there is no other bowel segment available (Gosalbez, Jr. et al. 1993). Auto-augmentation, (vesicomyotomy or vesicomyomectomy) increases bladder capacity by creating a large mucosal diverticulum avoiding the need for an intestinal segment. The results however are not very impressive (Duel et al. 1998). In a select group of patients megaureter has been successfully used to construct a ureterocystoplasty avoiding the complications of intestinal epithelium (Bellinger 1993; Syed et al 2001). Seromuscular cystoplasty using demucosalised bowel has been used in several animal models and in some limited human series. The results have been disappointing due to high rates of fibrosis and shrinkage of the intestinal patch which is seen in all cases (Shoemaker 1955). Work using other tissues including peritoneum, omentum, bowel serosa and skeletal muscle have been similarly affected by patch contraction (Duel et al. 1998).

Currently there is great interest in urothelial lined augmentation. Demucosalised bowel is placed onto intact urothelium as a seromuscular colocystoplasty, which has achieved good durable results (Gonzalez et al. 1995). To extend this procedure to patients with small

bladders where there is insufficient urothelium, for example bladder exstrophy, attempts have been made to place cultured urothelium onto a de-epithelialised bowel, stomach and uterus with some success in minipigs (Fraser et al. 2004; Frey et al. 1996; Merguerian et al. 1994). Various artificial materials have been used to cover bladder defects in the hope that they will provide a scaffold for healing to occur. Materials include polyvinyl and gelatin sponges, polytef felt, silicones, rice paper and polygalactins. Most cases have resulted in failure due to sepsis, peritonitis and leakage. Where healing has occurred the prosthetic material is extruded into the bladder and the healed area is composed of inflammatory and fibrous tissue with a thin lining of urothelium. Bioabsorbable materials including autogenous fascia (Duel et al. 1998), alcohol preserved fascia (Koontz 1929), pericardium (Kambic et al. 1992; Novick et al. 1977), fascia, dura (Kelami 1971), and bladder allografts (Tsuji et al. 1961) have proved more effective than artificial materials in other animals (Kambic et al. 1992; Kelami 1971; Koontz 1929; Novick et al. 1977; Tsuji et al. 1961), and limited human studies (Kelami 1971; Tsuji et al. 1961). Tissue engineering using cultured urothelium and smooth muscle cells on a polygalactin scaffold or preserved intestinal submucosa have also shown promising functional results (Atala et al. 1993; Baret et al. 1953; Kropp et al. 1995; Kropp et al. 1996; Vaught et al. 1996; Yoo et al. 1998), and this is the area being researched most extensively at the present time.

Despite extensive research into alternatives to conventional bladder augmentation and replacement it remains that very few techniques have shown significant benefits over large or small bowel augmentation with the exception of autoaugmentation using seromuscular colocystoplasty.

Diversion colitis

Diversion of the faecal stream by formation of a defunctioning stoma is performed for a number of conditions including diverticular disease, bowel perforation, abdominal malignancy, inflammatory bowel disease, bowel dysfunction, and trauma. The defunctioned colonic segment develops diversion colitis which is an inflammatory condition affecting the mucosa of colon and rectum excluded from the faecal stream.

First recognised by Morsen and Drenick as a condition affecting bypassed segments of bowel, it was Glotzer who gave the condition the name diversion colitis (Drenick et al. 1977; Glotzer et al. 1981; Morsen & Dawson 1972). Since then it has become well recognized as a condition with distinct histological features, and often asymptomatic clinical course and is always reversed by restoring bowel continuity. Prospective studies predict that all diverted bowel segments given enough time develop the condition to varying degrees (Ferguson & Siegel 1991; Grant et al. 1997; Haas & Haas 1988; Roe et al. 1993; Whelan et al. 1994), and onset is between 3 and 36 months of diversion (Korelitz et al. 1985). Although the condition is usually asymptomatic it may present with blood stained mucous discharge (Ma et al. 1990) or poorly localized abdominal pain (Ordein et al. 1992). Endoscopic appearances are diffuse and similar to mild ulcerative colitis with erythema, oedema, and petechial haemorrhages. The mucosa is granular, friable with nodularity, and aphthous ulceration (Ordein et al. 1992). On double contrast barium enema features of mucosal nodularity, superficial ulceration and inflammatory polyps have been demonstrated (Scott & Pinstein 1984), and in one case diversion colitis was delineated using a In-111 leucocyte scan (Stein et al. 1983).

A range of histological features are seen and the nature of these changes is dependent on the underlying disease before diversion. Features include acute and chronic inflammation, lymphoid follicular hyperplasia, abundant eosinophils, architectural distortion, goblet cell depletion, paneth cell metaplasia, aphthous ulcers, oedema, cryptitis, crypt abscesses and

fibrosis (Geraghty & Talbot 1991; Komorowski 1990; Vujanic & Dojcinov 2000). Diversion colitis in the normal bowel has less cryptitis, crypt abscesses and distortion of crypt architecture than diverted inflammatory bowel disease. In diverted ulcerative colitis transmural changes and granulomas may be present which makes the distinction with Crohn's disease difficult. Thirty percent of patients with diverted normal colon or Crohn's will be symptomatic with their diversion colitis compared with 87% of patients with diverted ulcerative colitis (Edwards et al. 1999; Ma et al. 1990).

Interestingly diversion colitis has not been reported in the setting of bladder reconstruction where bowel segments are diverted out of the faecal stream into the urinary tract, despite similar histological features being present including chronic inflammation, crypt atrophy and metaplasia. Diversion colitis has been reported in colovaginoplasty. Initial series reported little in the way of complication except mild mucus discharge (Hensle & Reiley 1998; Hitchcock et al. 1994; Martinez-Mora et al. 1992; Wesley & Coran 1992). However with longer follow up blood stained vaginal mucus discharge is more prevalent and problematic with endoscopic and histological features compatible with diversion colitis taking as long as 7 years to develop (Syed et al. 2001; Toolenaar et al. 1993).

Although endoscopic and histological changes have been reported in 70% of diverted colonic segments, the majority of cases are asymptomatic (Ferguson & Siegel 1991). Classification of diversion colitis on both morphological and clinical grounds into mild "diversion reaction" which is usually asymptomatic and more severe "diversion colitis" which is symptomatic has been proposed. All excluded colon may be affected by diversion reaction and the more severe cases have additional aetiological factors causing true diversion colitis. Also suggested is that diversion reaction should refer to the histological features and the term diversion colitis should be reserved for symptomatic disease (Edwards et al. 1999).

The pathogenesis of diversion colitis remains controversial. Hypotheses include bacterial overgrowth of normal flora or nutritional deficiency. Short chain fatty acids (SCFAs) produced by bacterial fermentation of dietary fibre in the bowel lumen are the preferred metabolic substrate of colonocytes (Roediger 1982). Acetic, propionic and n-butyric acid account for approximately 90% of the SCFAs present in the colonic lumen. Their absence from the excluded colon became the favoured aetiological factor in diversion colitis when Harig et al instilled a cocktail of fatty acids containing butyrate, propionate and acetate into the diverted colon and demonstrated resolution of the colitis (Harig et al. 1989). A similar beneficial effect has been demonstrated in children with diversion procto-colitis (Kiely et al. 2001). However in a much shorter 14 day double blind randomized control trial another group demonstrated saline enemas may produce a similar response (Guillemot et al. 1991). Enemas of 5-aminosalicylic and betamethasone have also been effective (Triantafillidis et al. 1991; Tripodi et al. 1992).

Another theory is that changes in the bacterial flora within excluded bowel segments may play a role. The diversity and number of strict anaerobic bacteria are reduced and there is an increase in aerobes of the enterobacteria variety such as *Proteus*, *Providencia*, and *Morganella* genera often found with gastrointestinal disease (Neut et al. 1989). Lymphoid hyperplasia one of the predominant histological features of diversion colitis is thought to be attributable to increased antigenic stimulation in the gastrointestinal tract and therefore evidence of a low grade pathogenic infection in the excluded colon leading to diversion colitis (Grant et al. 1997). To incorporate both the nutritional deficiency and bacterial pathogenic hypotheses with the spectrum of morphological features it has been proposed that a mild diversion reaction occurs due to nutritional deficiency in all excluded colon. Then in a subgroup, a superimposed second insult from a low grade pathogen leads to the more severe condition of diversion colitis (Haque et al. 1993). Whether nutritional deficiency reduces

host defenses and predisposes the excluded colon to pathogenic infection is not clear. Elevated numbers of nitrate reducing bacteria in the defunctioned colon have led to the suggestion that nitric oxide may also be involved (Neut et al. 1997). Hypoplasia and reduced rate of crypt cell proliferation, and mucosal height has been demonstrated when short chain fatty acids are excluded in defunctioned colon, providing evidence for nutritional deficiency as an aetiological factor (Appleton & Williamson 1989). Current areas of research into colonic epithelial cell kinetics, changes in the mucus barrier and the immune response in the defunctioned colon may uncover the link between short chain fatty acids, microflora alterations and other aetiological factors in diversion colitis. Short-chain fatty acid deficiency may play a role in the pathogenesis of other forms of colonic inflammation such as pseudomembranous colitis (Rombeau et al. 1995), ulcerative colitis (UC), and radiation proctitis. It has been postulated that UC is due to either exogenous (decreased luminal availability) or endogenous (impaired intracellular oxidation of butyrate) starvation factors limiting fatty acid oxidation in colonocytes (Roediger 1990; Scheppach et al. 1995). In a randomized double blind placebo controlled trial, oral sodium butyrate improved the efficacy of mesalazine in the treatment of ulcerative colitis (Vernia et al. 2000b). Also in both chronic and acute radiation proctitis SCFA enemas have caused sustained clinical, endoscopic and histological remission in randomized double blind placebo controlled trials (Pinto et al. 1999; Vernia et al. 2000a).

Short chain fatty acids (SCFAs)

Short chain fatty acids (acetate, propionate and butyrate) are C2-C5 organic acids (appendix 1), produced in the gastrointestinal tract of mammals by anaerobic fermentation of undigested non starch polysaccharides by the colonic bacterial flora, and are avidly absorbed by colonic epithelial cells (Velazquez et al. 1997). Collectively known as dietary fibre undigested plant

cell wall material includes celluloses, hemicelluloses, pectins, and gums. They are resistant to enzymatic digestion in the gastrointestinal tract until they reach the large bowel where they are fermented into carbon dioxide, methane, hydrogen, and short chain fatty acids. Acetate, propionate and butyrate, are the 2, 3, and 4 carbon SCFAs respectively, and they account for 90-95% of the SCFAs found in the colon. Isobutyrate, valerate, isovalerate and caproate account for the rest (Bugaut 1987). Proteins are preferentially fermented to valerate, isovalerate and 2-methylbutyrate (Macfarlane et al. 1992).

The total concentration of SCFAs in the colonic lumen ranges from 131mmol/l in the caecum to 80mmol/l in the distal colon. Human studies have shown the molar ratio of acetate, propionate and butyrate in the colon is 59:22:19 which changes to 68:17:13 when the diet is deprived of dietary fibre. The type of fibre consumed affects the molar ratio of SCFAs. Wheat bran, a slow fermentable fibre, produces higher levels of butyrate than oat bran, a completely fermentable fibre (Kashtan et al. 1992). Oligosaccharides in particular fructooligosaccharide has prebiotic properties, stimulating bifidobacteria which leads to increased levels of butyrate in the colon (Tsukahara et al. 2003). The luminal concentration of acetate and butyrate can reach as much as 60mmol/L and 30mmol/l respectively. The total SCFA output of the large bowel has been measured to be at least 300mmol/day (Cummings et al. 1987). SCFAs are weak acids with pKa of 4.75, 4.87, and 4.81 for acetate, propionate, and butyrate respectively. Greater than 90% exist in the dissociated anionic form constituting the major anions within the colonic contents (Engelhardt et al. 1998). Ninty five to ninty nine percent of SCFAs are rapidly absorbed from the colonic lumen by the colonocytes where they are either metabolised or transported to the liver via the portal circulation or directly to peripheral tissues (Bugaut 1987; Engelhardt et al. 1998; Ruppin et al. 1980). The SCFA concentrations found at different sites in the blood circulation vary (portal (375mmol/l),

hepatic (148mmol/l) and peripheral (79mmol/l)). The molar ratio at these sites indicate a preferential and greater uptake of butyrate by the colonocytes (Cummings et al. 1987).

From animal studies three mechanisms of absorption of SCFAs have been proposed (Harig et al. 1991; Harig et al. 1996; Mascolo et al. 1991; Velazquez et al. 1996; Wachtershauser & Stein 2000):

i. Passive absorption of undissociated lipid soluble forms probably coupled to Na^+-H^+ exchange;
ii. An energy dependent carrier mediated $SCFA^-$-HCO_3^- exchange;
iii. Diffusion of anionic forms through paracellular 'leaky spots'.

Absorption is independent of bulk lumen pH and SCFA carbon chain length. The permeability of proximal colon is higher for acetate, equal for propionate and lower for butyrate compared with the distal colon. These regional differences in absorption are due to higher activity of the ionic $SCFA^-$-HCO_3^- exchange mechanism in the proximal colon. Protonated SCFAs are lipid soluble and readily diffuse across the cell membrane, however 95% of SCFAs are in the ionized form at normal pH and therefore are not lipid soluble. These ionized SCFAs are exchanged for HCO3 at both the apical and basolateral membranes (Schroder et al. 2000). Within the colonocyte the protonated SCFAs rapidly dissociate causing an increase in intracellular protons. Oxidation of SCFAs generate CO_2 which by the action of carbonic anhydrase releases further protons and HCO_3^- (Roediger 1982). The basolateral sodium-proton exchangers are activated in response to the intracellular proton load to maintain intracellular pH constant (Wachtershauser & Stein 2000). Water follows down an osmotic gradient and sodium is pumped back into the circulation by a basolateral Na^+-K^+ ATPase (Binder & Mehta 1989; Rowe et al. 1994). SCFAs induce calcium, potassium and magnesium absorption from the colon by a similar proton linked exchange

mechanism (Lutz & Scharrer 1991; Scharrer & Lutz 1990). SCFA induced Na and water reabsorption (Roediger & Moore 1981) is also enhanced by up regulation of transcription of mRNA for the Na^+-H^+ exchanger (Bishop et al. 1992) and Na^+-K^+ ATPase α subunit (Chehab et al. 1987). Endocrine, paracrine and autocrine factors may also play a role (Velazquez et al 1996). These absorption mechanisms are shown in illustration 1.1.

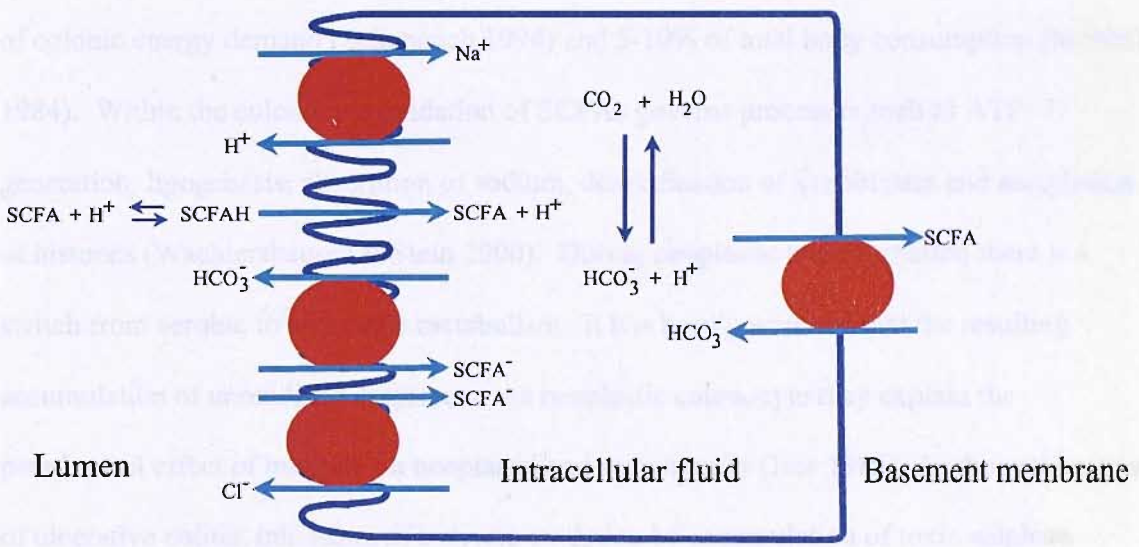


Illustration 1.1 Model of cellular mechanisms involved in absorption of short chain fatty acids (SCFAs) in the human colon.

Short chain fatty acid metabolism

SCFAs are an important energy source for the both the colonocyte and within the liver (Topping et al. 1993). They are metabolized to CO_2 and Ketones by a β–oxidation pathway which is coenzyme A dependent (Appendix 2). Inhibition of this pathway can induce ulcerative colitis in rats and patients with ulcerative colitis have been shown to have decreased coenzyme A levels (Ellestad-Sayed et al. 1976; Roediger & Nance 1986). Butyrate is the preferred SCFA to meet the colonocytes energy requirements followed by acetate then

propionate, which is least metabolized. Compared with other intestinal energy substrates butyrate is preferred over acetoacetate, glutamine and glucose by colonocytes in both adults (Cummings 1981; Roediger 1982; Topping et al. 1993), and neonates (Krishnan & Ramakrishna 1998). This preference becomes greater as you move from the caecum distally to the rectum (Roediger 1982) which is interesting as anatomically it follows the distribution of colorectal cancer and ulcerative colitis (Mortensen et al. 1996). SCFAs provide up to 80% of colonic energy demand (Scheppach 1994) and 5-10% of total body consumption (McNeil 1984). Within the colonocyte oxidation of SCFAs governs processes such as ATP generation, lipogenesis, absorption of sodium, detoxification of xenobiotics and acetylation of histones (Wachtershauser & Stein 2000). During neoplastic transformation there is a switch from aerobic to anaerobic metabolism. It has been postulated that the resulting accumulation of unoxidized butyrate in the neoplastic colonocyte may explain the paradoxical effect of butyrate on neoplastic and normal cells (Jass 1985). In the pathogenesis of ulcerative colitis, inhibition of butyrate oxidation by accumulation of toxic sulphate reduction products, generated by sulphate reducing bacteria may play a role (Roediger et al. 1997).

Mucosal nutrition and starvation

The gastrointestinal mucosa is unique in that it draws nutrition from both the vascular and luminal routes. Metabolic fuels include glucose, glutamine, ketone bodies and short chain fatty acids. The main substrate of small intestinal epithelial cells is glutamine which is derived from the lumen, but during starvation can be drawn from the blood stream from muscle catabolism or hepatic ketogenesis. Glucose and ketone bodies are derived from the same route. The colonic epithelium however derives 80% of its fuel from SCFAs which are only derived from the lumen as described earlier. During starvation or diversion the luminal

supply of SCFAs is absent (Illman et al. 1986). The colonocytes have to utilize a less efficient and less abundant supply of ketone bodies from the blood stream, which is insufficient to maintain colonic mucosal nutrition and function (Firmansyah et al. 1989). Various studies have demonstrated that deficiency of luminal SCFA causes mucosal atrophy, and reduced colonic mucosal weight, crypt height, colonocyte proliferation rate and DNA content. Functionally the starved colon is converted from an absorptive to a secretory epithelium leading to diarrhoea. Bacterial load and mucosal capacity to metabolise SCFAs is impaired. This is demonstrated during periods of famine where severe diarrhoea initially resistant to refeeding occurs (Kenny 1945). Conversely luminal instillation of SCFAs or diets high in wheat fibre increase colonic mucosal weight, crypt height, colonocyte proliferation rate and DNA content. Of the three main SCFA butyrate followed by propionate have the strongest trophic effects on the colonic mucosa (Kripke et al. 1989; Scheppach et al. 1992). Increases in colonic motility (Yajima 1985), mucosal blood flow (Rombeau et al. 1990) and colonic anastomotic strength and improved healing have been demonstrated by both intra luminal and intravenous infusion of SCFAs in animal models (Rolandelli et al. 1986; Rolandelli et al. 1997; Topcu et al. 2002).

Role of short chain fatty acids in differentiation, apoptosis and prevention of colorectal carcinogenesis

Apoptosis is a physiological process by which cells effectively commit suicide, it is characterized by loss of cell contact, loss of cell matrix adhesion, cell shrinkage, loss of microvilli and chromatin condensation (Arends & Wyllie 1991). In tissues like the gut undergoing rapid turnover apoptosis is the mechanism by which homeostasis is maintained. A balance between proliferation at the base of the colonic crypt and apoptosis at the upper crypt is thought to be important in preventing carcinogenesis and in fact when the gut is

exposed to dietary carcinogens or γ radiation cells undergo apoptosis to eliminate damaged cells and protect the tissue from neoplastic change (Merritt et al. 1994; Potten et al. 1992). SCFAs, particularly butyrate, exert a multitude of antineoplastic effects on transformed cells *in vitro* including modulation of cell proliferation, differentiation, and apoptosis and the expression of many proteins. Propionate and acetate on the whole have similar effects but less pronounced, and of great interest is the contrasting effect of butyrate on normal and neoplastic cells known as the butyrate paradox. *In vitro* and *in vivo* butyrate stimulates a physiological pattern of cell proliferation in the colonic crypt, whereas it inhibits proliferation in colon cancer cells (Scheppach & Weiler 2004). A diet supplemented with dietary fibre increased butyrate levels and reduced colonic cell proliferation *in vivo* (Boffa et al. 1992). In a model of chemically induced colon cancer, aberrant crypts representing early changes in the adenoma-carcinoma sequence where reduced by 61% in rats fed fructooligosaccharide (a butyrate precursor) and the cyclooxygenase-2 inhibitor celecoxib (Buecher et al. 2003). In a another model of colon cancer using deoxycholic acid in non inflamed colonic mucosa, butyrate itself when instilled into the colon antagonised upper crypt hyperproliferation (Bartram et al. 1993). Conversely in pigs with normal colonic crypts fed raw potato starch to increase colonic butyrate concentration apoptosis was reduced and shifted to the basal stem cell compartment (Mentschel & Claus 2003). In the European Prospective Investigation into Cancer and Nutrition (EPIC), a study of the effect of dietary fibre on the incidence of colorectal cancer in 520,000 individuals, dietary fibre intake was found to be inversely related to the relative risk of colorectal cancer (Bingham et al. 2003).

In vitro butyrate inhibits cell proliferation and induces a more differentiated phenotype reflected by increased expression of brush border glycoproteins, alkaline phosphatase and carcinoembryonic antigen in colo-rectal tumour cell lines (Augeron & Laboisse 1984; Deng et al. 1992; Gamet et al. 1992; Whitehead et al. 1986). Apoptosis induced by SCFAs in

colonic adenoma and carcinoma cell lines is thought to be the terminal event in the pathway of colonic epithelial cell proliferation and subsequent differentiation. This is supported by the appearance of increased levels of differentiation markers such as alkaline phosphatase and e-caderin in apoptotic cells collected from the medium of butyrate treated colonic cell lines (Heerdt et al 1994). In studies of colonic adenoma cell lines butyrate reversibly down regulates both mutant and wild type p53 (Palmer et al. 1997). However SCFA induces apoptosis in cells which have no p53 indicating that apoptosis via terminal differentiation is separate from p53 dependent apoptosis in response to DNA damage (Hague et al. 1993; Hague et al. 1995).

In normal colonocytes however butyrate shows a paradoxical effect as it inhibits expression of differentiation markers *in vitro* (Gibson et al. 1992), and when butyrate is instilled into the rectum of humans with normal colonic mucosa, reduced mucosal urokinase activity which stabilizes cell adhesion and reduces cell death is seen, but with no changes in epithelial cell kinetics or differentiation (Gibson et al. 1998).

The actions of SCFAs may be due to increased expression of genes that favour apoptosis such as Bax and Bak and concomitantly reduced expression of genes which prevent apoptosis such as Bcl-2 and Bcl-X_L (Hague et al. 1996; Scheppach et al. 2001).

Again there would appear to be a paradox between normal and neoplastic cells when gene expression induced by butyrate is investigated. In normal colonic mucosa a lack of butyrate results in increased expression of the Bax gene, a pro apoptotic protein expressed largely in the upper half of the colonic crypt leading to subsequent apoptosis (Hass et al. 1997). However in malignant cell lines the addition of butyrate induces expression of the Bax gene and subsequent apoptosis and in premalignant colonic crypts with hyperproliferation, a lack of butyrate reduces apoptosis. Therefore it would appear that butyrate promotes normal physiological proliferation but when proliferation is abnormal apoptosis is induced. Thus

butyrate and to a lesser extent propionate and acetate counteract any disruption of the balance between cell proliferation and cell death by apoptosis which is thought to be an important event in colonic carcinogenesis (Garewal et al. 1996).

In addition to the control of proliferation, butyrate may help prevent carcinogenesis by improving the detoxification of dietary carcinogens. This is achieved by inducing expression of the Glutathione-S-transferase family of proteins which catalyses the conjugation of carcinogens to glutathione (Ebert et al. 2003).

The intracellular mechanism by which butyrate inhibits malignant cell proliferation, modulates gene expression and induces differentiation culminating in apoptosis is thought to be by histone hyperacetylation within the cell nucleus. Butyrate is a non competitive inhibitor of the enzyme histone deacetylase, reversibly binding to a separate site from that of the substrate acetate and resulting in changes in transcriptional activity, phosphorylation and methylation of the DNA-histone complex and possibly inhibition of DNA synthesis itself. Expression of various oncogenes are affected including c-myc important in tumour cell growth and cytoskeleton associated tyrosine kinase involved in cellular responses to cytokines (e.g TGF-β_1) leading to tumour growth inhibition (Topping & Clifton 2001). Cell cycle analysis has shown butyrate induces G0/G1 and G2/M cell cycle arrest in SW620 colonic cancer cells (Heerdt et al. 1997)

Butyrate has similar pro-apoptotic and anti-neoplastic activity in cell lines originating from other human cancers including neuroblastoma, breast cancer, prostate cancer and lung cancer, and the clinical potential of butyrate derivatives and other histone deacetylase inhibitors have been demonstrated by Phase I trials in patients with solid tumours and leukaemias. Butyrate and other histone deacetylase inhibitors (HDI's) may have a role as sensitizing agents making cancers more susceptible to other chemotherapeutic agents and form a valuable adjunct in cancer treatment strategies.

Rationale for the luminal provision of SCFAs in the neobladder

Short chain fatty acids play a central role in maintaining the mucosal barrier in the lower gastrointestinal tract. In the context of bladder augmentation the intestinal segment, in the majority of cases colon or ileum is starved of its preferred metabolic substrate when removed from the faecal stream to augment or create a neobladder (Roediger 1990). A process of mucosal crypt atrophy (Treiger & Marshall 1991), chronic inflammation and excess mucous production occurs with accompanying changes in mucin expression (Aragona 2001; N'dow et al. 2000; N'dow et al. 2004). Clinically patients experience blocked catheters, urinary tract infection, bladder calculi and occasionally spontaneous perforation (Bertschy et al. 2000). In the long term there is an increased risk of malignancy. The lack of exogenous SCFAs that occurs distal to any stoma (Appleton & Williamson 1989; Haas & Haas 1988), or in colovaginoplasty (Hensle & Reiley 1998; Martinez-Mora et al. 1992; Syed et al. 2001; Toolenaar et al. 1993; Wesley & Coran 1992) results in similar histological and clinical manifestations termed diversion colitis and has been successfully treated with SCFA instillations (Harig et al 1989; Kiely et al. 2001). Likewise ulcerative colitis in part due to failure to utilize luminal SCFAs (endogenous starvation) responds to increasing the luminal SCFA availability (Sandborn 1998; Vernia et al. 2003).

At a biochemical and cellular level the mucosal barrier separates the oxidative environment of the lamina propria from the reductive environment of the lumen. Processes such as substrate metabolism, membrane assembly, detoxification of luminal toxins and maintenance of the redox balance across the mucosal barrier are performed by the enterocyte to maintain good barrier function. Inadequate provision of SCFAs causes enterocyte starvation and subsequently impaired barrier function. The host may then be exposed to various toxic, bacterial and immunological insults as well as physiological consequences of failure to maintain the redox balance (Wachtershauser & Stein 2000). The mucosa becomes a

secretory epithelium resulting in diarrhoea. Morphologically there is hypoplasia, reduced mucosal height and decreased crypt cell proliferation in the defunctioned colon (Appleton & Williamson 1989).

In the long term there is a risk of malignancy in enterocystoplasty, the aetiology of which is unknown. Patterns of mucin expression are similar to those associated with colorectal adenocarcinoma (Moorcraft et al. 1983; Nurse & Mundy 1989a), and the diverted colon exhibits features considered to be pre-neoplastic including crypt hyperproliferation, and chronic inflammation. Short chain fatty acids are known to be antiproliferative, and promote cellular differentiation and apoptosis in a wide variety of colonic carcinoma and adenoma cell types and alter gene expression towards a less neoplastic phenotype (Scheppach & Weiler 2004). In clinical trials dietary fibre the precursor of SCFAs is protective against colorectal adenocarcinoma (Bingham et al. 2003).

We postulate that the process of mucosal starvation and its sequelae seen in the diverted or starved colon occurs in those segments incorporated into the urinary tract, and would benefit in a similar way to intravesical instillations of SCFAs. The resulting provision of the enterocytes preferred metabolic substrate would help restore mucosal health including epithelial and immunological barrier function, trophic improvements, and reduced secretory function and potential for neoplastic transformation. With improved barrier function and reduced secretions we would hope that problems with urinary tract infection and excess mucous production would diminish. The strength of the anastomosis and bowel segment would improve reducing the risk of spontaneous perforation and anastomotic leak. The pro-apoptotic and anti-neoplastic benefits of butyrate would promote a more differentiated phenotype in the bowel segment in particular at the anastomosis thus reducing the risk of neoplastic transformation.

There is little doubt that SCFAs have a beneficial effect on the native gastrointestinal tract including the colon and to a lesser extent the small intestine, and their absorption, distribution and metabolism outside the gastrointestinal tract is well understood. However before a clinical trial of intravesical SCFA instillations in enterocystoplasty can be performed, their effect on the native urothelium should be investigated especially in view of the cytotoxic effects on urothelial cells seen with other fatty acids including meglumine gamma linolenic acid (Crook et al. 2002), and N3 and N6 long chain fatty acid (Diggle et al. 2000b; Diggle et al. 2000a).

Summary of study design and objectives

This study set out to investigate the effect of SCFAs on urothelial cells. There were a number of malignant urothelial and colonic cell lines available in house which using simple standard techniques were established in culture. Development of primary urothelial cell cultures however presented a greater challenge. In chapter 3, details of the technique originally developed in Leeds by Dr Southgate's group with my own modifications are described. By simplifying the Southgate method I was able to achieve greater cell yield. However all primary urothelial cell cultures had a short usable life span, making the technique very labour intensive and dependent on a regular fresh supply of human urothelium.

With the cell culture techniques established, the first set of experiments set out in chapter 4 looked at the effect of SCFAs on cell growth. Using the MTT assay the effect of both the cocktail of butyrate, propionate and acetate and the effect of each SCFA individually was investigated. Malignant and primary urothelial cells and two colonic cancer cell lines were investigated for comparison.

In chapter 5 flow cytometry was used to investigate the mechanism of cell growth inhibition. Again the effect of the combined cocktail as well as each individual SCFA was investigated in primary and malignant urothelial cells. Initially the dose response and incubation time was investigated in all three SCFAs, but then using malignant urothelial cells the effect of butyrate on synchronized cells was investigated. This aimed to demonstrate the site of butyrate's action in the cell cycle.

Finally in Chapter 6, to complete the cell culture work, direct fluorescence microscopy was employed to investigate the presence of apoptosis in butyrate treated cells.

In chapter 4, 5 and 6 the effect of SCFAs on cell growth and the cell cycle were investigated *in vitro*, so in chapter 7 a rodent model was employed to investigate how significant these effects were *in vivo*. Tolerance to both a single instillation and multiple instillations into the

urinary bladder of a SCFA cocktail currently used at Great Ormond Street Hospital for the treatment of diversion colitis was investigated. Histological and immunohistochemical assessment was performed.

Chapter 2

Materials and Methods

Introduction

This chapter contains details of all materials, and general methodology related to cell culture. Specific methodology relating to each set of experiments is described in detail at the beginning of the appropriate chapter.

Materials

All materials were purchased from Sigma, Poole, Dorset unless otherwise stated.

Short chain fatty acids

Individual component SCFAs were purchased in desiccated form and rehydrated as a concentrated stock solution in phosphate buffered saline (PBS). The sodium salt of butyric acid was purchased from Sigma, Poole, UK. Sodium propionate and sodium acetate were purchased from BDH chemicals (appendix 1)

A concentrated stock solution of SCFA cocktail was prepared in PBS from a formulation used by Great Ormond Street Hospital for Children NHS Trust (Table 2.1). The concentration of butyrate was 1600mM.

For animal experiments a pre packaged mixture of SCFA cocktail which when 50mls of sterile water was added contained 40mM butyrate, 30mM propionate, and 60mM acetate at pH 7.0 (Special Products Ltd, Addlestone, UK).

Separate stock solutions of butyrate (1600mM), propionate (2400mM) and acetate (2400mM) were prepared in PBS. Stock solutions were filter sterilized through a 0.22 μm filter and stored at 4°C in the fridge. Immediately before use appropriate dilutions of SCFA were

prepared with complete medium, either Dulbecco's Modified Eagle's Medium (DMEM) for cell line experiments or keratinocyte serum free medium (KSFM) for primary urothelial cell experiments.

Sodium Hydroxide(powdered) [BDH]	0.8000g
Butyric acid [BDH]	1.7622g
Sodium acetate [Fluka]	2.4609g
Sodium propionate [Fluka]	1.4409g
Distilled water to make up to	500mls

Table 2.1 Great Ormond Street Hospital SCFA cocktail formulation

Cell Culture

Primary cells

Normal urothelial cells were obtained from paediatric samples of bladder, ureter and renal pelvis obtained at open operation with ethics approval and informed consent (Appendix 3) The tissue was transported in sterile Hanks' balanced salt solution (HBSS) containing 10 mmol/l HEPES, pH 7.6 (Sigma) and 20 KIU/ml of aprotinin (Trasylol, Bayer, Newbury). The tissue was washed twice in fresh transport medium and excess stromal and fatty tissue was removed. The sample was divided into 0.5cm pieces and incubated overnight at 4°C in 0.02% EDTA (Sigma).

The following morning the urothelium was gently removed from the underlying stroma with fine forceps (later with a scalpel blade) and washed twice in Hanks balanced salt solution. The sheets of cells were then digested to a single cell suspension with 200 U/ml of collagenase type IV (Sigma) for 10 minutes. This step was later removed from the technique

because cell yield appeared to be improved without it. Cells were then washed in keratinocyte serum free medium (KSFM) containing cholera toxin (Sigma) at a concentration of 30ng/ml and 100μg/ml of both penicillin / streptomycin solution. Cells were then placed into 25cm^2 culture flasks at an average density of 4 x 10^4/cm^2 (Southgate et al. 1994). Cultures were maintained at 37°C in an incubator with humidified 5% CO_2 in air. The medium was changed initially after 24 hours then subsequently every 48 hours.
Cells were passaged for experiments or to maintain in culture either when cells were >75% confluent or up to 10 days after initial plating. If cells were not passaged regularly they became increasingly adherent resulting in low passage efficiency. Cells were incubated with 0.02% EDTA (Sigma) at room temperature for 10minutes followed by 60 seconds with a minimal volume (1 ml) of trypsin/0.02%EDTA (Sigma) just enough to coat the flask. Excess was removed immediately and the flask was gently agitated to disaggregate the cells. Soybean trypsin inhibitor (Sigma) was added to the flask at a concentration of 1mg/ml in 3mls of KSFM to neutralize residual trypsin. The cell suspension was centrifuged at 1000rpm and the pellet resuspended in KSFM. For experimental use the cell density was adjusted to100 000 cells/ml using a haemocytometer. (See cytotoxicity assay)

Cell lines

The following cell lines were kindly donated by Dr John Masters of University College Hospital, London, UK:

1. MGHU-1 human female superficial (T2/3 G3) transitional cell carcinoma cell line (TCC) in wild type form.
2. RT112 human female transitional cell carcinoma cell line in wild type form
3. HT-29 human female colon cancer cell line
4. CaCo-2 human male colon cancer cell line

The MGH-U1 human bladder cancer cell line is thought to be a multiclonal cell line, originally derived in America from a T3 bladder cancer metastasis which has since been contaminated with the T-24 clone. It grows as an adherent monolayer (McGovern et al. 1988; Povey et al. 1976).

The RT112 cell line is a moderately well differentiated transitional cell carcinoma cell line derived from a human urinary bladder TCC histological grade G2, excised from a female patient with untreated primary bladder carcinoma in 1973. It is morphologically an epithelial cell which grows as an adherent monolayer (Marshall et al. 1977; Masters et al. 1986).

HT29 is a human Caucasian colon adenocarcinoma grade II cell line, isolated from a primary tumour in a 44 year old Caucasian female. It forms an adherent monolayer in culture (Marshall et al. 1977; von Kleist et al. 1975).

Caco-2 is a well differentiated colon cancer cell line derived from a primary caecal carcinoma in a 72 year old Caucasian male using the explant culture technique. It forms moderately well differentiated adenocarcinomas consistent with the colonic primary grade II (Fogh et al. 1977).

All cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 2mM L-glutamine, foetal bovine serum (FBS at 10% v/v) and 100μg/ml of both penicillin / streptomycin solution (complete medium). Medium was changed every 48 hours and cells were passaged when 75% confluent. Cultures were maintained in a humidified incubator with 5% CO_2 at 37°C. Reserves of each cell line were maintained by viably freezing aliquots in a liquid nitrogen freezer. Freezing medium composed DMEM with 10% Dimethylsulphonate (DMSO) and 10% FBS.

Cells were passaged for experiments or to maintain in culture by initially removing existing culture medium with a Pasteur pipette followed by a rinse with a solution of EDTA 0.02% in phosphate buffered saline (PBS/EDTA). Cells were then disaggregated with 1mg/ml trypsin

in PBS/EDTA. The rinsing process removes residual serum which can neutralize the action of trypsin. EDTA removes calcium which helps break cells adhesions. PBS/EDTA and trypsin solutions were kept in the fridge and aliquots were warmed to room temperature before use. For experimental purposes cells were harvested once they had achieved >75% confluence.

Data analysis and statistics

In each chapter the specific method of data analysis is described, but in general Microsoft Excel spread sheets were employed to process data. Statistical advice was taken from Professor Peter Thomas at Bournmouth University and where data was amenable to statistical analysis this was performed using SPSS software version 12.

Chapter 3

Development and characterization of primary urothelial cell cultures

Introduction

Primary urothelial cells were cultured from paediatric specimens of ureter, renal pelvis and bladder using a method developed by Southgate et al (Southgate et al. 1994). This is described in detail in chapter 2. This technique was modified to improve cell yield after experiencing initial difficulties with the original method. The cultures were then characterized to confirm that despite modifications to the technique, they still contained epithelial cells rather than stromal or fibroblast elements from the specimen

Cell culture method

The essential steps in the method are listed below:

1. Remove tissue specimen with minimal trauma.
2. Transfer to laboratory in transport medium.
3. Remove excess adventitial tissue and divide tubular specimens longitudinally under sterile conditions. Large specimens can be divided into 1cm pieces.
4. Rinse specimen in 0.02% EDTA to remove debris and blood cells and place in fresh 0.02% EDTA overnight at 4°C.
5. Dissect the urothelium from the specimen using fine forceps.
6. Wash cells in Hank's balanced salt solution
7. Incubate in 200U/ml collagenase type VI for 10minutes at 37°C
8. Wash cells in growth medium
9. Plate in to culture flasks.

The yield of cells using this method was very variable, and despite having a large piece of good quality urothelium after step 5, separation by enzymatic digestion with collagense IV failed to produce a satisfactory cell suspension and after 24 hours in culture the yield was in the region of 10 %. With in house experience of urothelial explants using rat bladder which can generate large skirts of urothelial cell monolayers around the full thickness explant in culture (Crook et al. 2000) and methods used by other groups (Mothersill et al. 1994; Petzoldt et al. 1994), modifications to the Southgate technique were developed.

Modified technique

1. No use of diathermy. Macroscopically normal specimens and microscopically normal uroethelial cell suspensions failed to grow if they had been removed using diathermy.

2-4 No change

5 Use a 22 gauge scalpel blade to remove urothelium. Draw across urothelial surface at 45°. Helps divide the sheets of urothelium into small clumps of urothelial cells.

6 Rinse cells from scalpel blade directly into KSFM.

7 Shake cells suspended in KSFM vigorously if necessary for a few seconds to disperse cell clumps and plate directly into culture flasks.

Results

The cell suspension produced by this technique contained single cells, and variable sized clumps of cells. By plating cells out in a minimal volume of about 3mls into a 25 cm^2 flask and leaving undisturbed for 24 hours allowed greater than 50 % of cell clumps to settle down and adhere to the base of the flask. Cell clumps behaved as miniature explants. Once

adherent, cells appeared to migrate from the colony producing a monolayer skirt of urothelial cells around the explant as illustrated in figure 3.1.

This simplified technique consistently produced a greater yield of cells, less cell debris and greater plating efficiency. Reducing the number of steps also minimized the risk of introducing infection to the culture.

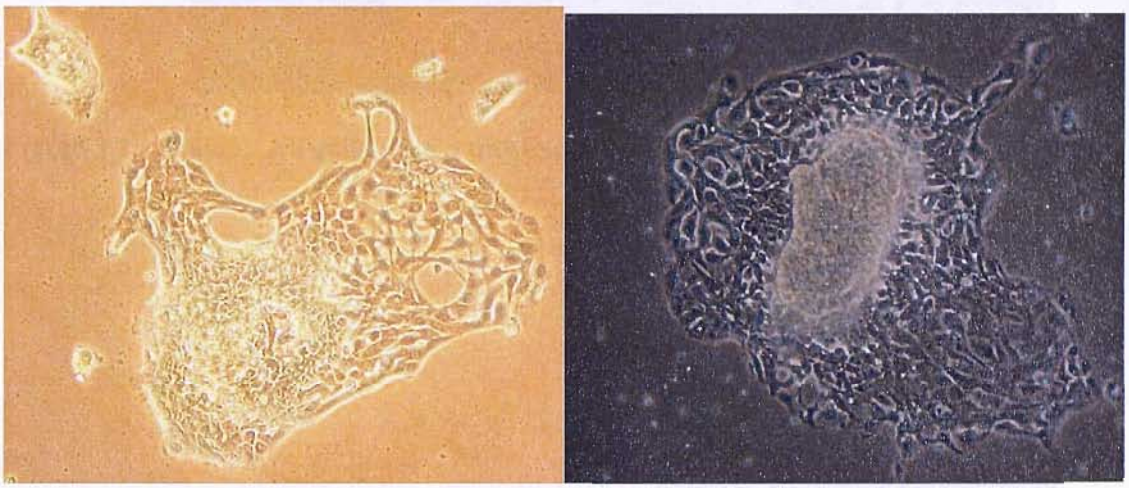


Figure 3.1: Day 2. Central clumps of urothelial cells with surrounding skirt of adherent monolayer

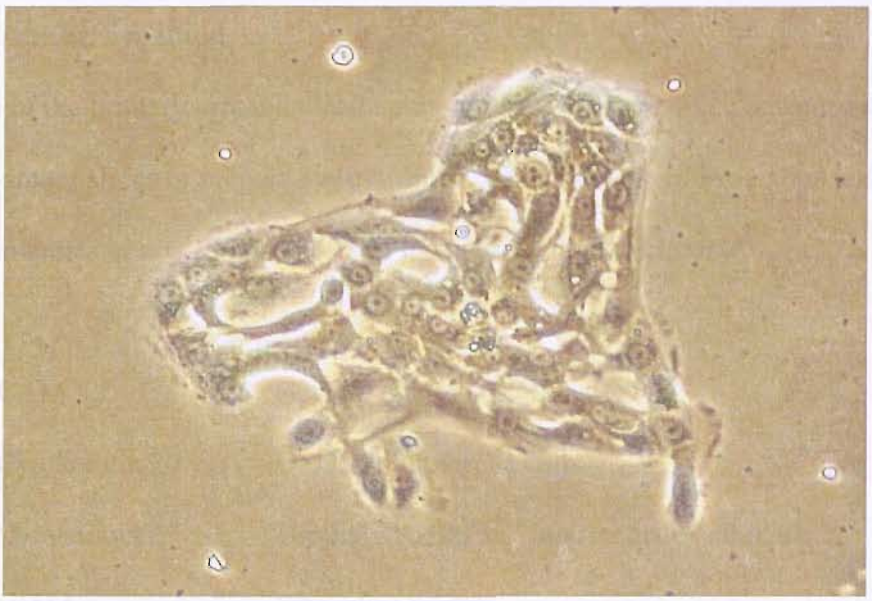


Figure 3.2: Day 3. All cells from a tiny explant have become adherent as a monolayer

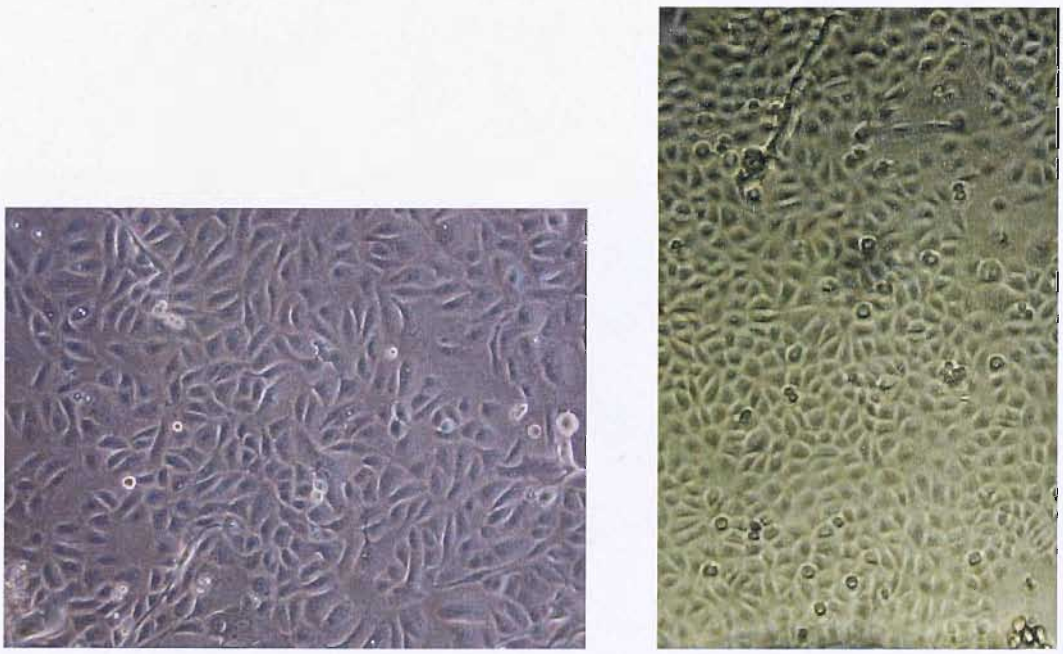
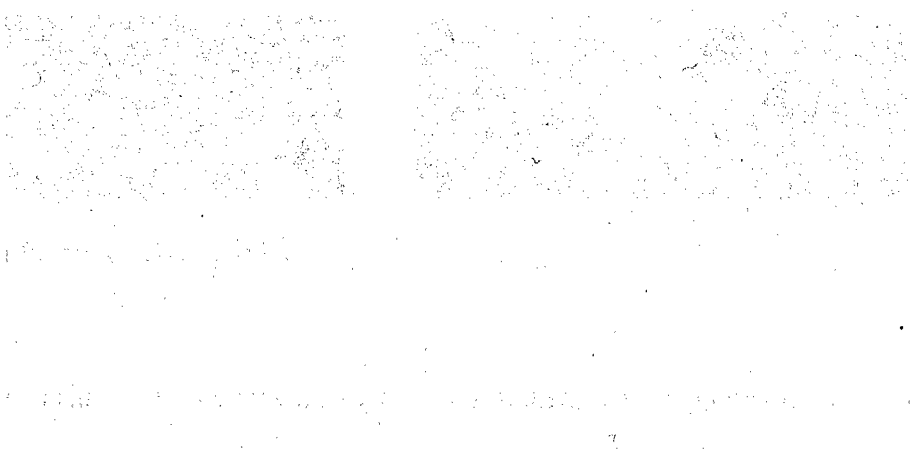


Figure 3.3: Day 6. Confluent monolayer of urothelial cells

Immunohistochemistry method

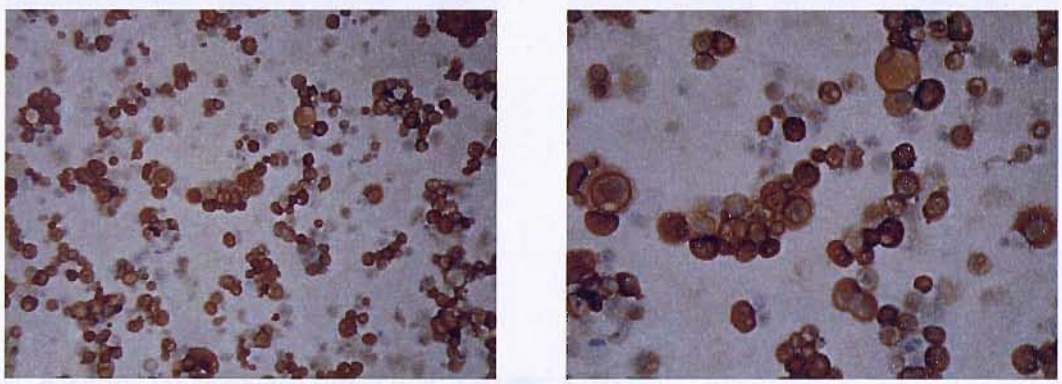
During passage of the primary urothelial cell cultures a small volume of cells were seeded on to eight well chamber slides or prepared as cytospins. They were fixed in acetone and stained with various antibodies in order to characterize the cells within the culture.

Antibodies:	CAM 5.2	General epithelial cell marker
	Cytokeratin 20	Urothelial umbrella cells
	Cytokeratin 17	Urothelial basal and intermediate cell marker
	αSMA	Fibroblast marker
	HB 95	HLA class 1 (nucleated cells)
	Alkaline phosphatase	Negative control
	Mouse irrelevant	Negative control



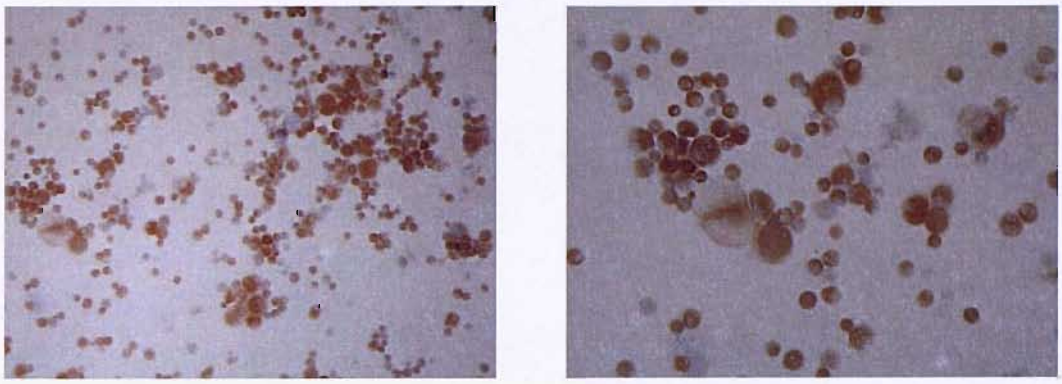
Results

All urothelial cell cultures stained positively for CAM 5.2 and HB 95 and negatively for αSMA, alkaline phosphatase, and mouse irrelevant antibody (figure 3.4). Staining for cytokeratin 20 and cytokeratin 17 varied considerably between specimens (figure 3.5). About 75% of specimens stained with some positivity to cytokeratin 20 but the number of positive cells in each field varied from about 5% to 50%. Further work done by others within the department have shown that the density of cytokeratin 20 expression decreased and the density of cytokeratin 17 expression increased with increasing number of passages. This observation would require further detailed experiments to prove statistically, however the ratio of cytokeratin 20 to cytokeratin 17 expression may offer a means of assessing urothelial cell differentiation in culture (Varatharaj et al. 2003).

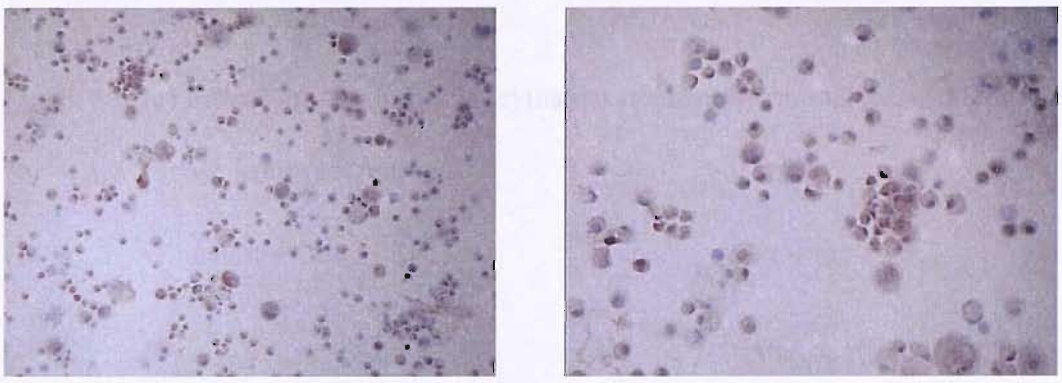


a. Cam 5.2 (objective: x10 and x20)

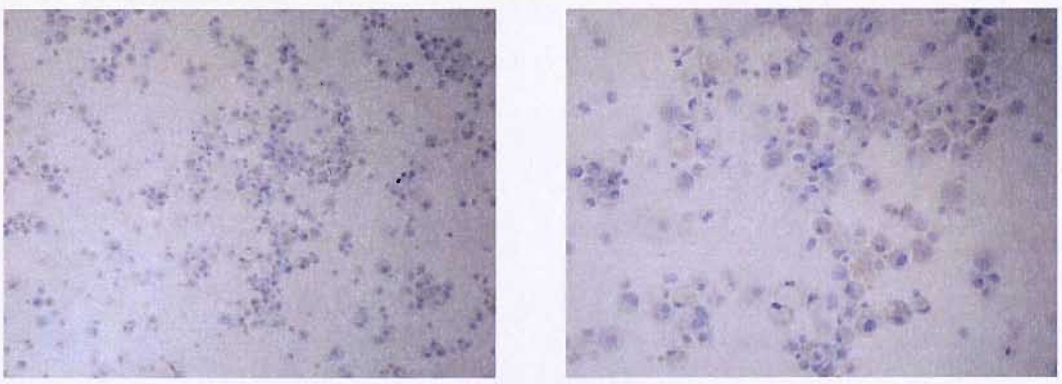
Figure 3.4 (a) Primary urothelial cells (cytospins) stained by immunohistochemistry with cam 5.2



b. HB 95 (objective: x10 and x20)

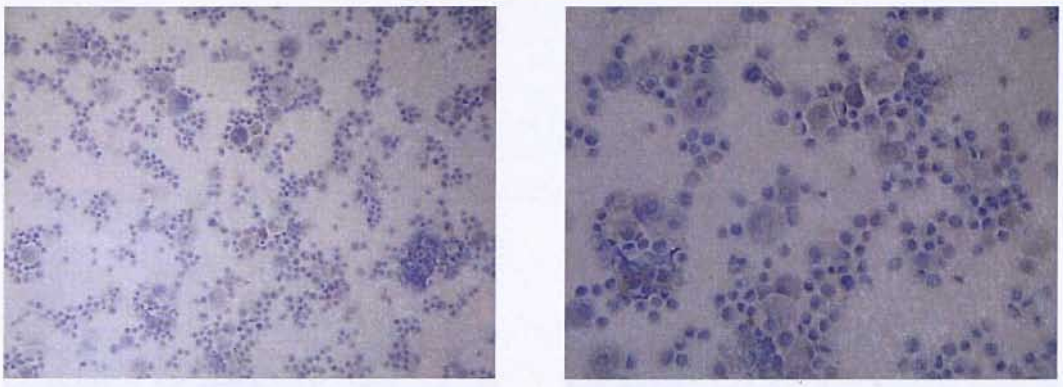


c. αSMA (objective: x10 and x20)



d. Alkaline phosphatase (objective: x10 and x20)

Figure 3.5 (b-d) Primary urothelial cells (cytospins) stained by immunohistochemistry with (b) HB 95, (c) αSMA, (d) Alkaline phosphatase



e. Mouse irrelevant antibody (objective: x10 and x20)

Figure 3.6 (e) Primary urothelial cells (cytospins) stained by immunohistochemistry with mouse irrelevant antibody

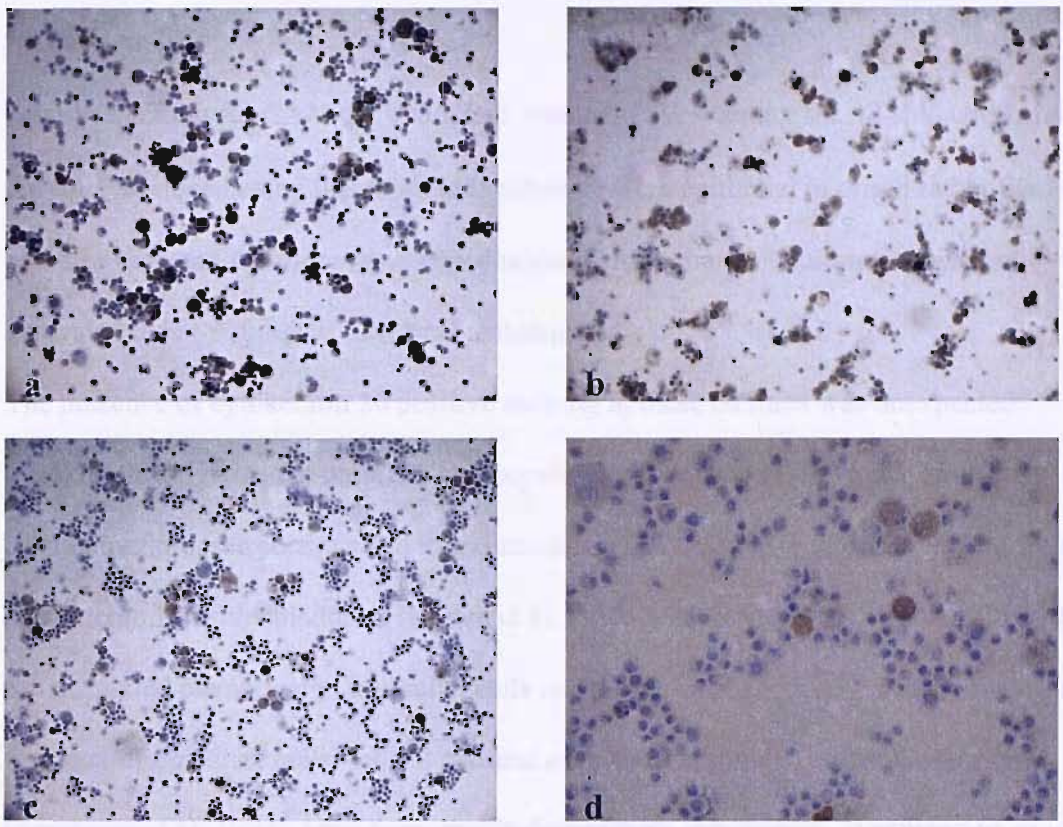


Figure 3.7 (a-d) Primary urothelial cells (cytospins) stained for cytokeratin 20 (a-c: objective x10, d: objective x20)

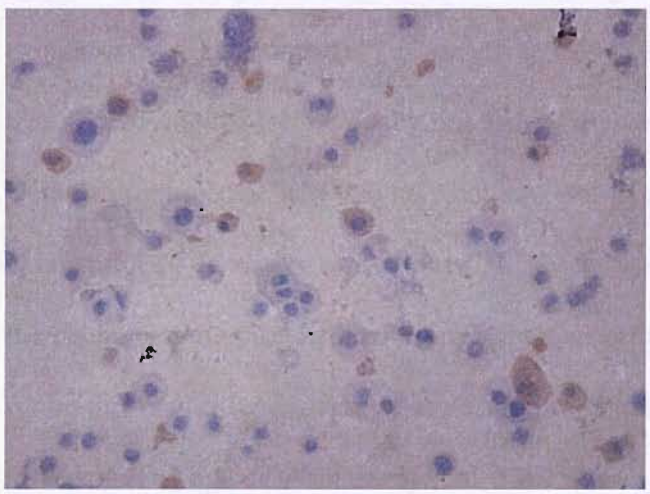


Figure 3.8 Primary urothelial cells (cytospins) stained for cytokeratin 17 (objective x20)

Discussion

Positive staining for CAM 5.2 and HB95 with negative staining for αSMA, confirmed that the cells produced using this modified technique were epithelial in origin rather than stromal or fibroblasts and therefore represented primary urothelial cell cultures equivalent to those generated using Southgate's original technique.

The presence of cytokeratin 20 positive staining in these cultures was unexpected. Cytokeratin 20 stains the umbrella cell population in the superficial layer of normal urothelium in tissue sections and its expression is increased in well differentiated transitional cell carcinoma of the bladder (Harnden et al. 1999; Moll et al. 1992). It identifies a fully differentiated phenotype in urothelial cells and therefore only stains the umbrella cells and a few late intermediate cells. In cell cultures of primary urothelial cells other groups (Southgate et al. 1994) have been unable demonstrate cytokeratin 20 expression suggesting that urothelial cell cultures do not represent all levels of the urothelium or degrees of stratification. The presence of cytokeratin 20 in cultures using this modified tissue preparation technique may indicate that this method promotes urothelium in culture to express a more normal pattern of growth and differentiation. An alternative explanation is that simplifying the technique, by not using enzymatic digestion may promote survival of well differentiated cells which may not have survived otherwise. The observation that cytokeratin 20 decreased and cytokeratin 17 increased with further passages is consistent with the phenomenon of de-differentiation which is observed in most cell culture systems. In the future this may offer a means of assessing urothelial cell culture stratification in response to differentiating agents (Varatharaj et al. 2003).

Chapter 4

Assessment of the cytotoxicity of short chain fatty acids

Introduction

The effect of a cocktail of short chain fatty acids and each component butyrate, propionate, and acetate on cell proliferation was investigated in primary urothelial cells and both urothelial and colonic tumour cell lines.

Method

Cell proliferation in culture was assessed using the MTT (3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) assay (Freshney 1994). This is a calorimetric dye assay based on the ability of viable cells to sequester MTT and reduce it to a purple formazan dye. Experiments were performed using primary urothelial cells, transformed urothelial cell lines (MGH-U1 and RT112) and colonic cell lines (CaCo-2 and HT29) in culture. For each experiment cells were disaggregated from culture flasks with Trypsin EDTA and seeded into 96 well microtitre plates in complete medium at a density of 10^3 cells per well for tumour cells and 10^4 cells per well for primary cells. 100µl of cell suspension was aliquoted into each well then placed in the incubator for 24-36 hours allowing the cells to settle down and become adherent. Various solutions of SCFAs in complete medium were then added and the plates transferred back to the incubator for between 30 minutes and 72 hours. Drug solution was then removed, wells were rinsed and 100µl of fresh warmed culture medium was added. Plates were incubated for a total of 5 days or until the control wells became confluent. The residual viable biomass was measured using the MTT assay. Fifty microlitres of 1.25mg/ml MTT prepared in fresh medium from a stock solution (5mg/ml in PBS) was added to each well and plates were incubated for a further 3 hours. The

supernatant was removed and 150 μl dimethyl sulphoxide was added to each well. This dissolved the purple formazin crystals allowing the optical density of the purple liquid to be measured using a microtitre plate reader at 570nm. A hard copy of the data was produced by a printer attached to the plate reader.

Data analysis

Data was processed in Microsoft Excel. The mean optical density (a measure of viable biomass) was calculated for each drug dilution. All values were expressed as percentage of control. The mean value and 95% confidence intervals were plotted in figures 4.1 to 4.18, and mean optical density and 95% confidence intervals in figures 4.19 and 4.20. Statistical analysis was performed in SPSS software version 12, using a 2 way analysis of variance to compare mean percentage of control or optical density between concentration, different fatty acids and incubation time, and their interactions. The analysis was performed for each curve separately, comparing all values within each curve. Then a combined analysis was performed (fatty acid* time* concentration) to assess overall statistical significance. P values are included with each figure and values below 0.05 have been interpreted as being statistically significant.

Experimental plate set up

Cells seeded into 96 well plates were incubated with freshly prepared SCFA dilutions in complete medium. A standard lay out of dilutions and controls were used in each experiment. Tables 4.1 and 4.2 illustrate the arrangement of wells for butyrate, propionate and acetate dilutions for tumour cells. The arrangement and number of wells utilized for each primary cell experiment was dependent on the number of cells available which was often

insufficient to seed a complete plate. Butyrate concentrations between 0.16mM and 80mM, and propionate and acetate concentrations between 0.46mM and 240mM were investigated.

						MTT	MTT	MTT	MTT	MTT	MTT
control	80	40	20	10	5	2.5	1.25	0.625	0.31	0.16	control
control	80	40	20	10	5	2.5	1.25	0.625	0.31	0.16	control
control	80	40	20	10	5	2.5	1.25	0.625	0.31	0.16	control
control	80	40	20	10	5	2.5	1.25	0.625	0.31	0.16	control
control	80	40	20	10	5	2.5	1.25	0.625	0.31	0.16	control
control	80	40	20	10	5	2.5	1.25	0.625	0.31	0.16	control
MTT	MTT	MTT	MTT	MTT	MTT						

Table 4.1 Distribution of butyrate dilutions (mM), and reagent and experimental controls in the 96 well plate.

						MTT	MTT	MTT	MTT	MTT	MTT
control	240	120	60	30	15	7.5	3.75	1.87	0.93	0.46	control
control	240	120	60	30	15	7.5	3.75	1.87	0.93	0.46	control
control	240	120	60	30	15	7.5	3.75	1.87	0.93	0.46	control
control	240	120	60	30	15	7.5	3.75	1.87	0.93	0.46	control
control	240	120	60	30	15	7.5	3.75	1.87	0.93	0.46	control
control	240	120	60	30	15	7.5	3.75	1.87	0.93	0.46	control
MTT	MTT	MTT	MTT	MTT	MTT						

Table 4.2 Distribution of acetate or propionate dilutions (mM), and reagent and experimental controls in the 96 well plate

Results

Immediate effect of SCFAs on cells in culture

Initial experiments were designed to assess the immediate cytotoxic effect of SCFAs. In these experiments cells were incubated with drug for 1 hour then assayed with MTT. Figure 4.1 and 4.2 show the viable biomass of normal and transformed urothelial cells is unchanged immediately after 1 hours incubation with butyrate, propionate and acetate. Each graph illustrates the mean percentage of control with 95% confidence intervals plotted against SCFA concentration.

Figure 4.1 Viable biomass of primary urothelial cells immediately after incubation with SCFA

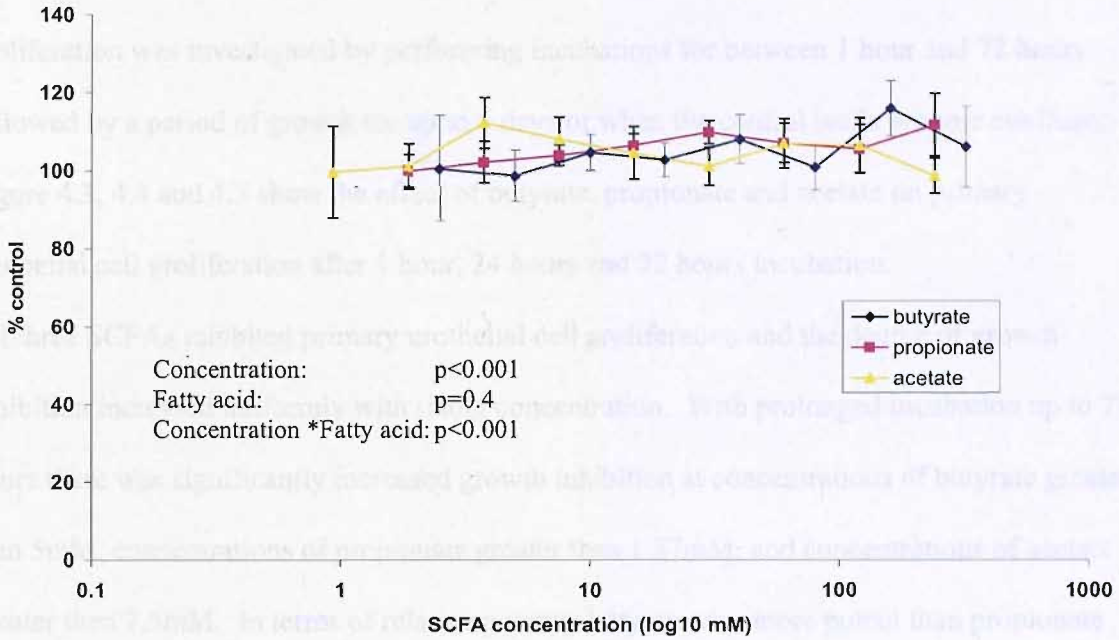
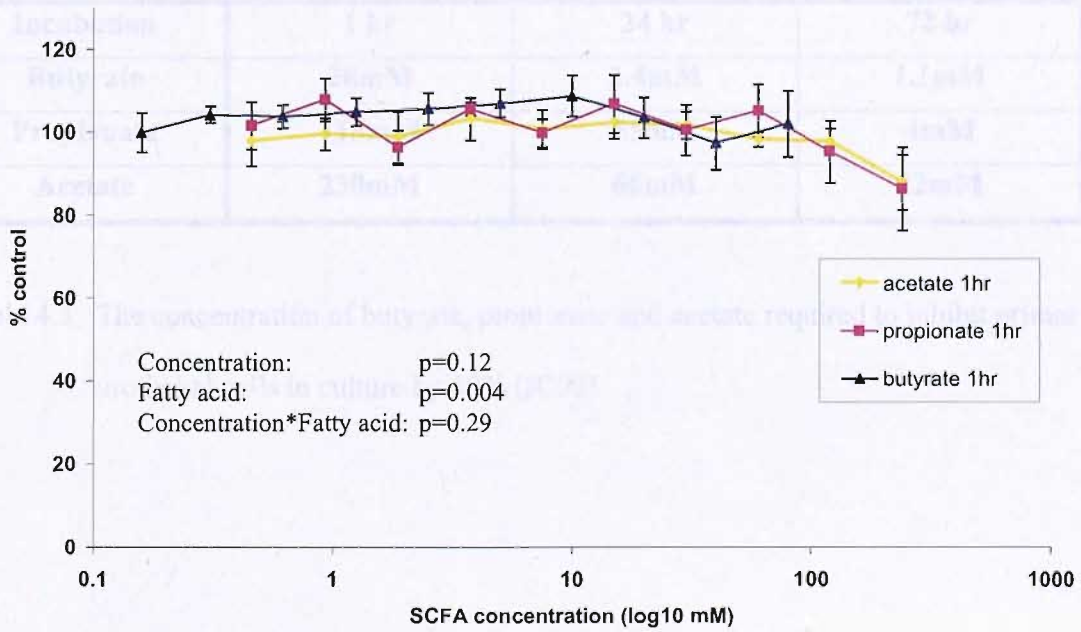


Figure 4.2 Viable biomass of RT112 urothelial cells immediately after incubation with SCFA



Effect of butyrate, propionate and acetate on primary urothelial cells in culture

Having established that exposure to SCFAs was not immediately cytotoxic, the effect on cell proliferation was investigated by performing incubations for between 1 hour and 72 hours followed by a period of growth for up to 5 days or when the control wells became confluent. Figure 4.3, 4.4 and 4.5 show the effect of butyrate, propionate and acetate on primary urothelial cell proliferation after 1 hour, 24 hours and 72 hours incubation.

All three SCFAs inhibited primary urothelial cell proliferation and the degree of growth inhibition increased uniformly with rising concentration. With prolonged incubation up to 72 hours there was significantly increased growth inhibition at concentrations of butyrate greater than 5mM, concentrations of propionate greater than 1.87mM, and concentrations of acetate greater then 7.5mM. In terms of relative potency butyrate was more potent than propionate which was more potent than acetate. This trend was maintained throughout all periods of incubation. The IC50 of butyrate, propionate and acetate are illustrated in table 4.3

SCFA	Primary Urothelial Cell Monolayers		
Incubation	**1 hr**	**24 hr**	**72 hr**
Butyrate	**20mM**	**2.4mM**	**1.1mM**
Propionate	**118mM**	**55mM**	**4mM**
Acetate	**230mM**	**66mM**	**22mM**

Table 4.3 The concentration of butyrate, propionate and acetate required to inhibit primary urothelial cells in culture by 50% (IC50).

Figure 4.3 Viable biomass of primary urothelial cells after incubation with butyrate

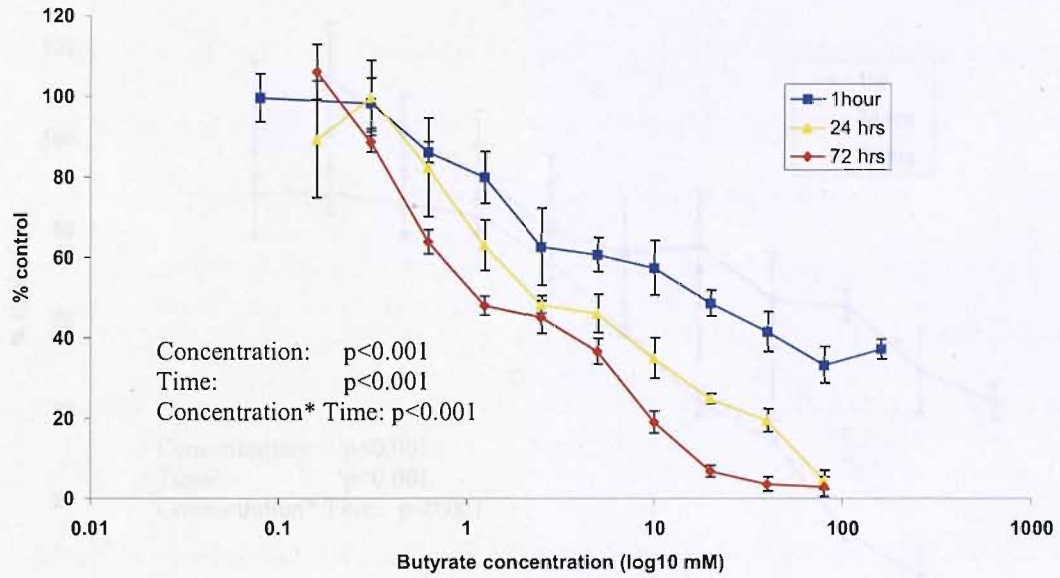


Figure 4.4 Viable biomass of primary urothelial cells after incubation with propionate

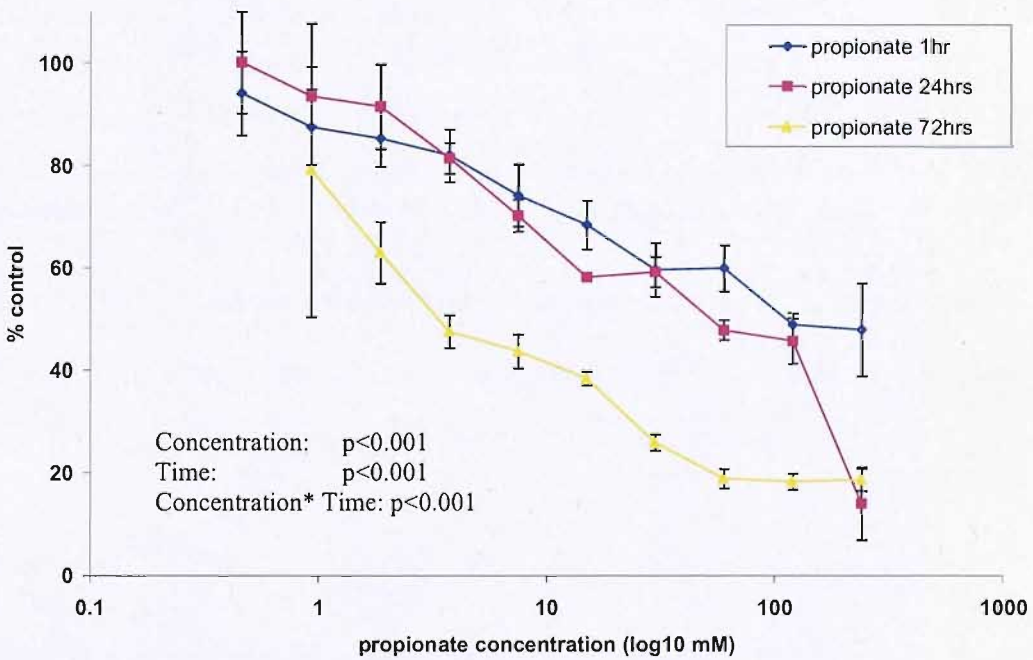
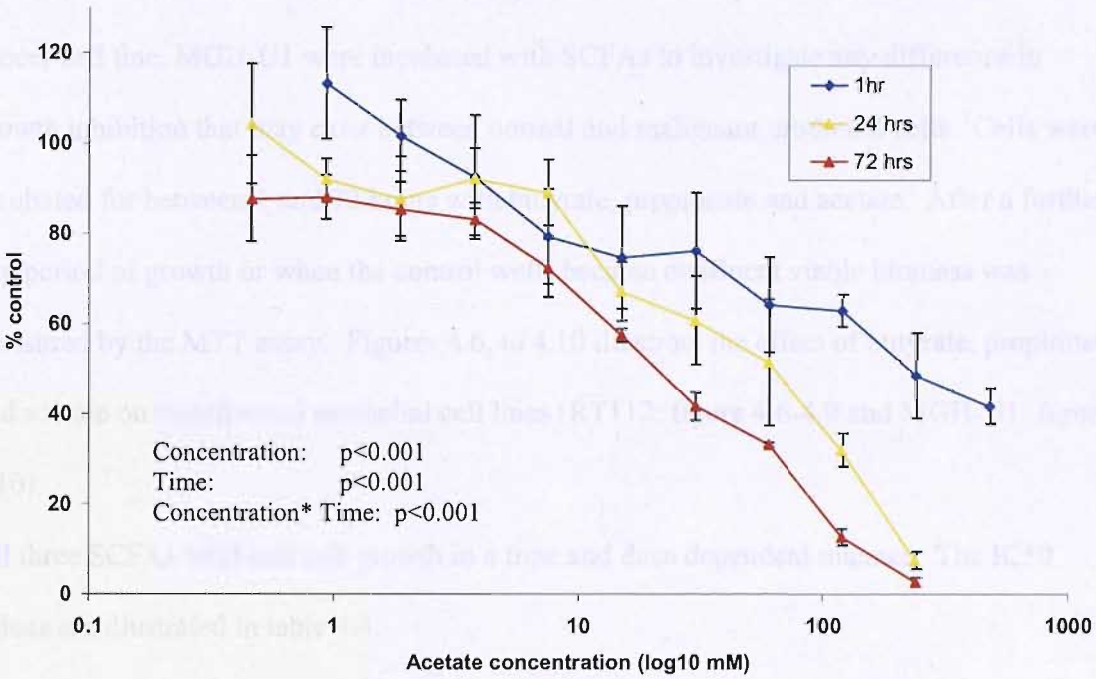


Figure 4.5 Viable biomass of primary urothelial cells after incubation with acetate



Effect of butyrate, propionate and acetate on urothelial tumour cell lines

A well differentiated urothelial cancer cell line, RT112 and a poorly differentiated urothelial cancer cell line, MGH-U1 were incubated with SCFAs to investigate any difference in growth inhibition that may exist between normal and malignant urothelial cells. Cells were incubated for between 1 and 72 hours with butyrate, propionate and acetate. After a further 5 day period of growth or when the control wells became confluent viable biomass was measured by the MTT assay. Figures 4.6, to 4.10 illustrate the effect of butyrate, propionate and acetate on transformed urothelial cell lines (RT112: figure 4.6-4.9 and MGH-U1: figure 4.10).

All three SCFAs inhibited cell growth in a time and dose dependent manner. The IC50 values are illustrated in table 4.4.

SCFA	RT112 urothelial cell line			MGH-U1 urothelial cell line	
Incubation	**1 hour**	**24 hour**	**72 hour**	**1 hour**	**24 hour**
Butyrate	**32mM**	**3mM**	**2mM**	**35mM**	**16mM**
Propionate	**105mM**	**30mM**	**12mM**	**120mM**	**52mM**
Acetate	**240mM**	**103mM**	**75mM**	**240mM**	**128mM**

Table 4.4: The concentration of butyrate, propionate and acetate required to inhibit transformed urothelial cells in culture by 50% (IC50) after incubation for 1 hour, 24 hours and 72 hours.

Both urothelial cell lines demonstrated similar degrees of growth inhibition as shown by their comparable IC50 values. Compared with primary urothelial cells they were almost half as sensitive to butyrate after 1 hour incubation with IC50 values of 32mM and 35mM for RT112

and MGH-U1 cell lines compared with 20mM for primary cells. This increase in sensitivity of primary cells to butyrate was lost after longer incubation periods and there was no difference in sensitivity to propionate or acetate between primary and transformed urothelial cells.

Figure 4.6 Viable biomass of RT112 urothelial cells after incubation with butyrate

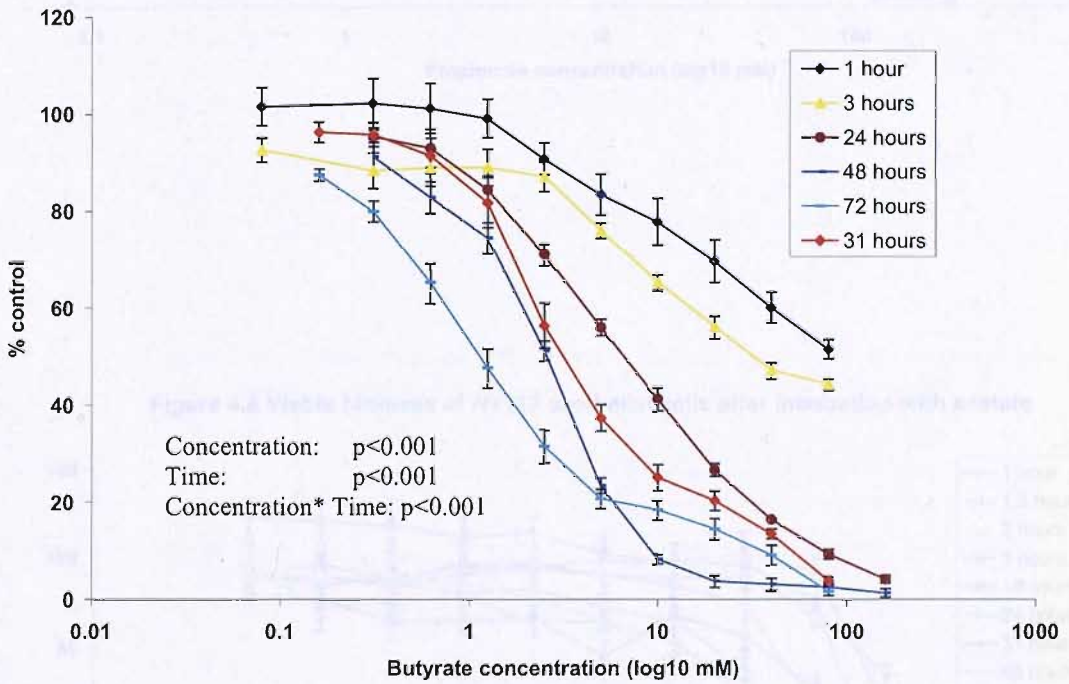


Figure 4.7 Viable biomass of RT112 urothelial cells after incubation with propionate

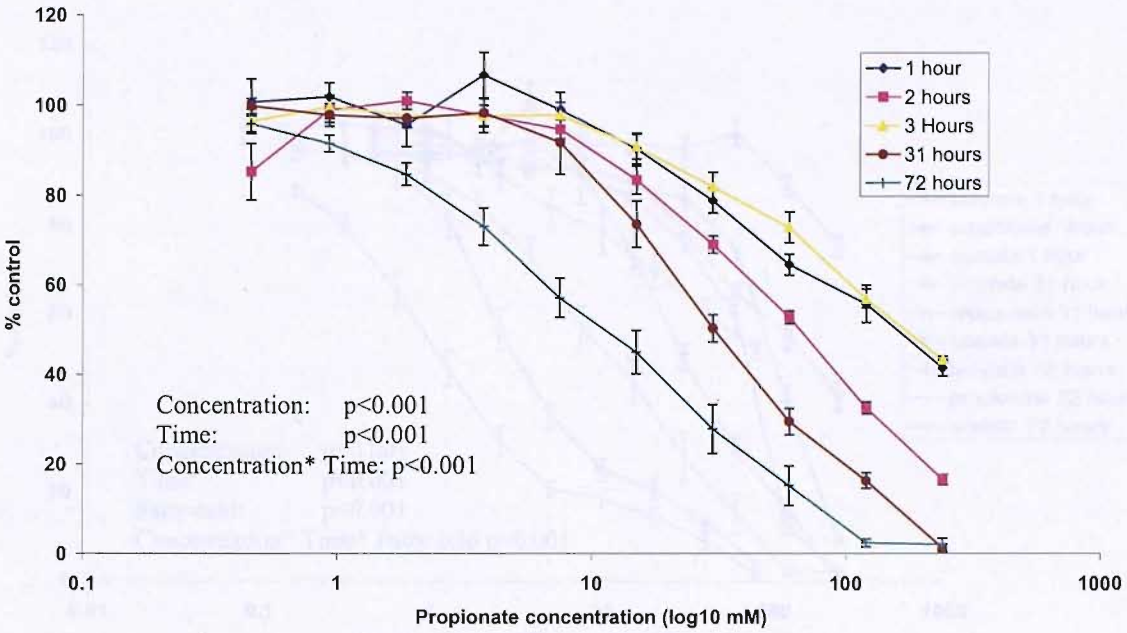


Figure 4.8 Viable biomass of RT112 urothelial cells after incubation with acetate

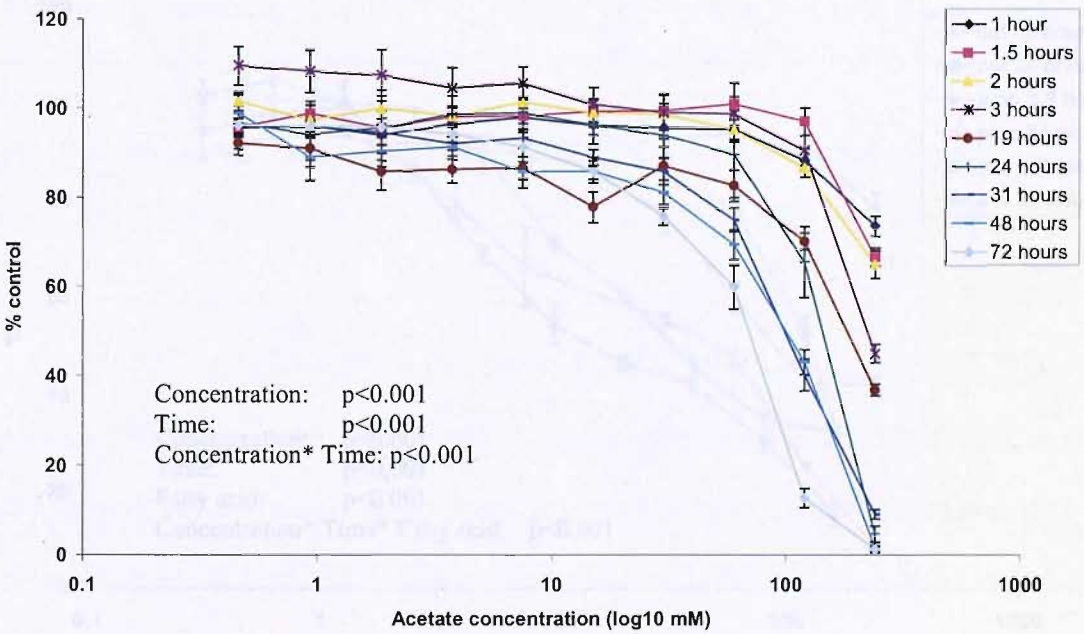


Figure 4.9 Viable biomass of RT112 urothelial cells after incubation with SCFAs. Comparison between butyrate, propionate and acetate

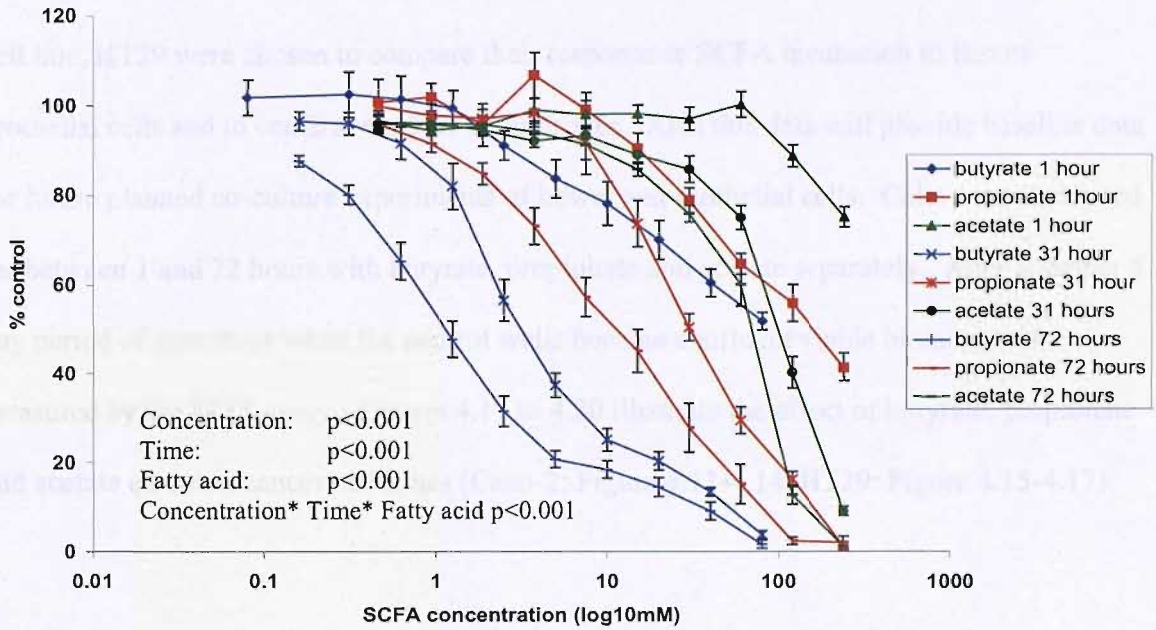
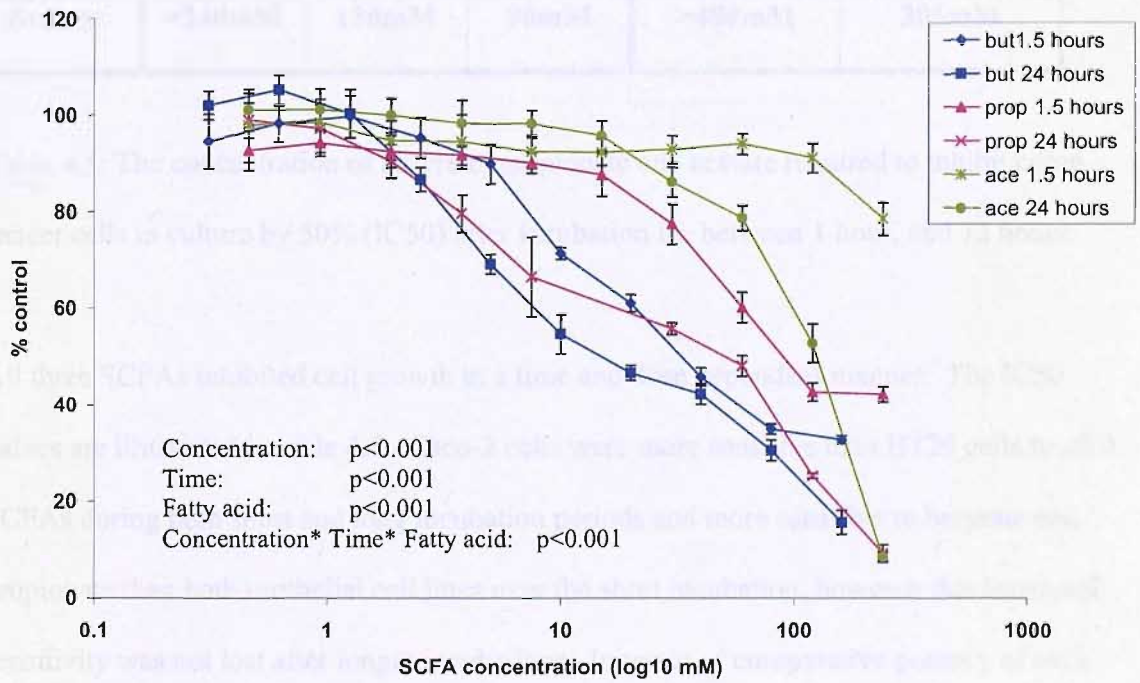


Figure 4.10 Viable biomass of MGH-U1 urothelial cells after incubation with SCFAs. Comparison between butyrate, popionate and acetate.



Effect of butyrate, propionate and acetate on colonic tumour cell lines

A well differentiated colonic cancer cell line, Caco-2 and a poorly differentiated colon cancer cell line, HT29 were chosen to compare their response to SCFA incubation to that of urothelial cells and to confirm work of other groups. Also this data will provide baseline data for future planned co-culture experiments of bowel and urothelial cells. Cells were incubated for between 1 and 72 hours with butyrate, propionate and acetate separately. After a further 5 day period of growth or when the control wells became confluent viable biomass was measured by the MTT assay. Figures 4.11 to 4.20 illustrate the effect of butyrate, propionate and acetate on colon cancer cell Lines (Caco-2: Figure 4.11-4.14, HT29: Figure 4.15-4.17)

	Caco-2 Colon Cancer Cell Line			HT29 Colon Cancer Cell Line	
Incubation	**1 hour**	**30 hours**	**72 hours**	**1 hour**	**24 hours**
Butyrate	**20mM**	**3.8mM**	**3.8mM**	**52mM**	**33mM**
Propionate	**75mM**	**33mM**	**17mM**	**210mM**	**157mM**
Acetate	**>240mM**	**150mM**	**90mM**	**>480mM**	**305mM**

Table 4.5: The concentration of butyrate, propionate and acetate required to inhibit colon cancer cells in culture by 50% (IC50) after incubation for between 1 hour, and 72 hours.

All three SCFAs inhibited cell growth in a time and dose dependent manner. The IC50 values are illustrated in table 4.5. Caco-2 cells were more sensitive than HT29 cells to all 3 SCFAs during both short and long incubation periods and more sensitive to butyrate and propionate than both urothelial cell lines over the short incubation, however this increased sensitivity was not lost after longer incubations. In terms of comparative potency of each

SCFA the pecking order was the same in colon cancer cell lines as it was in the urothelial cell cultures with butyrate being the most potent followed by propionate then acetate. Interestingly at low concentrations of both butyrate and propionate during longer incubations there was enhancement of growth in Caco-2 cells of up to 20% at concentration below 1.25mM of butyrate and 3.75mM of propionate.

Figure 4.11 Viable biomass of Caco-2 colonic cancer cells after incubation with butyrate

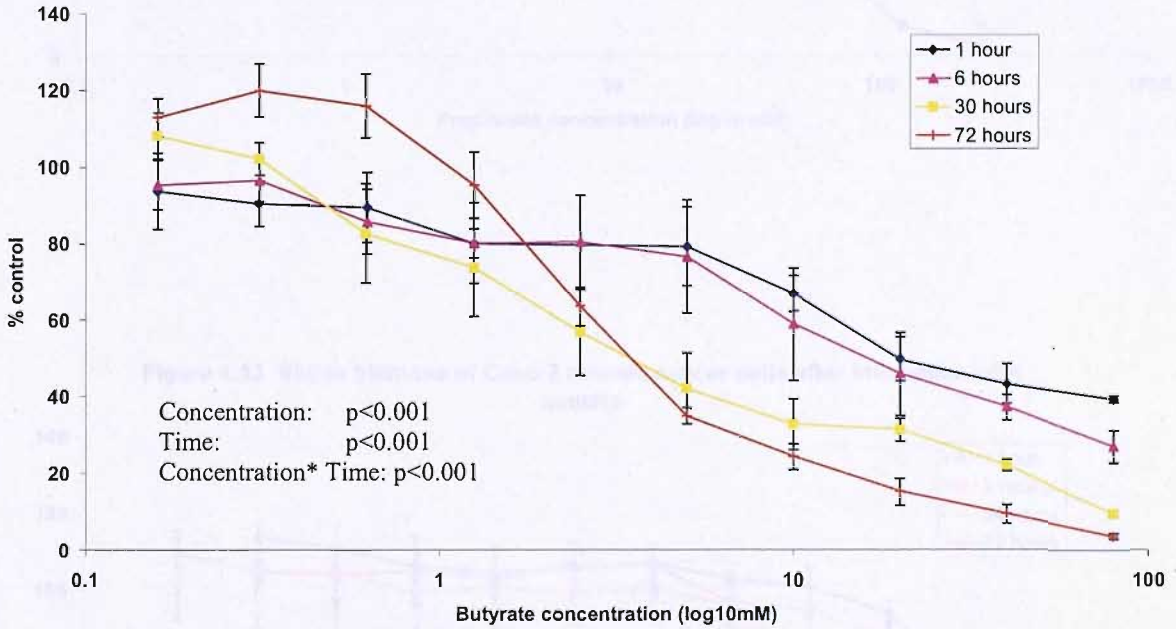


Figure 4.12 Viable biomass of Caco-2 colonic cancer cells after incubation with propionate

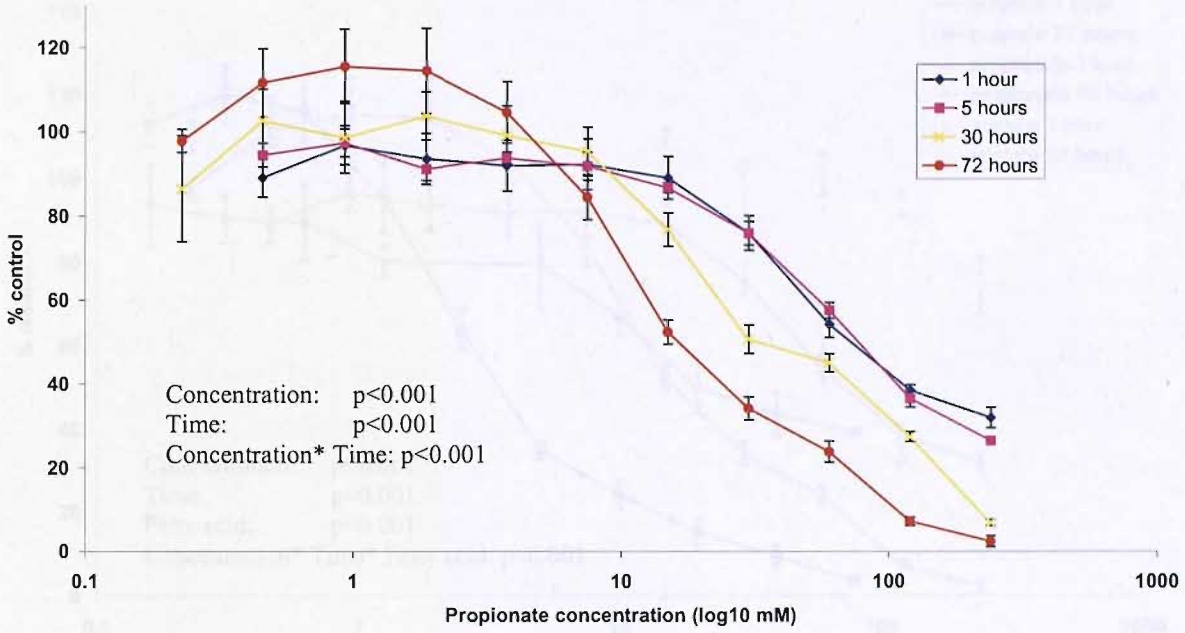


Figure 4.13 Viable biomass of Caco-2 colonic cancer cells after incubation with acetate

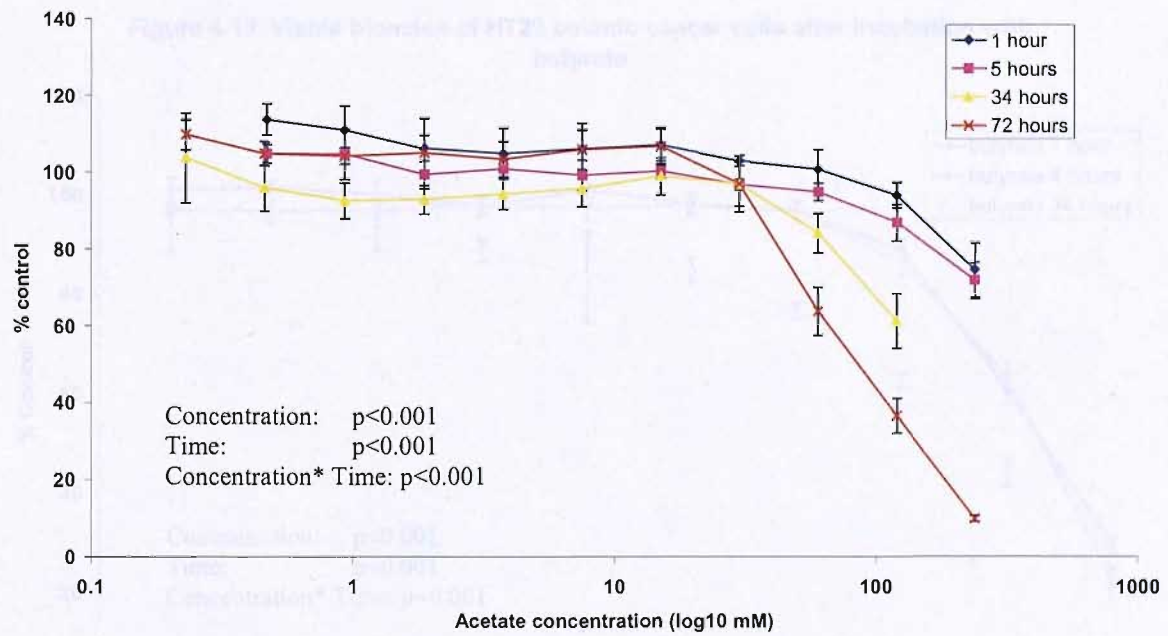


Figure 4.14 Viable biomass of Caco-2 colonic cancer cells after incubation with SCFA. Comparison between butyrate, propionate and acetate

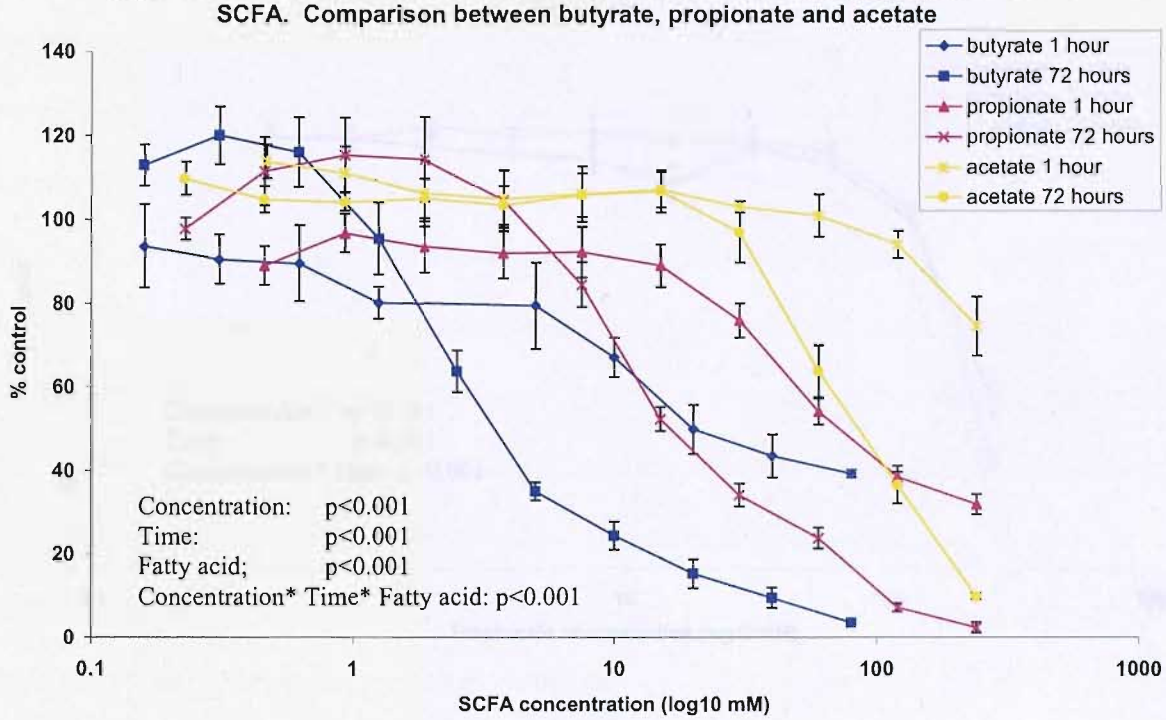


Figure 4.15 Viable biomass of HT29 colonic cancer cells after incubation with butyrate

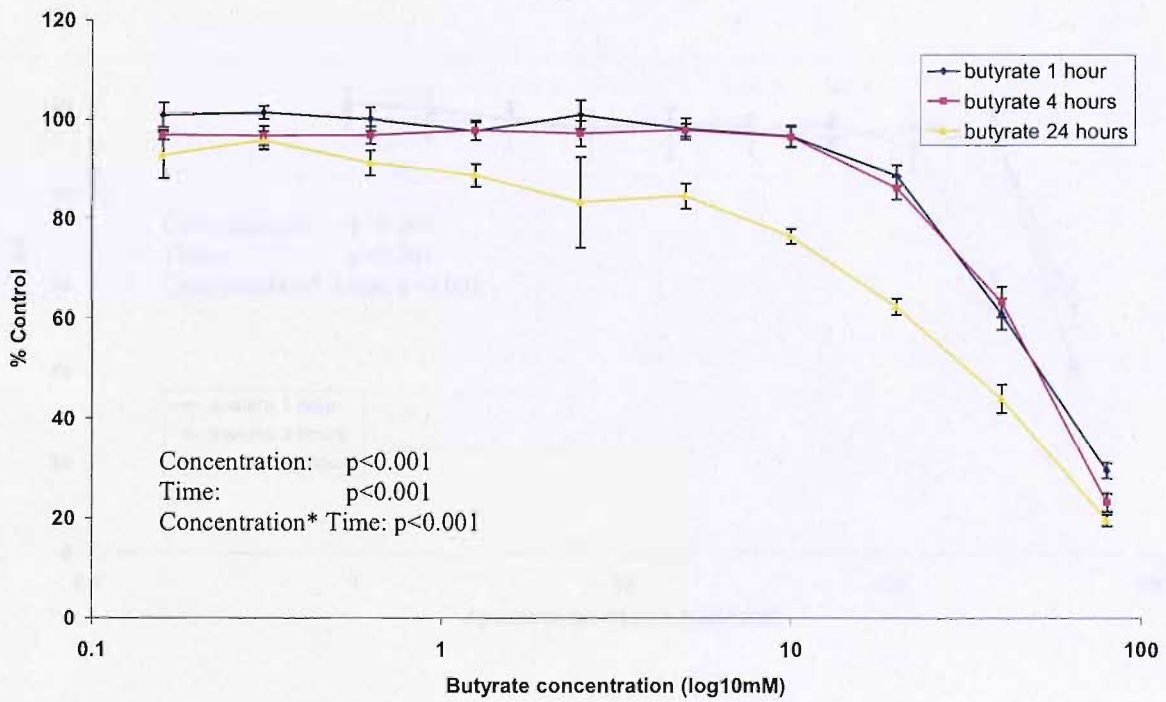


Figure 4.16 Viable biomass of HT29 colonic cancer cells after incubation with propionate

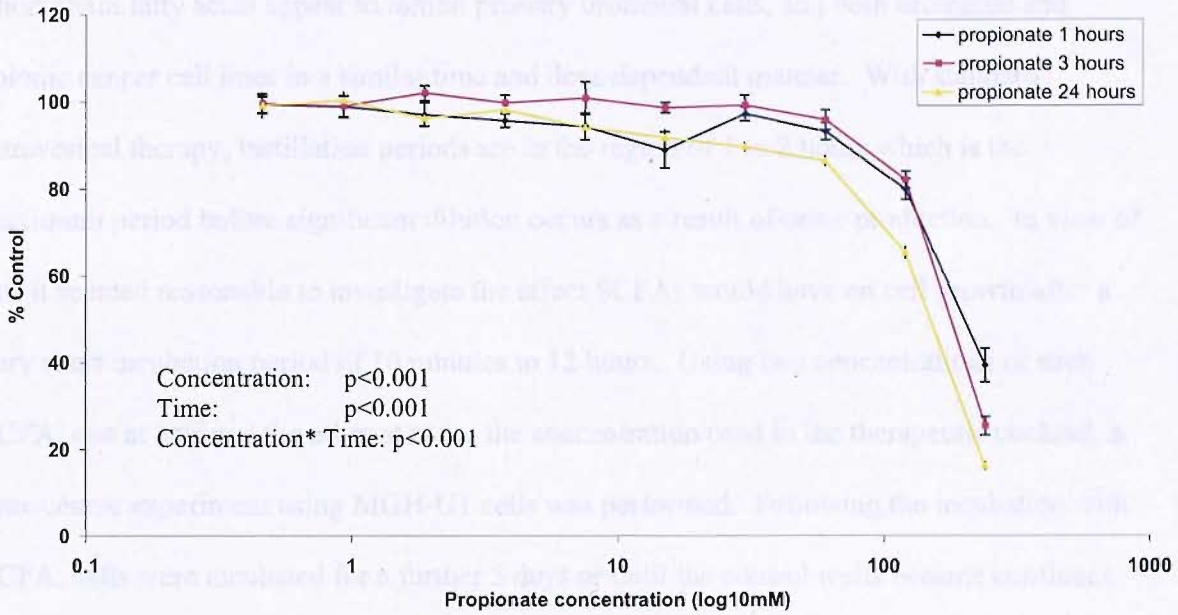
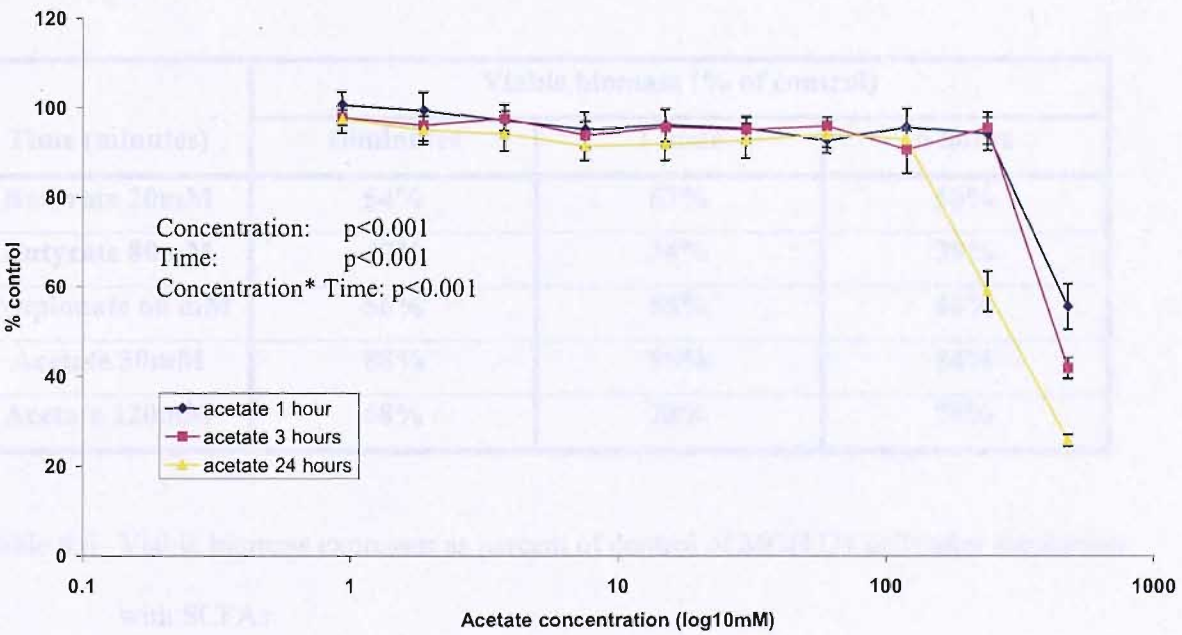


Figure 4.17 Viable biomass of HT29 colonic cancer cells after incubation with acetate



Short exposure to SCFAs

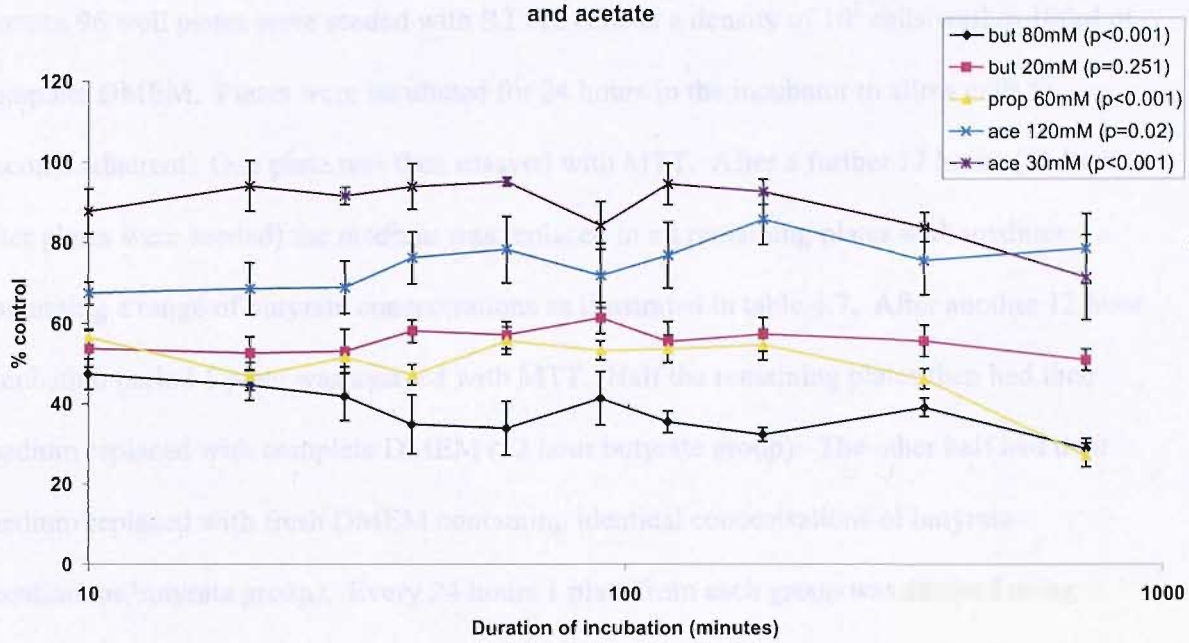
Short chain fatty acids appear to inhibit primary urothelial cells, and both urothelial and colonic cancer cell lines in a similar time and dose dependent manner. With standard intravesical therapy, instillation periods are in the region of 1 to 2 hours which is the maximum period before significant dilution occurs as a result of urine production. In view of this it seemed reasonable to investigate the effect SCFAs would have on cell growth after a very short incubation period of 10 minutes to 12 hours. Using two concentrations of each SCFA, one at half and the other at twice the concentration used in the therapeutic cocktail, a time course experiment using MGH-U1 cells was performed. Following the incubation with SCFA, cells were incubated for a further 5 days or until the control wells became confluent. MTT assay was performed as previously described.

The growth inhibition produced by the shortest incubation period (10 minutes) did not increase significantly with incubation periods up to 6 hours demonstrating that what ever effect SCFAs are having on cell growth is initiated almost immediately after exposure. (Figure 4.18)

Time (minutes)	Viable biomass (% of control)		
	10minutes	1 hour	6 hours
Butyrate 20mM	54%	57%	56%
Butyrate 80mM	47%	34%	39%
Propionate 60 mM	56%	55%	46%
Acetate 30mM	88%	95%	84%
Acetate 120mM	68%	78%	76%

Table 4.6 Viable biomass expresses as percent of control of MGH U1 cells after incubation with SCFAs.

Figure 4.18 Viable biomass of MGH-U1 urothelial cells after incubation with SCFAs. Comparison of incubation period (10mins to 12 hours) between butyrate, propionate and acetate



Investigation of the duration of short chain fatty acid induced inhibition of cell growth

Introduction

It has been established that all cell types investigated behaved in a similar manner when exposed to SCFAs and that when compared with the concentration in the therapeutic cocktail, butyrate was potentially the most clinically significant. Therefore the RT112 urothelial cell line and butyrate were chosen to investigate the duration of the inhibitory effect of SCFAs. It was considered unnecessary to duplicate these experiments in all cell types or with propionate and acetate.

Method

Sixteen 96 well plates were seeded with RT112 cells at a density of 10^2 cells/well in 100µl of complete DMEM. Plates were incubated for 24 hours in the incubator to allow cells to become adherent. One plate was then assayed with MTT. After a further 12 hours (36 hours after plates were seeded) the medium was replaced in all remaining plates with medium containing a range of butyrate concentrations as illustrated in table 4.7. After another 12 hour incubation period 1 plate was assayed with MTT. Half the remaining plates then had their medium replaced with complete DMEM (12 hour butyrate group). The other half had their medium replaced with fresh DMEM containing identical concentrations of butyrate (continuous butyrate group). Every 24 hours 1 plate from each group was assayed using MTT and in the remaining plates the medium was replenished every 48 hours. Results were combined to generate growth curves for each butyrate concentration in both the 12 hour butyrate group and the continuous butyrate group.

Empty	empty	empty	empty	empty	empty	null	null	null	null	null	null
Control	control	control	control	control	control	control	control	control	control	control	control
Control	control	control	control	control	control	control	control	control	control	control	control
Control	control	control	control	control	control	control	control	control	control	control	control
Control	0.16	0.31	0.62	1.25	2.5	5	10	20	40	80	control
Control	0.16	0.31	0.62	1.25	2.5	5	10	20	40	80	control
Control	0.16	0.31	0.62	1.25	2.5	5	10	20	40	80	control
Null	null	null	null	null	null	empty	empty	empty	empty	empty	empty

Table 4.7: Distribution of butyrate concentrations (mM) within the 96 well plate.

Null: wells containing medium only.

Control: wells containing cells incubated with complete medium without butyrate.

Results

In control wells exponential growth was maintained throughout the 9 days of the experiment. In cells exposed to butyrate for 12 hours there was significant inhibition of growth 24 hours after exposure with concentration above 2.5mM. Reduction in growth rate was demonstrated for 5 days after butyrate exposure (day 7 of experiment). Beyond this period the growth rate returned to control levels in wells exposed to butyrate concentrations less than or equal to 40 mM (figure 4.19).

In cells exposed to butyrate throughout the experiment there was significant inhibition of growth 48 hours after commencing incubation with butyrate above 1.25mM. Butyrate induced inhibition was maintained for 5 days after which there was recovery of growth rate to control levels in cells still incubated with butyrate concentrations less than 2.5mM (Figure 4.20).

Continuous incubation with butyrate at concentrations above 2.5mM inhibited any increase in viable biomass for the duration of the experiment and concentrations above 10mM produced a fall in viable biomass. With 12 hours incubation the minimum butyrate concentrations required to inhibit an increase in viable biomass was 20mM. There was a fall in viable biomass 48 hours after incubation with 80mM of butyrate for 12 hours.

Figure 4.19 Time course of viable biomass of urothelial cells after incubation with butyrate

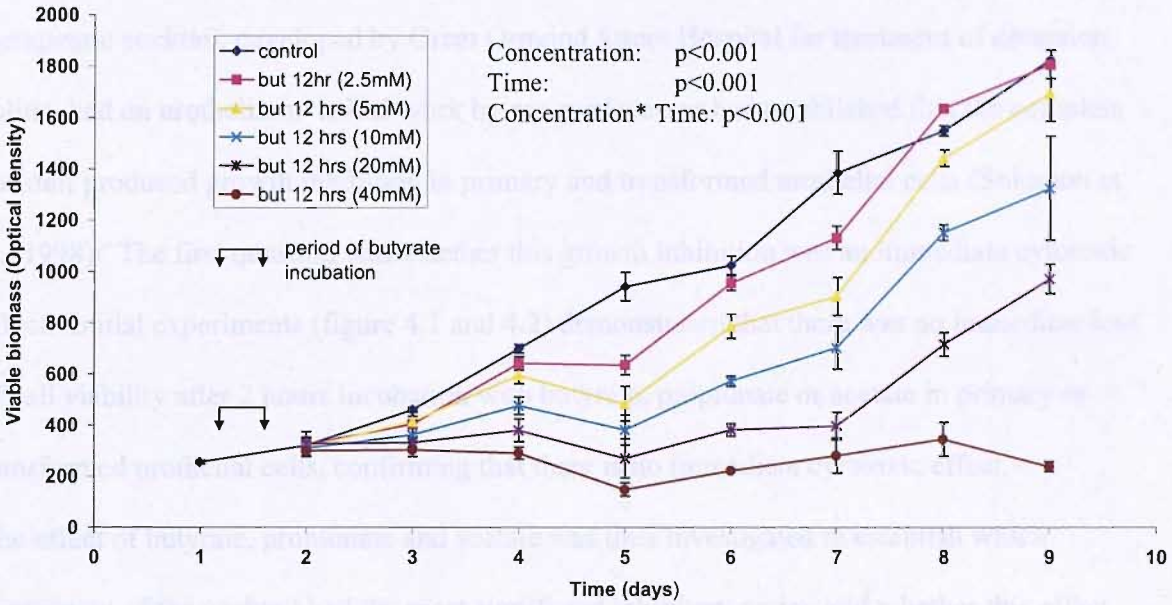
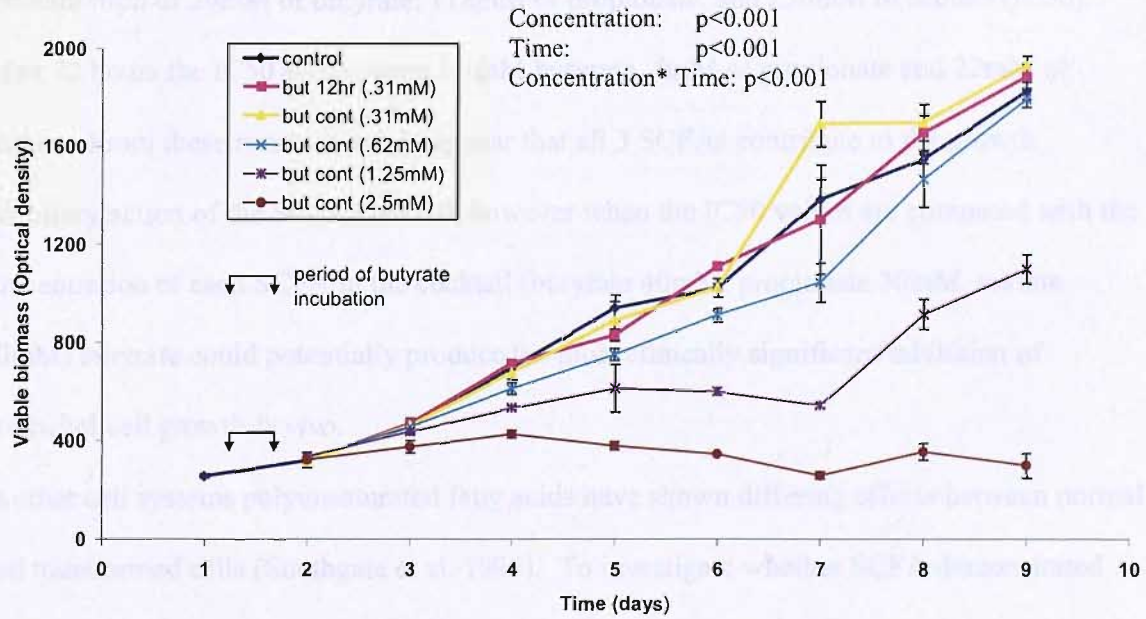


Figure 4.20 Time course of viable biomass of urothelial cells after incubation with butyrate



Discussion

This chapter set out to investigate the effect each component short chain fatty acid of the therapeutic cocktail, developed by Great Ormond Street Hospital for treatment of diversion colitis, had on urothelium. Initial work by my predecessor had established that the complete cocktail produced growth inhibition in primary and transformed urothelial cells (Solomon et al. 1998). The first question was whether this growth inhibition was an immediate cytotoxic effect. Initial experiments (figure 4.1 and 4.2) demonstrated that there was no immediate loss of cell viability after 2 hours incubation with butyrate, propionate or acetate in primary or transformed urothelial cells, confirming that there is no immediate cytotoxic effect.

The effect of butyrate, propionate and acetate was then investigated to establish which component of the cocktail had the most significant inhibitory action and whether this effect was universal amongst normal and transformed urothelial cells. In primary urothelial cells there was both time and dose dependent inhibition of cell growth by all 3 SCFAs (figure 4.3-4.5). Primary cells incubated for 1 hour with SCFA produced 50% inhibition of growth at a concentration of 20mM of butyrate, 118mM of propionate, and 230mM of acetate (IC50). After 72 hours the IC50 values were 1.1mM butyrate, 4mM of propionate and 22mM of acetate. From these results it would appear that all 3 SCFAs contribute to the growth inhibitory action of the SCFA cocktail, however when the IC50 values are compared with the concentration of each SCFA in the cocktail (butyrate 40mM, propionate 30mM, acetate 60mM) butyrate could potentially produce the most clinically significant inhibition of urothelial cell growth *in vivo*.

In other cell systems polyunsaturated fatty acids have shown differing effects between normal and transformed cells (Southgate et al. 1996). To investigate whether SCFA demonstrated similar selectivity, and if selectivity did exist whether it was dependent on the degree of differentiation of the cells, a well differentiated urothelial cell line, RT112 and a poorly

differentiated urothelial cell line, MGH-U1 were selected. Over a similar range of concentrations and incubation periods all SCFAs demonstrated similar time and dose dependent inhibition of cell growth (figures 4.6-4.10 and table 4.4). From the IC50 values it appears that malignant urothelium was less sensitive to butyrate at least over shorter incubation periods than primary urothelial cells, however the relative potency between butyrate, propionate and acetate was the same.

It is well known that colon cancer cell lines are inhibited by SCFAs in culture, numerous investigators have demonstrated this often as part of investigations into the role of SCFAs in prevention of colorectal cancer. As both a comparison with the urothelial cells and as baseline work for further co-culture experiments, a well differentiated colon cancer cell line, Caco-2 and a poorly differentiated colon cancer cell line were investigated.

Both colonic cell lines demonstrated time and dose dependent inhibition of cell growth when incubated with SCFAs (figure 4.11-4.17 and table 4.5), and the relative potency of each SCFA was the same.

Having established that SCFA induced inhibition was not due to immediate cytotoxicity and that both primary and malignant urothelial cells were inhibited in a similar manner to colon cancer cell lines the next step was to find out what the mechanism of growth inhibition could be.

During traditional intravesical therapy, the therapeutic agent is instilled into the bladder for up to 2 hours before it is either eliminated by normal voiding or drained by releasing an indwelling catheter. Beyond this 2 hour treatment period urine output significantly dilutes the concentration of intravesical agent and more practically patients find it difficult to retain it for any longer. With this in mind the effect of SCFA incubations over more clinically relevant incubation periods were investigated. Results showed no difference in the degree of growth inhibition induced from 10 minutes up to 6 hours of incubation (figure 4.18 and table

4.6). Therefore SCFAs were having an immediate effect on the cell which was not a cytotoxic effect as shown by Figures 4.1 and 4.2. This raised a number of questions, including whether this inhibition was a terminal cytotoxic event or a cytostatic effect from which the cells could recover, and if so what was the duration of the effect. To answer this question the most clinically relevant SCFA, butyrate was chosen and the urothelial tumour cell line RT112 was used as the number of cells required prohibited the use of primary cells in these experiments. Results demonstrated that after an incubation period of 12 hours with ≤20mM butyrate, cell growth was inhibited for up to 5 days, beyond which growth rate recovered to control levels. Therefore the mechanism of growth inhibition was reversible at lower concentrations of butyrate.

Continuous incubation with butyrate demonstrated similar inhibition of growth for 5 days with concentrations ≥0.62mM. This was followed by recovery of the growth curve at a growth rate greater than or equal to the control curve with concentrations <2.5mM implying that cells develop a degree adaptation to butyrate in their medium, perhaps even utilizing butyrate as a source of nutrition which would account for the steeper curve in some cases. This would be supported by findings in Caco-2 cells which demonstrated up to 20% greater growth than controls when incubated with butyrate (<0.62mM)(figure 4.11) and propionate (<3.75mM)(figure 4.12). An alternative mechanism to explain this apparent adaptation to butyrate could be growth of a selected resistant subpopulation of cells within the culture.

Butyrate has been shown to induce apoptosis in human colon cancer and adenoma cells by promoting differentiation which is thought to be one of the mechanisms involved in protection against colorectal cancer (Hague et al. 1996). Butyrate has also been shown to reduce cell growth in a number of different human colon cancer cell lines by blocking stages of the cell cycle including G1/S transition and G2/M (Kobayashi et al. 2003). To investigate

the mechanism of SCFA induced growth inhibition and potential effects on the cell cycle of urothelial cells flow cytometry was employed.

Chapter 5

The effect of short-chain fatty acids on urothelial cells: Cell cycle analysis

Introduction

Flow cytometry was employed to investigate the mechanism of SCFA induced inhibition of cell proliferation. Using propidium iodide stained cells both apoptosis and cell cycle arrest could be demonstrated by measurement of the proportion of cells within each phase of the cell cycle.

Experimental method

The effect of butyrate, propionate and acetate was measured in both primary and transformed urothelial cell populations. Cells were cultured in 12.5cm flasks until they were 50% confluent. They were then incubated with medium containing each SCFA over a range of concentrations and incubation periods from 1 to 90 hrs. Cells were fixed and stained with propidium iodide before FACS analysis which was performed up to 100 hrs after incubation. Each cell population was distributed on a dot plot (figure 5.1) of forward scatter (FSC) against side scatter (SSC). This is essentially a plot of cell granularity (SSC) against size (FSC). The dense central area represents the majority of single cells, to the right are larger particles and doublets and to the left are smaller particles of debris. Gating is performed to remove interference from debris and larger particles, allowing analysis to focus on the population of single cells within the defined gate. The cell DNA is labeled with propidium iodide which produces fluorescence in the red/orange spectrum measured best by the FL2 channel of the flow cytometer. A plot of fluorescence area (FL2A) against fluorescence height (FL2H) generates a plot that discriminates cells in the G2/M phase from doublets. Further gating is performed around the cell population to exclude doublets using this plot

(figure 5.2). A histogram of propidium iodide DNA fluorescence of the gated population of cells is generated to analyse the distribution of cells within the cell cycle (figure 5.3). Labels G0/1, S, G2/M represent cells in the dormant (Go) and first growth phase (G1), the Synthesis phase, and the second growth and mitosis phase of the cell cycle respectively. Cells in G2/M have twice the quantity of DNA within the nucleus and therefore twice the fluorescence of cells in G1. Cells undergoing apoptosis lose small fragments of DNA and therefore appear to the left of the G1 peak on the histogram, known as the sub-G1 region (Tounekti et al. 1995).

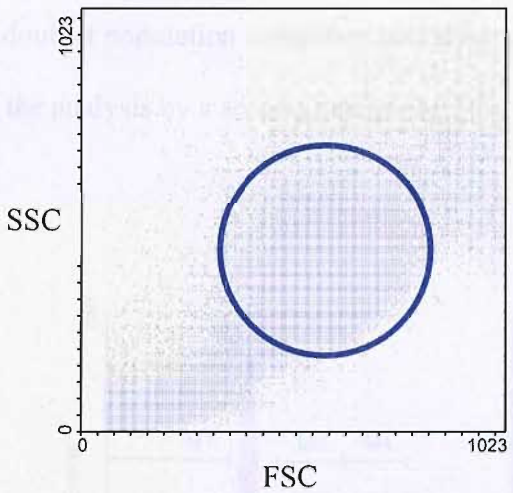


Figure 5.1 Flow cytometry dot plot of forward scatter (FSC) against side scatter (SSC). The dense central area within the A gate represented by the blue circle is used to select the cell population and exclude cell debris and larger particles from the analysis.

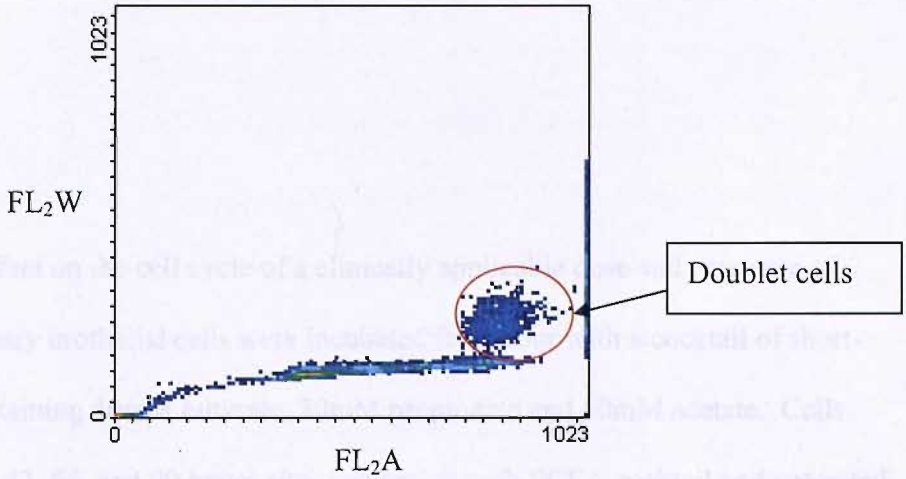


Figure 5.2 Flow cytometry density plot of fluorescence area (FL_2A) against fluorescence width (FL_2W) allows discrimination between cells in the G2/M phase from doublets. The doublet population comprises two adherent cells. They are excluded from the analysis by a second gate.

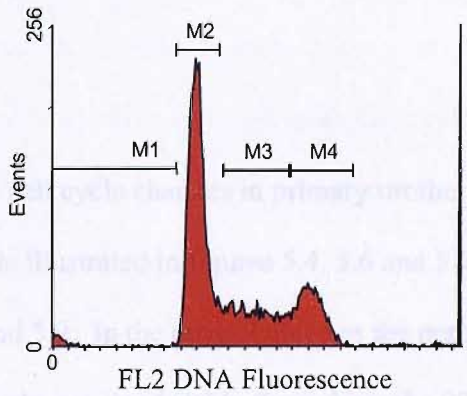


Figure 5.3 Histogram of DNA fluorescence in FL2.

M1 = Sub-G1 region, M2 = G1 peak, M3 = S phase, M4 = G2/M peak

Cell cycle analysis of primary urothelial cells incubated with short chain fatty acid cocktail

Method

To investigate the effect on the cell cycle of a clinically applicable dose and exposure of SCFA cocktail, primary urothelial cells were incubated for 1 hour with a cocktail of short-chain fatty acids containing 40mM butyrate, 30mM propionate and 60mM acetate. Cells were analysed at 22, 42, 64, and 90 hours after incubation with SCFA cocktail and untreated controls were analysed after 42 and 90 hours incubation with standard medium. After fixation cells were stored at 4°C until the end of the experiment when they were labeled with propidium iodide and analysed by flow cytometry. A particularly good cell yield on this occasion allowed the experiment to be replicated. To avoid wasting cells identical experiments (series 1 and 2) were performed in parallel using the same controls.

Results

Figures 5.4 to 5.9 illustrate the cell cycle changes in primary urothelial cells after incubation with SCFA cocktail. Series 1 is illustrated in figures 5.4, 5.6 and 5.7, and series 2 is illustrated in figures 5.5, 5.8 and 5.9. In the control cultures the percentage of cells in G1, S, and G2/M phases of the cell cycle remained stable throughout the 90 hour period of the experiment. During the experiment the control sub-G1 population increased from 17% at 42 hours to 30% at 90 hours. In the treated cells there was a reduction in the percentage of cells in the G1 phase throughout the experiment compared with controls. The the percentage of cells in the S-phase population remained relatively stable and similar to controls in both series 1 and series 2, with a small rise at 42 hours and 64 hours in series 1 and series 2

respectively. In both series the percentage of cells in S phase at 90 hours were lower than controls. In both series the percentage of cells in G2/M phase was higher than controls at 22 hours and delined to below control levels by 90 hours, however there was a difference in the rate of decline between the two series. In series 1 the level of G2/M remained stable until 64 hours then declined to less than 5%, however in series 2 after 22 hours the G2/M peak declined steadily to below 5% at 90 hours. The percentage of cells in the sub-G1 region was higher than controls in both series at 22 hours and at 90 hours represented 65% and 75% of the cell population in series 2 and series 1 respectively. This experiment demonstrated SCFA induced alterations in the cell cycle of primary urothelial cells. In summary the most striking findings were a fall in the percentage of cells in G1 phase, an initial rise in the number of cells in G2/M phase and a large increase in the SubG1 phase compared to controls. These results may suggest that SCFA's induce a degree of cell cycle blockade in primary urothelial cells at G2/M which then undergo apoptosis rather than return to G1. Also of note, were the differences in the results between series 1 and 2 (figures 5.4 and 5.5). Every effort was made to make the experiment as similar as possible, by using primary urothelial cells from the same specimen, which had been cultured together and passaged identically. The differences in the results probably reflect the heterogeneous nature of primary cultures, and their relatively slow growth and poor adaptability to cell culture conditions.

Figure 5.4 Time course of cell cycle changes in primary urothelial cells after 1 hour incubation with SCFA cocktail (series1)

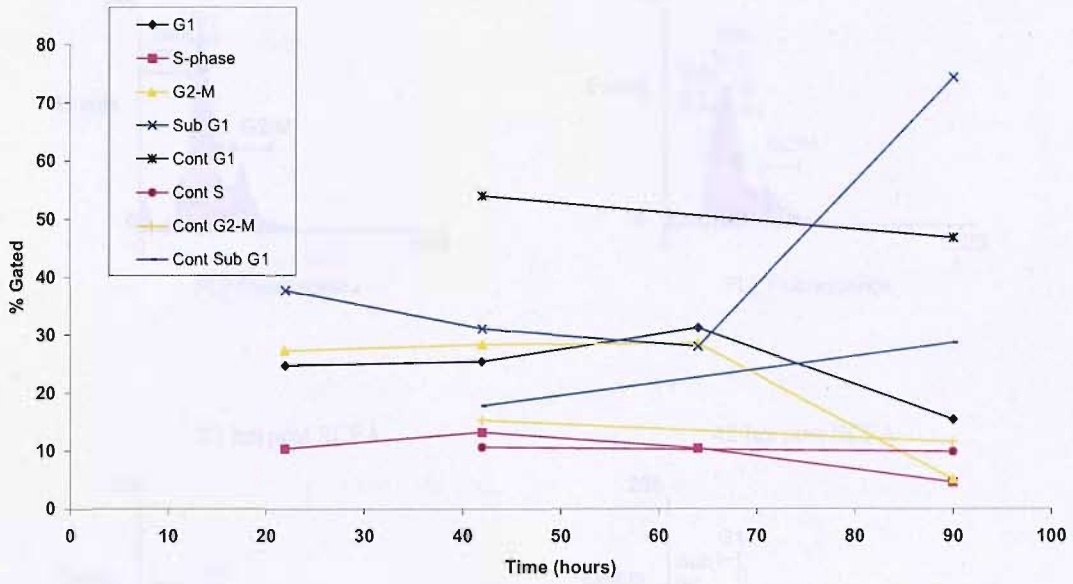
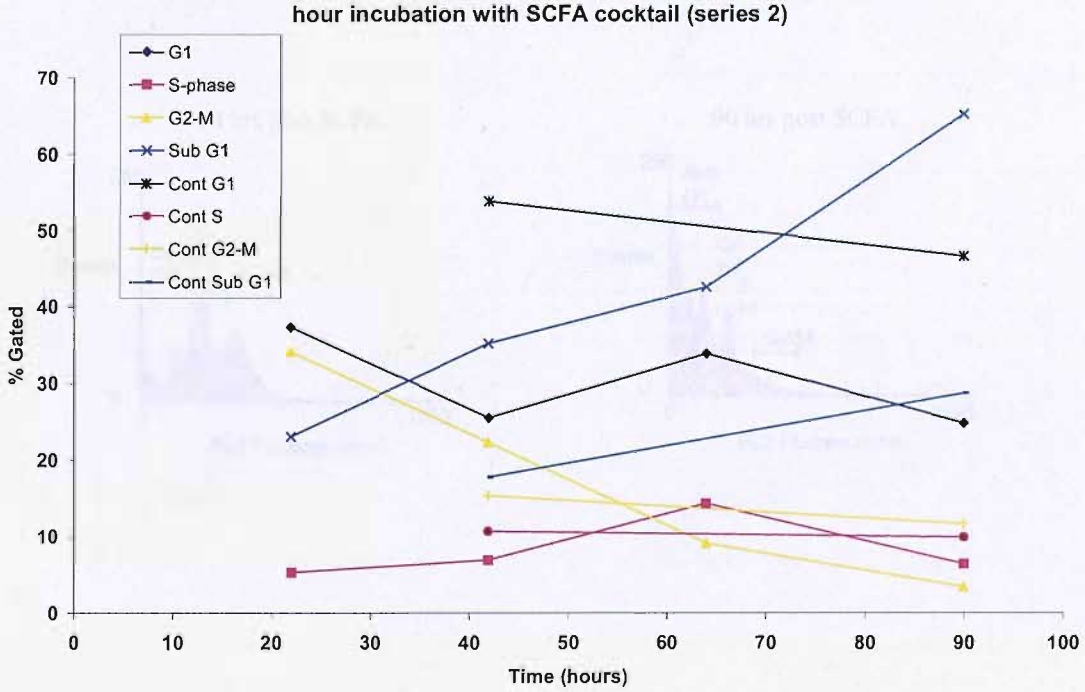


Figure 5.5 Time course of cell cycle changes in primary urothelial cells after 1 hour incubation with SCFA cocktail (series 2)



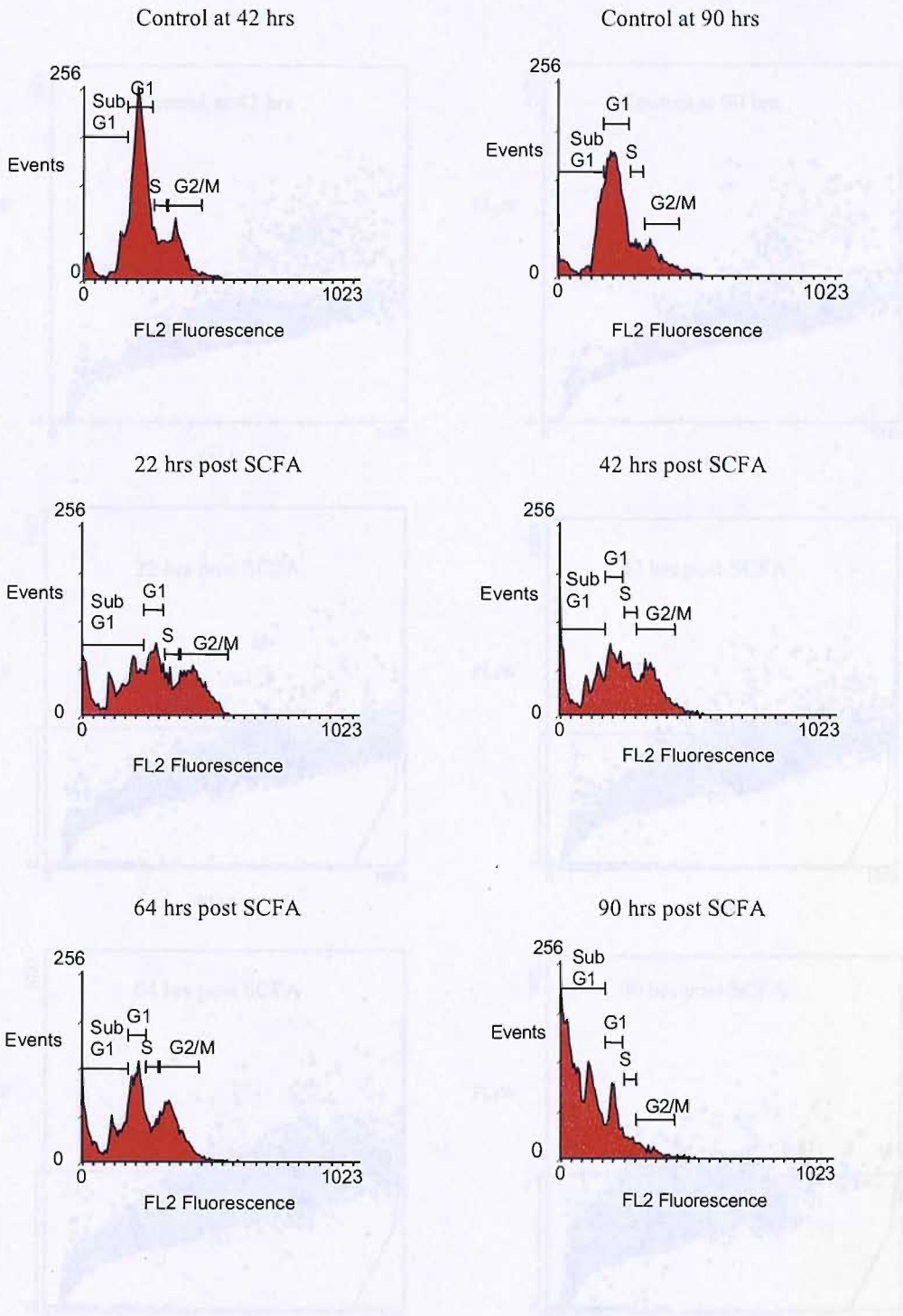


Figure 5.6 Time course of cell cycle changes in primary urothelial cells after 1 hour incubation with short chain fatty acid cocktail. (Series 1)

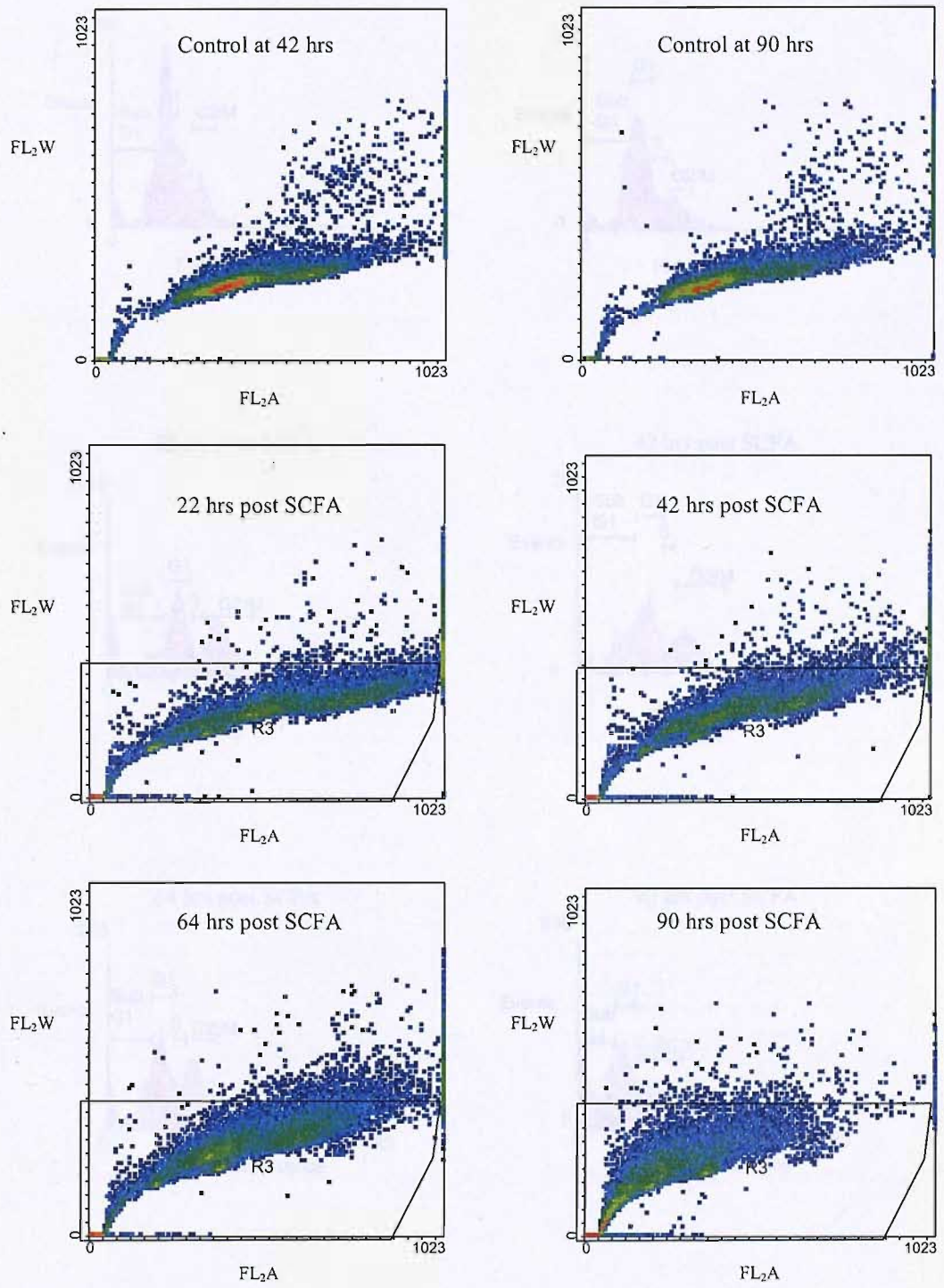


Figure 5.7 Time course of cell cycle changes in primary urothelial cells after 1 hour incubation with short chain fatty acid cocktail. Doublet discrimination plot. (Series 1)

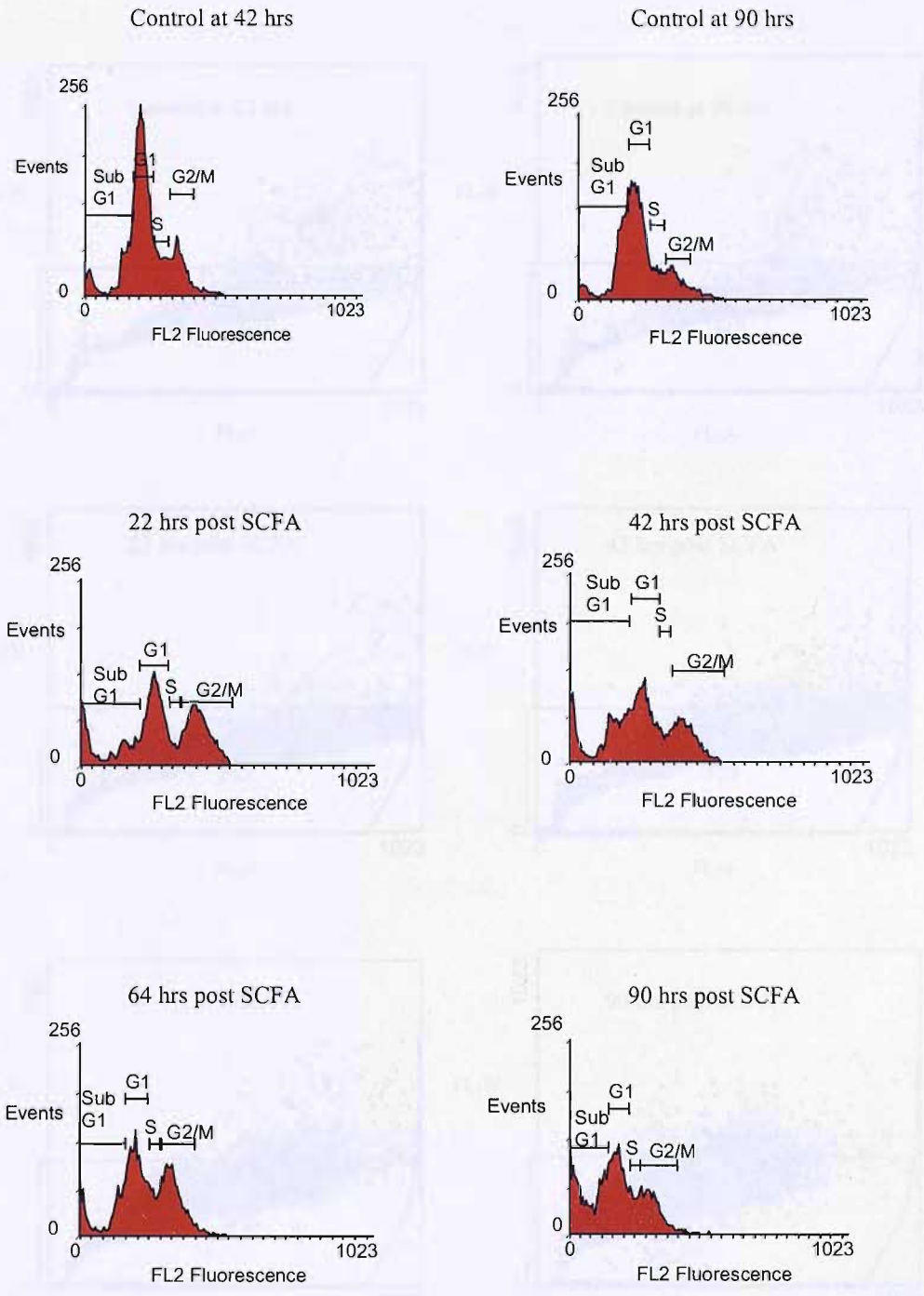


Figure 5.8 Time course of cell cycle changes in primary urothelial cells after 1 hour incubation with short chain fatty acid cocktail. (Series2)

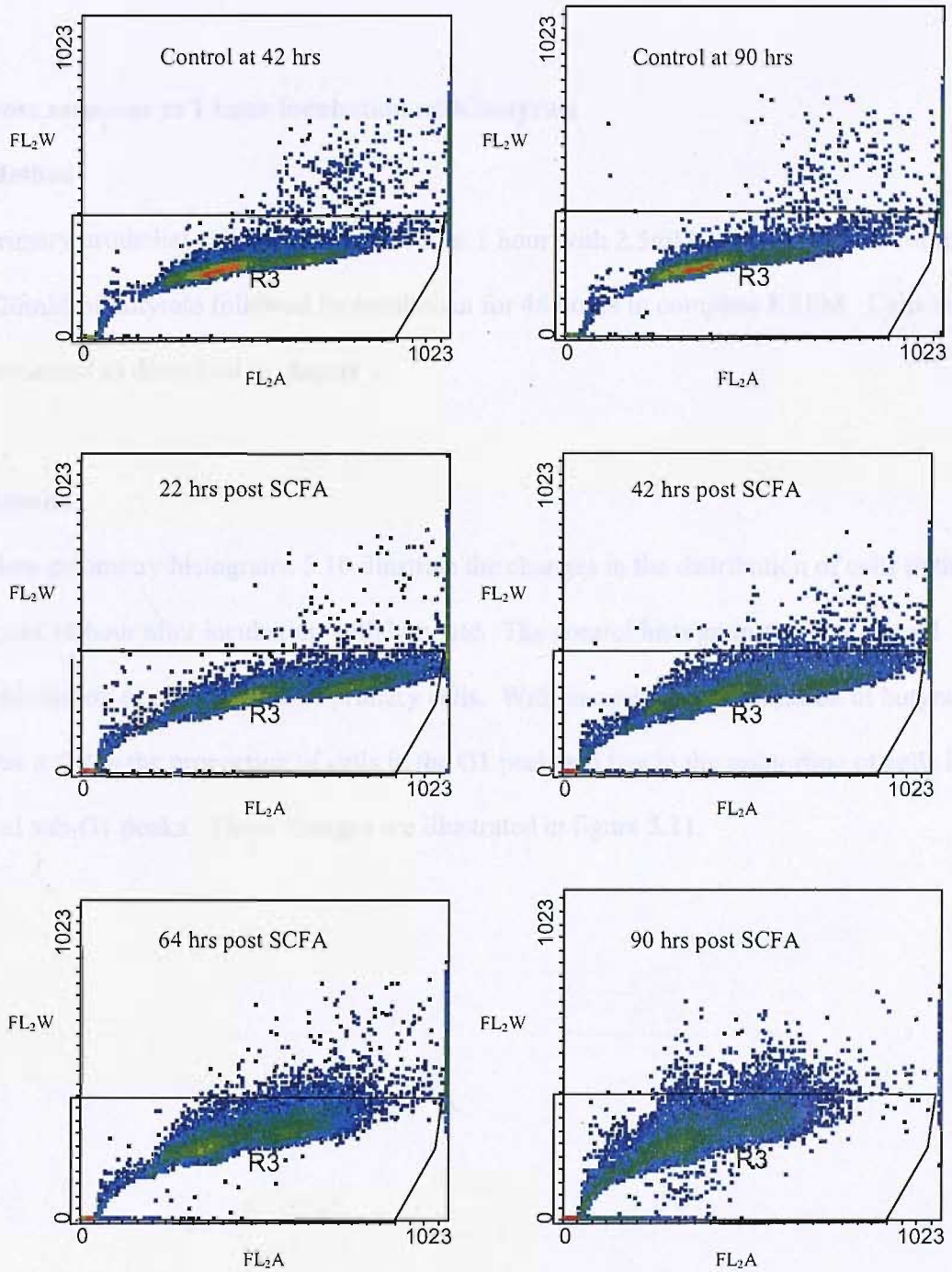


Figure 5.9. Time course of cell cycle changes in primary urothelial cells after 1 hour incubation with short chain fatty acid cocktail. Doublet discrimination plot. (Series 2)

Cell cycle analysis of primary urothelial cells incubated with butyrate

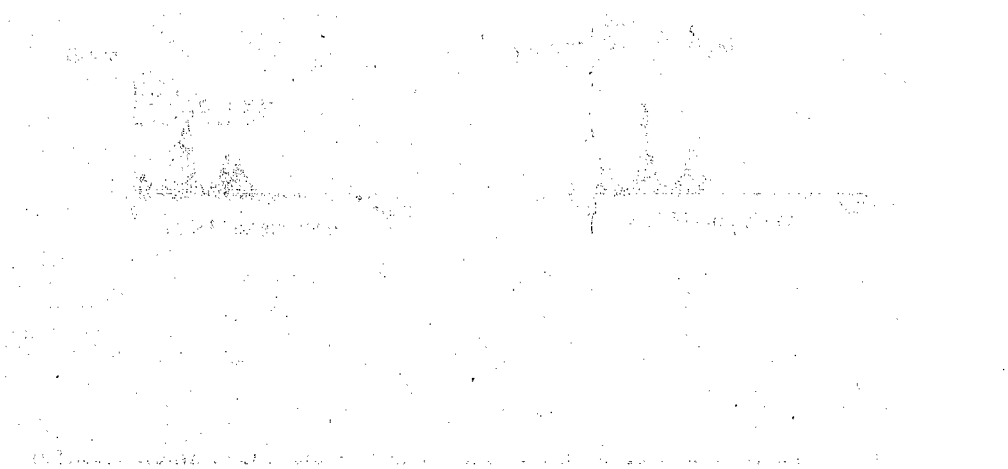
Dose response to 1 hour incubation with butyrate

Method

Primary urothelial cells were incubated for 1 hour with 2.5mM, 10mM, 40mM, 160mM, 320mM of butyrate followed by incubation for 48 hours in complete KSFM. Cells were then processed as described in chapter 2.

Results

Flow cytometry histograms 5.10 illustrate the changes in the distribution of cells in the cell cycle 48 hour after incubation with butyrate. The control histogram shows a normal distribution of a population of primary cells. With increasing concentration of butyrate there was a fall in the proportion of cells in the G1 peak and rise in the proportion of cells in G2/M and sub-G1 peaks. These changes are illustrated in figure 5.11.



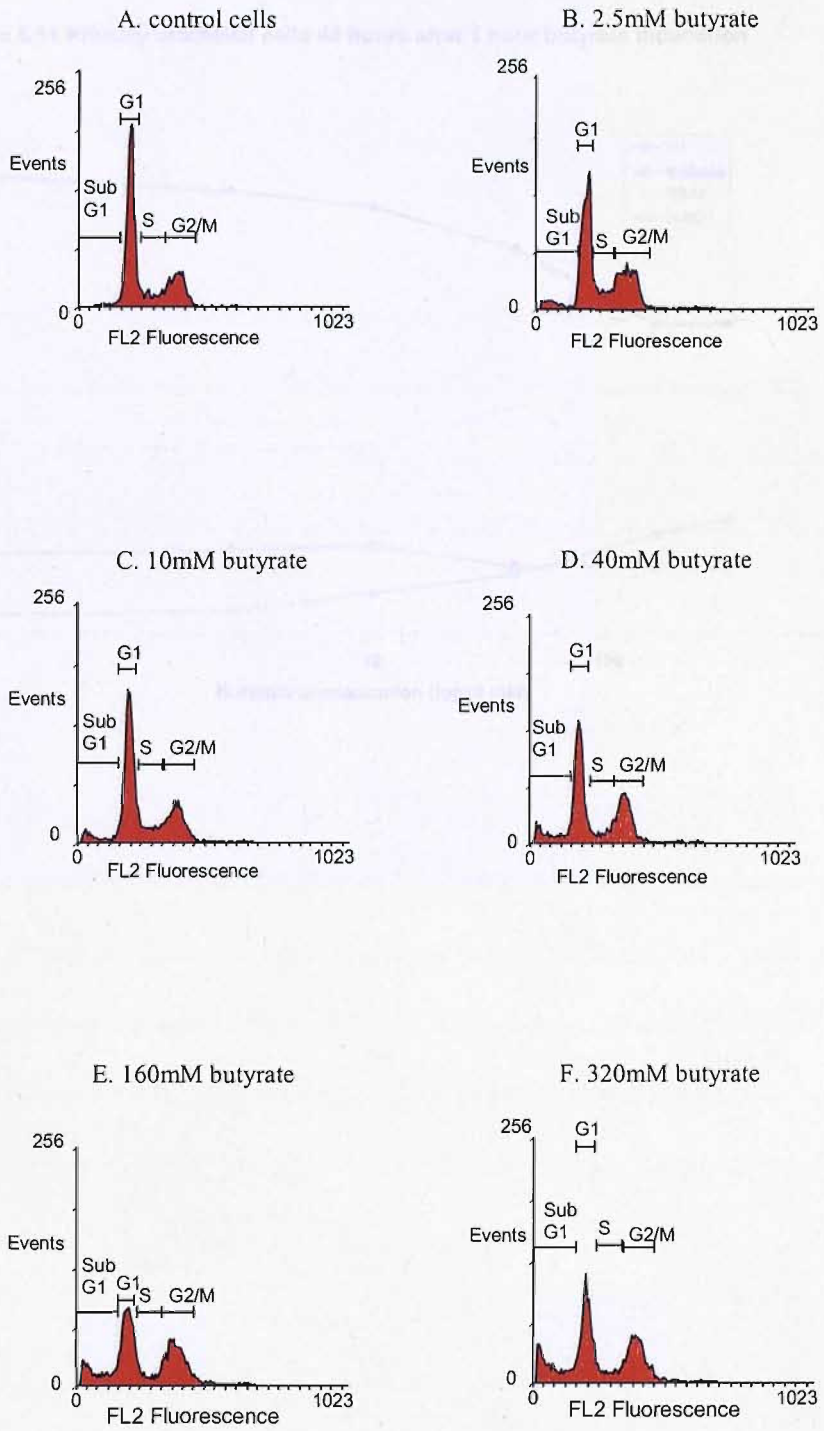
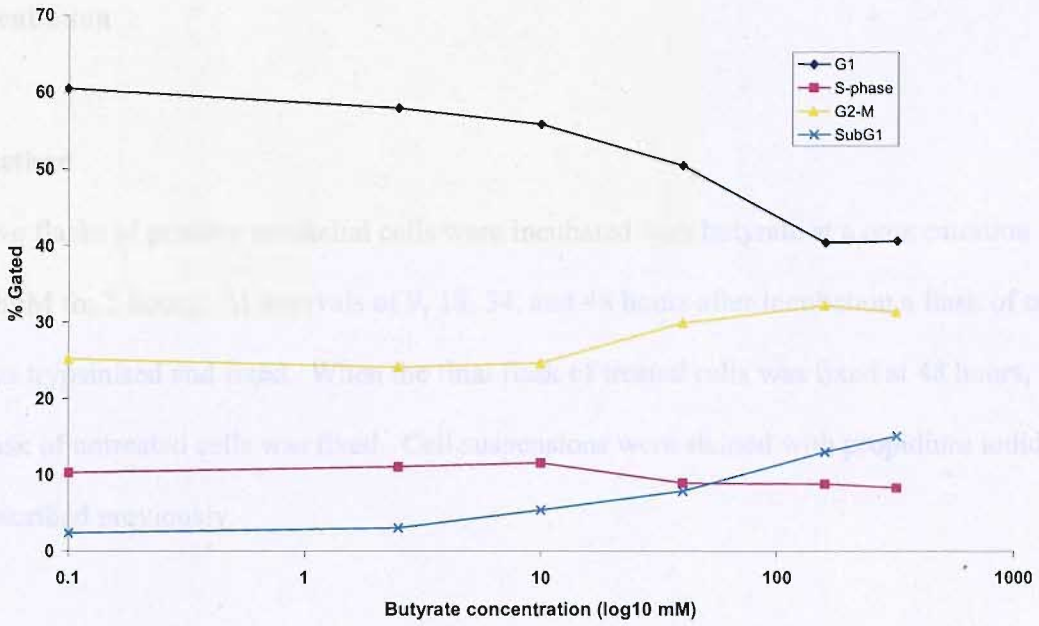


Figure 5.10. Primary urothelial cells 48 hours after incubation with butyrate for 1 hour.

Figure 5.11 Primary urothelial cells 48 hours after 1 hour butyrate incubation



Time course of cell cycle changes in primary urothelial cells after 2 hours butyrate incubation

Method

Five flasks of primary urothelial cells were incubated with butyrate at a concentration of 40mM for 2 hours. At intervals of 9, 18, 34, and 48 hours after incubation a flask of cells was trypsinised and fixed. When the final flask of treated cells was fixed at 48 hours, the flask of untreated cells was fixed. Cell suspensions were stained with propidium iodide as described previously.

Results

Flow cytometry histograms 5.12 and figure 5.13 illustrate the time course of cell cycle changes in primary urothelial cells after 2-hour incubation with 40mM butyrate. Compared with the control histogram there is a fall in the proportion of cells in the G1 peak, which occurs within 9 hours of exposure (figure 5.13). The proportion of cells in the G2/M peak increases from 13% to 22% then falls after 36 hours, and the proportion of cells in the sub-G1 region gradually increases to 40 % within 48 hours. There was no change in the number of cells in the S-phase region.

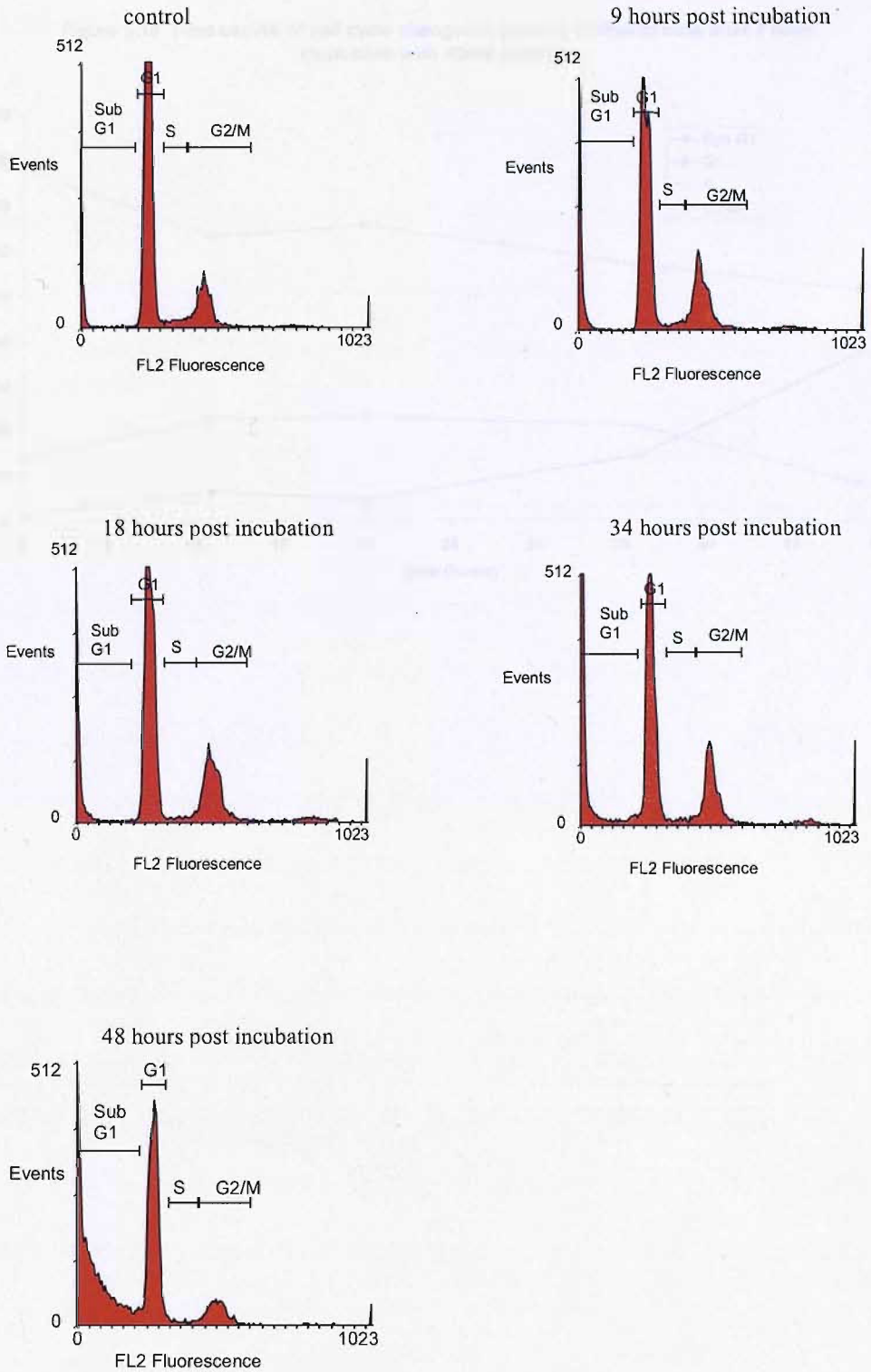
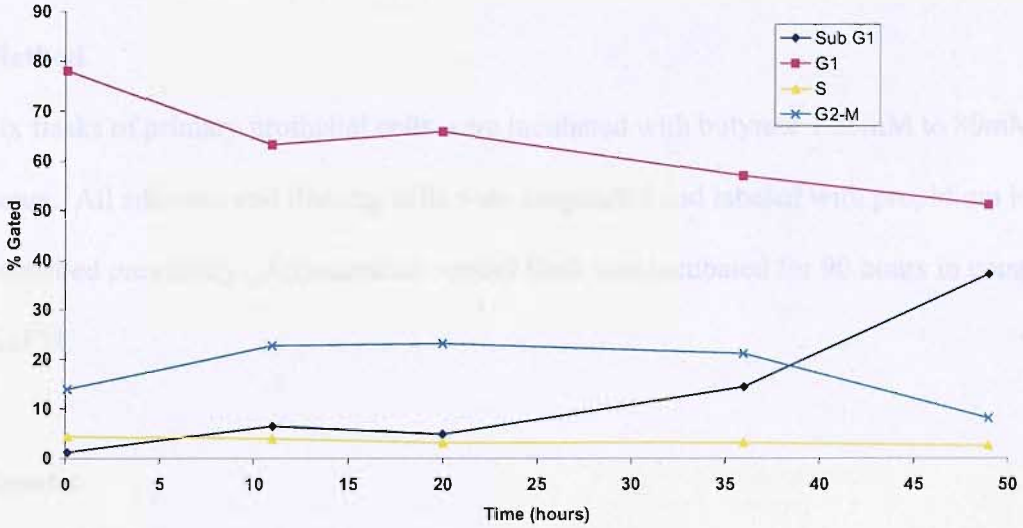


Figure 5.12 Time course of cell cycle changes in primary urothelial cells after 2 hours incubation with 40mM butyrate.

Figure 5.13 Time course of cell cycle changes in primary urothelial cells after 2 hour incubation with 40mM butyrate



The effect of prolonged incubation of primary urothelial cells with butyrate

Method

Six flasks of primary urothelial cells were incubated with butyrate 1.25mM to 80mM for 90 hours. All adherent and floating cells were suspended and labeled with propidium iodide as described previously. An untreated control flask was incubated for 90 hours in complete KSFM.

Results

Figure 5.14 illustrates the dot plots of FSC against SSC and figure 5.15 illustrates the DNA fluorescence histograms. Prolonged incubation with butyrate at 1.25mM and 5mM increased the side scatter of the cell population, which reflects increased cell granularity. The corresponding histograms demonstrated reductions in the G1 and G2/M peaks and rise in the proportion of cells in the sub-G1 region. At higher concentrations of 20, 40 and 80mM it was noted that a significant number of cells had detached from the base of the flask and where floating in the medium. On the dot plot the cell population in general was smaller and less granular reflected by their distribution closer to the x-y intersection. The histograms and graph (figures 5.15 and 5.16) demonstrate the large rise in the proportion of cells in the sub-G1 region representing apoptotic cells, DNA fragments and apoptotic bodies, with very small numbers in the other phases of the cell cycle.

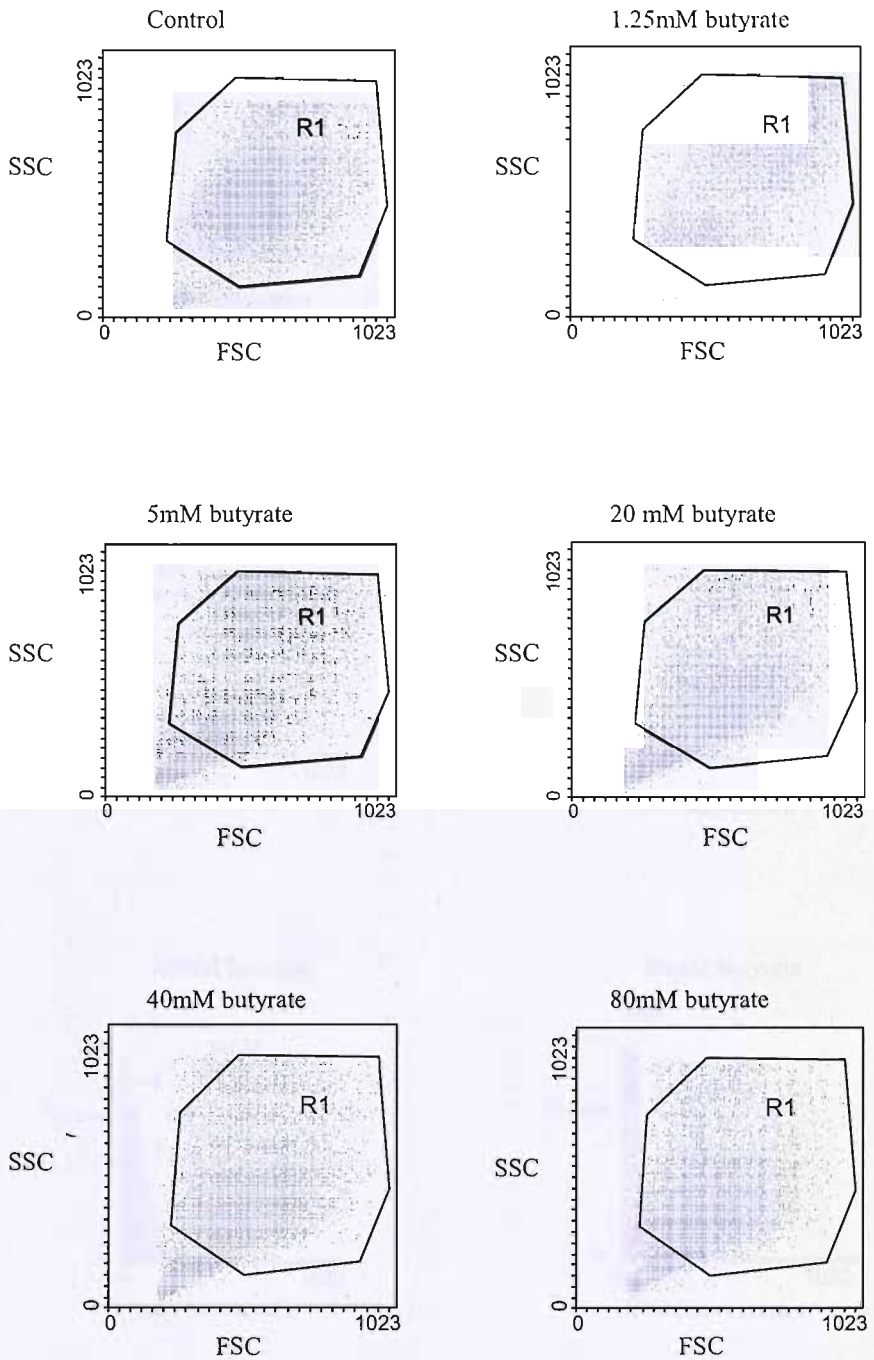


Figure 5.14 Dot plot of primary urothelial cells incubated with butyrate for 90 hours.

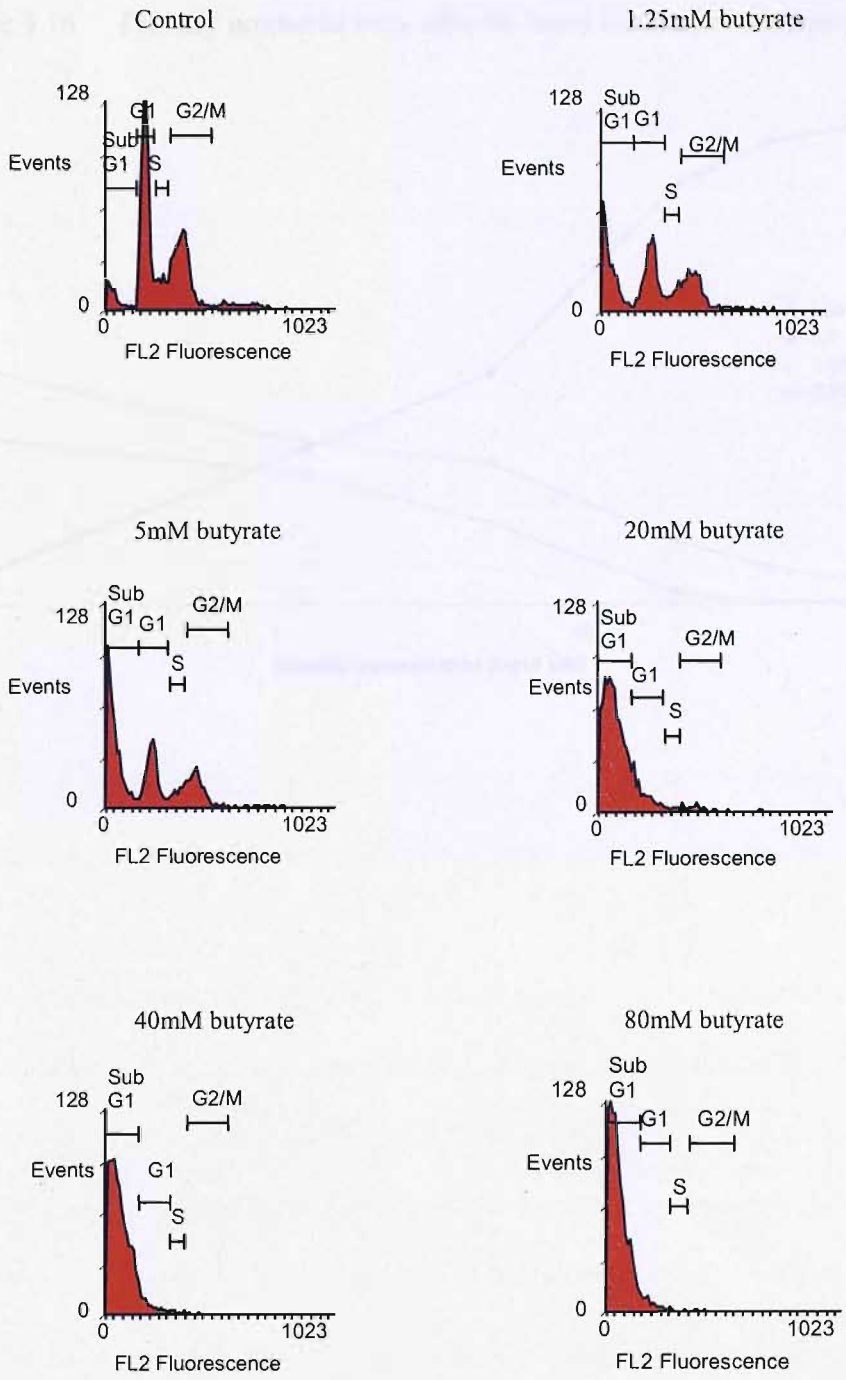
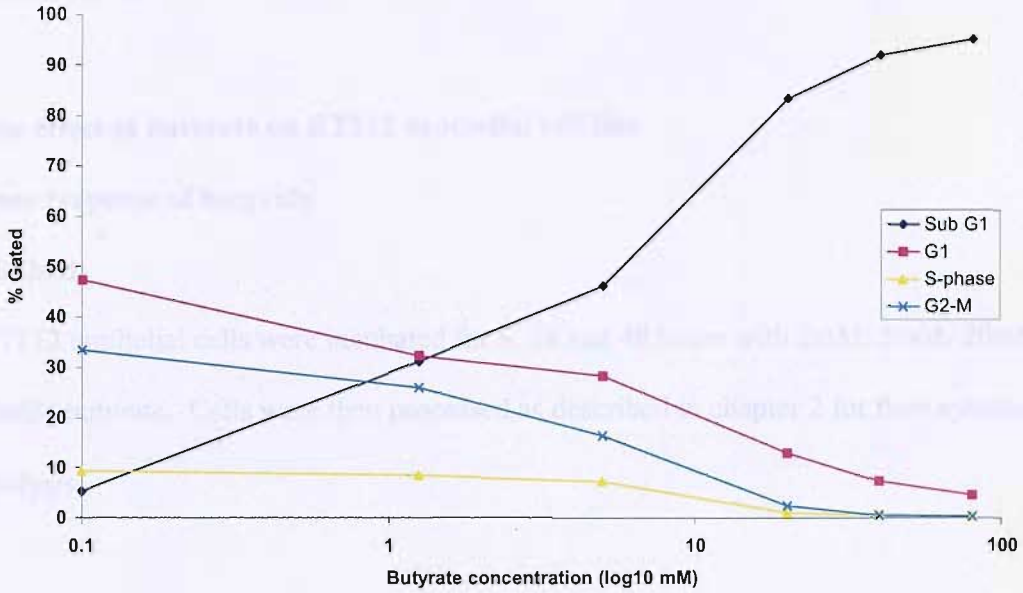


Figure 5.15 Primary urothelial cells after 90 hours incubation with butyrate

Figure 5.16 Primary urothelial cells after 90 hours incubation with butyrate



Cell cycle analysis of the effect of butyrate, propionate and acetate on malignant urothelial cell lines

The effect of butyrate on RT112 urothelial cell line

Dose response of butyrate

Method

RT112 urothelial cells were incubated for 6, 24 and 48 hours with 1mM, 5mM, 20mM, and 80mM butyrate. Cells were then processed as described in chapter 2 for flow cytometry analysis.

Results

There was no alteration in the distribution of cells in the cell cycle after 6 hours incubation with butyrate over a range of concentrations from 5mM to 80mM as illustrated in Figures 5.17 and 5.19.

Over longer incubation periods of 24 and 48 hours changes in the distribution of cells in the cell cycle occurred with increasing concentration of butyrate, including a fall in the G1 and rise in the sub-G1 populations. After 24 hours these changes occurred with all concentrations of butyrate (figures 5.20 to 5.22), and were more pronounced at 48 hours, as illustrated in figures 5.23 to 5.24. With the 24 hour incubation at 5mM and 20mM of butyrate there was a rise in the early S-phase peak seen on histograms A and B in figure 5.20. In both prolonged incubations there was a gradual dose dependent loss of G2/M and late S-phase populations.

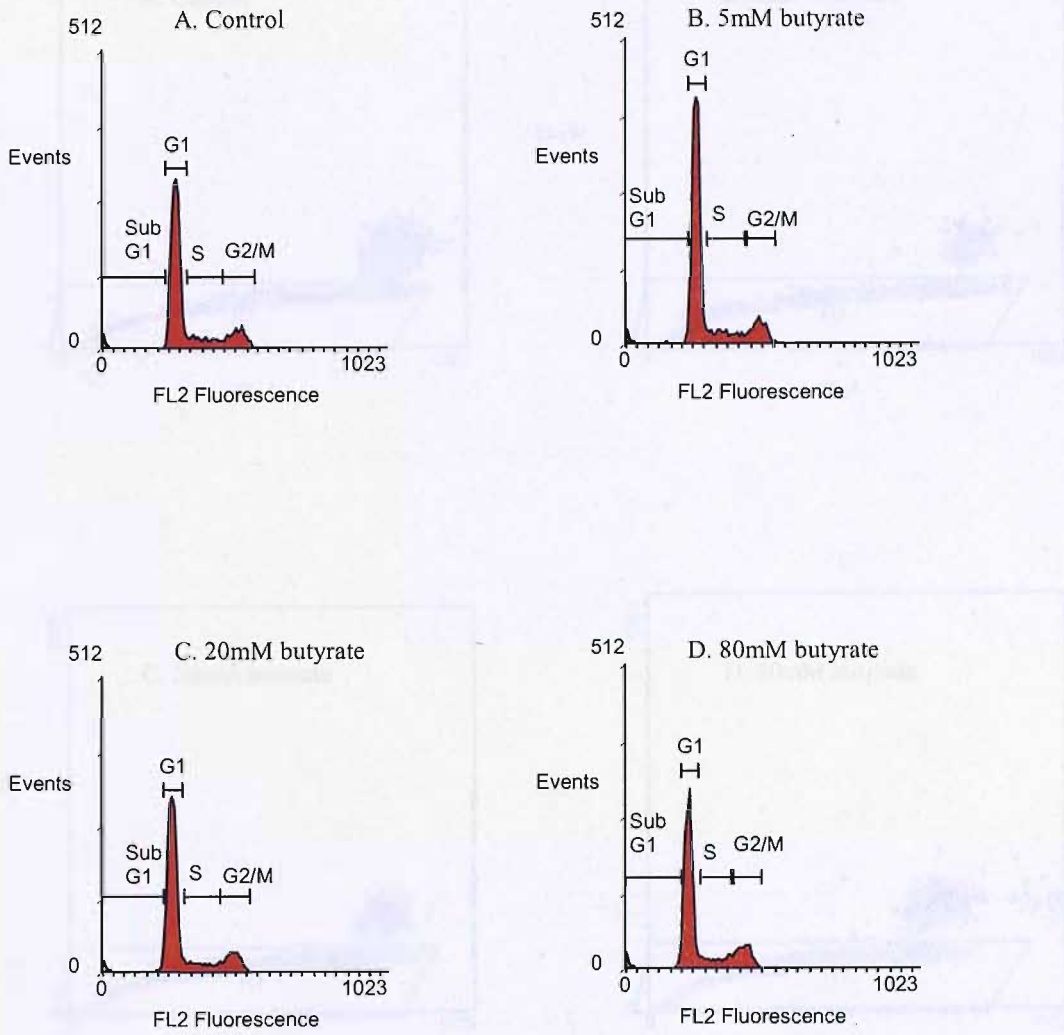


Figure 5.17 RT112 urothelial cells after 6 hours incubation with butyrate

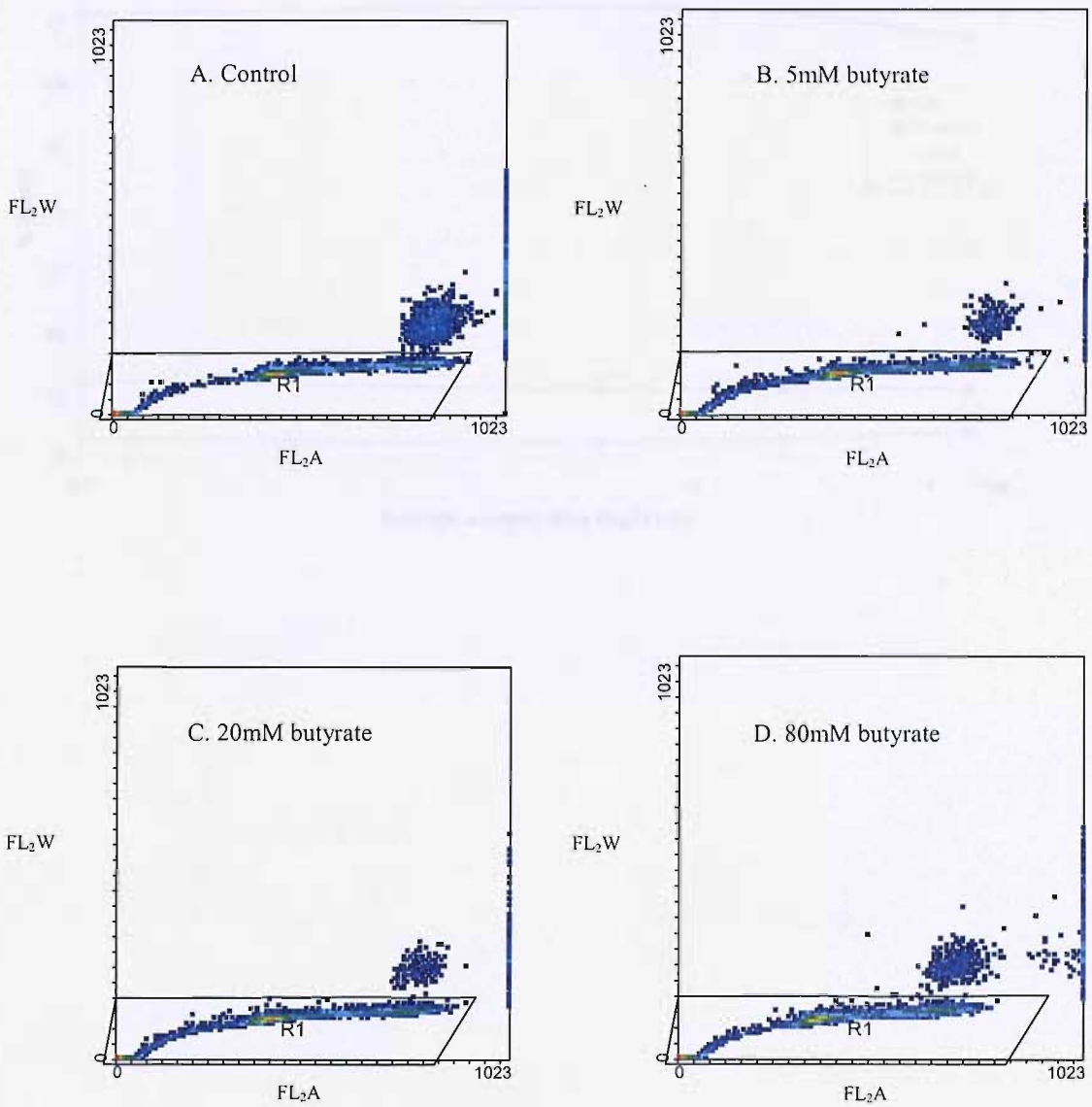
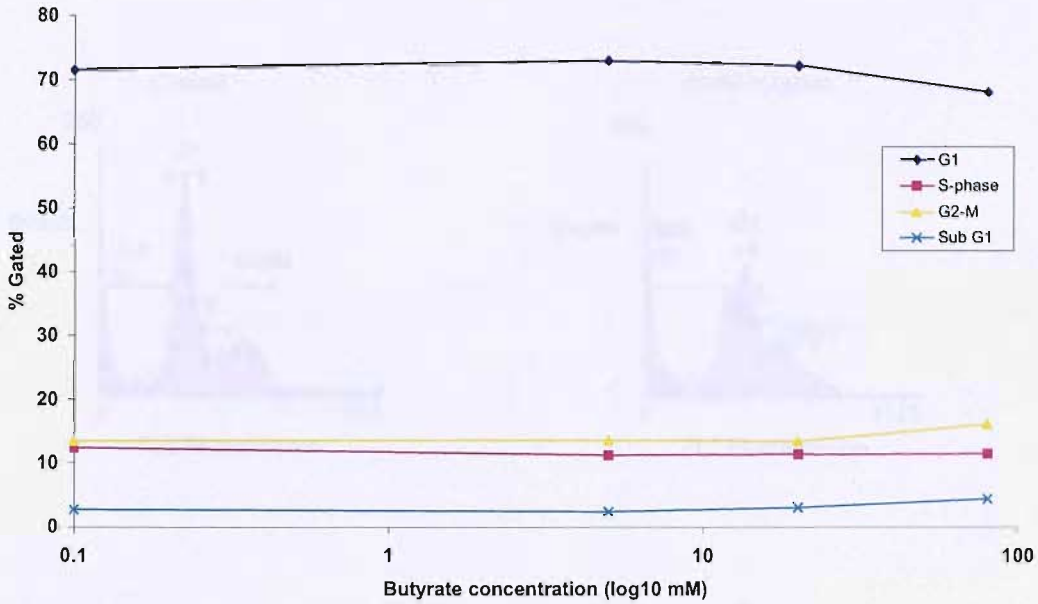


Figure 5.18 RT112 urothelial cells after 6 hours incubation with butyrate (doublet discrimination)

Figure 5.19 Cell cycle changes in RT112 urothelial cells after 6 hours incubation with butyrate



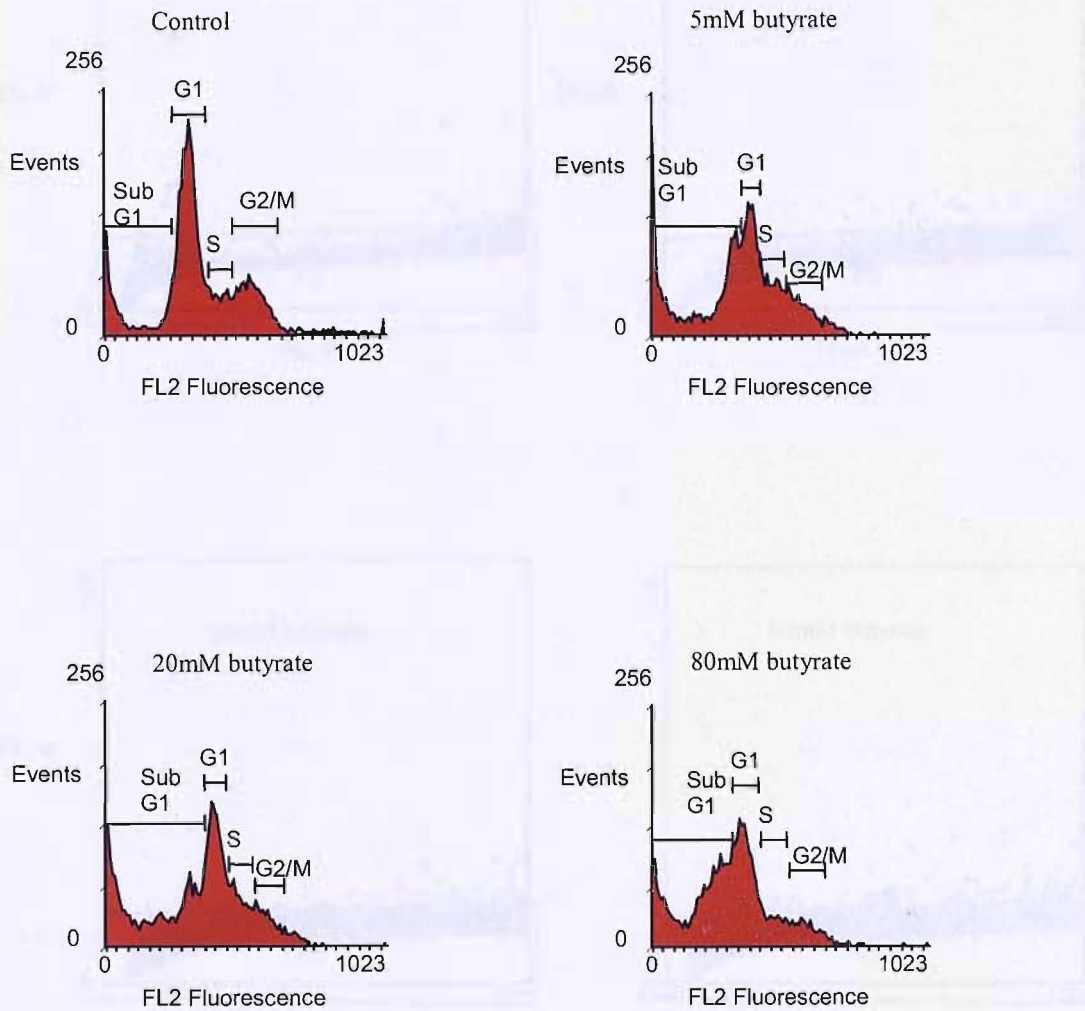


Figure 5.20 RT112 urothelial cells after 24 hours incubation with butyrate

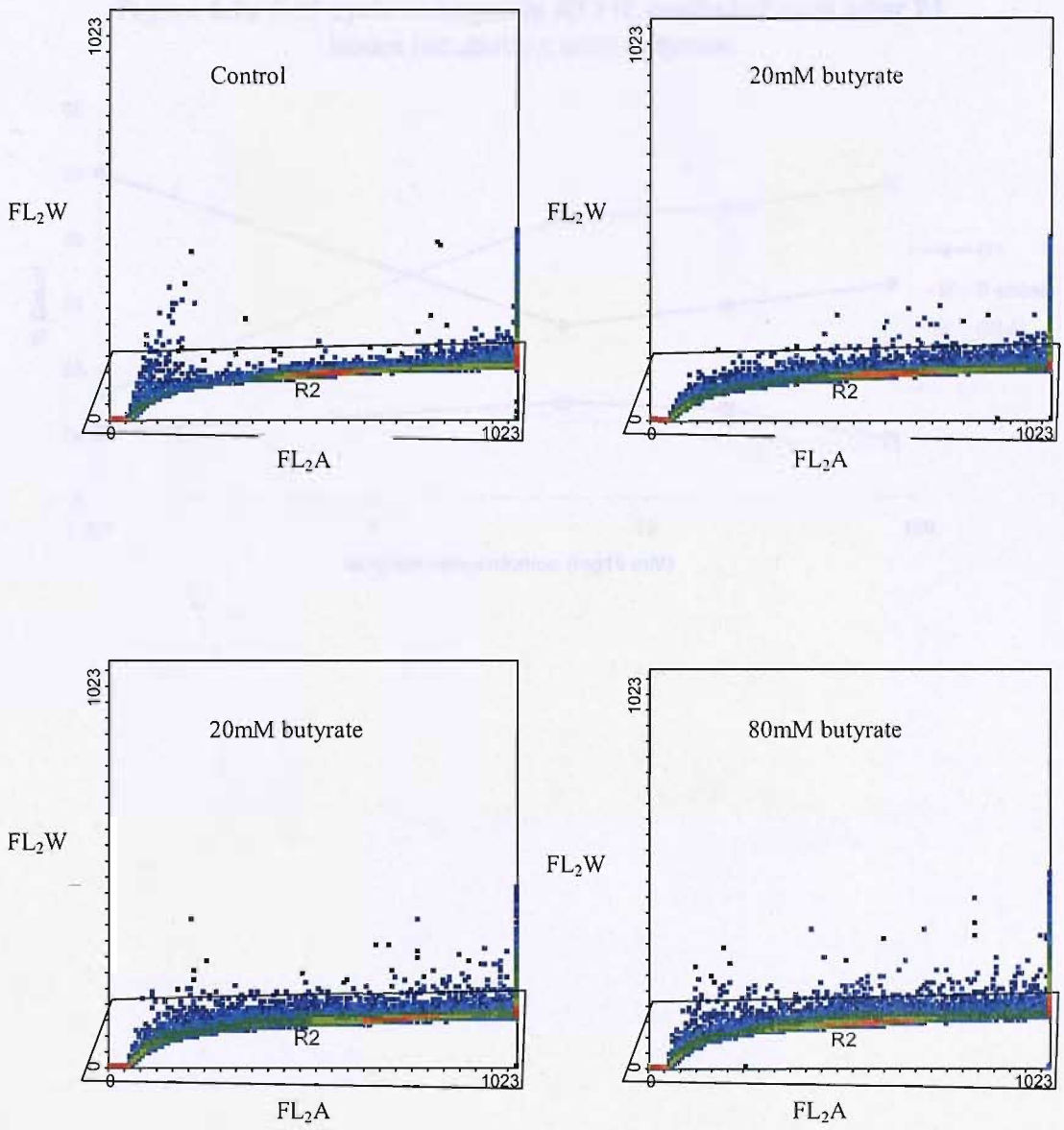
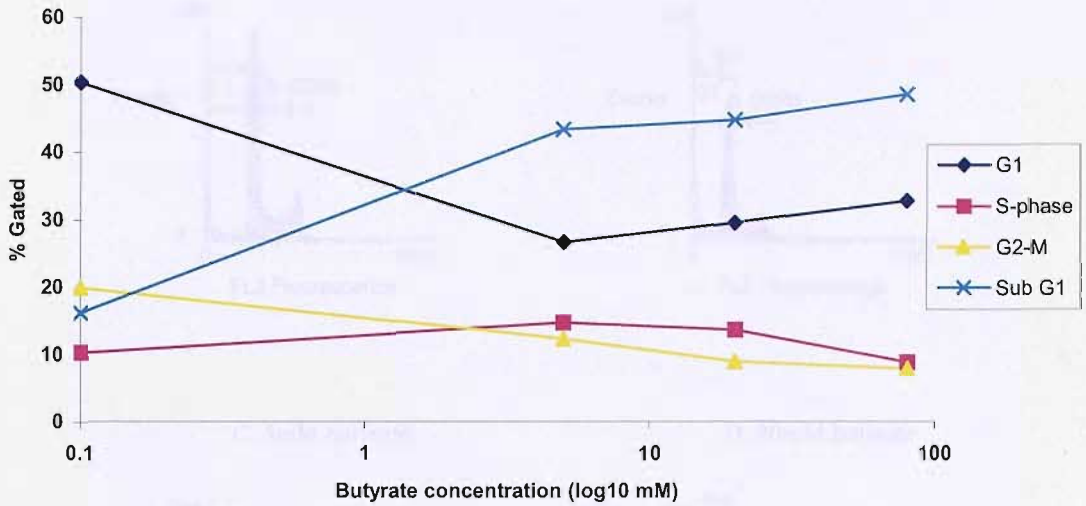


Figure 5.21 RT112 urothelial cells after 24 hours incubation with butyrate (doublet discrimination)

Figure 5.22 Cell cycle changes in RT112 urothelial cells after 24 hours incubation with butyrate



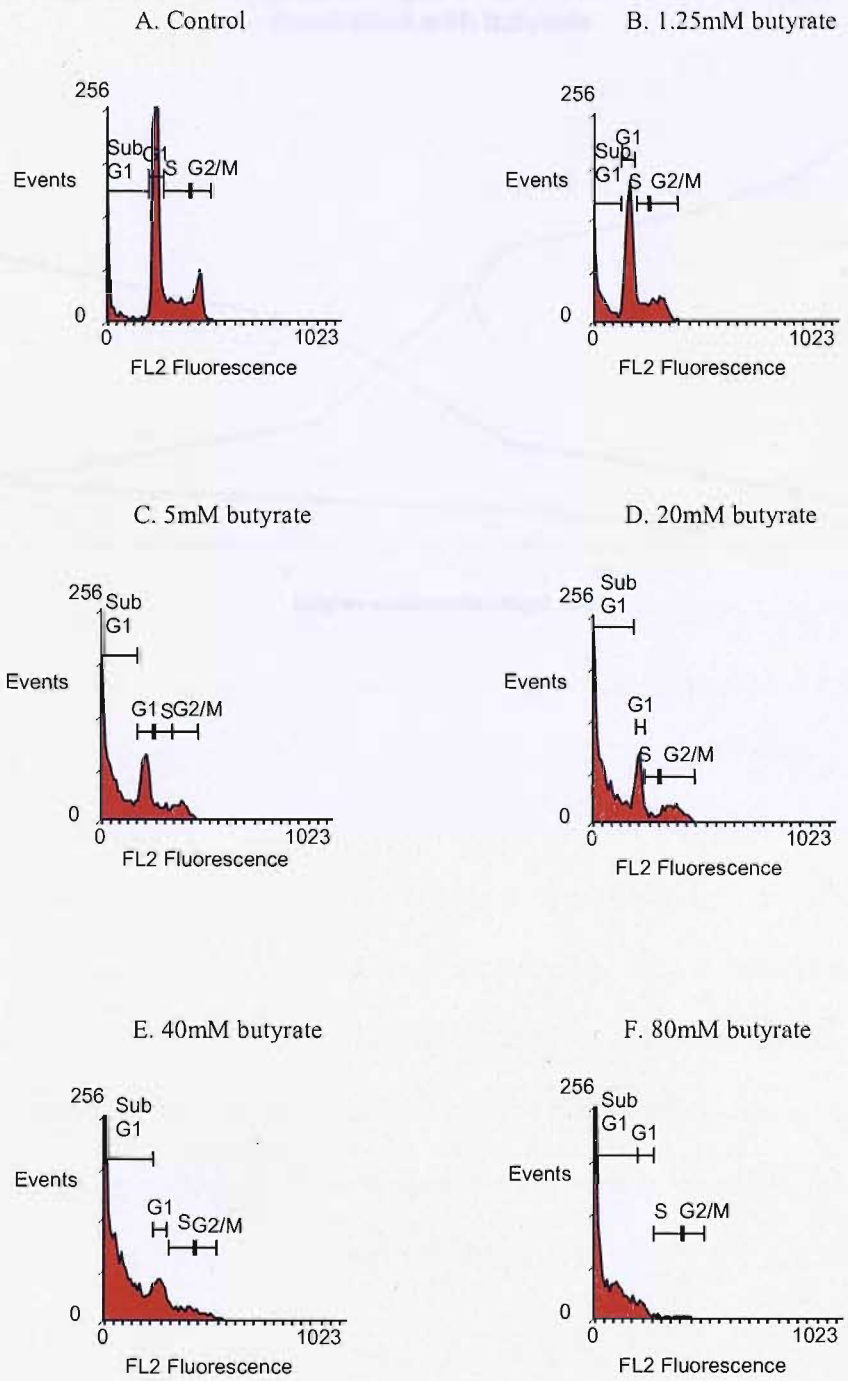
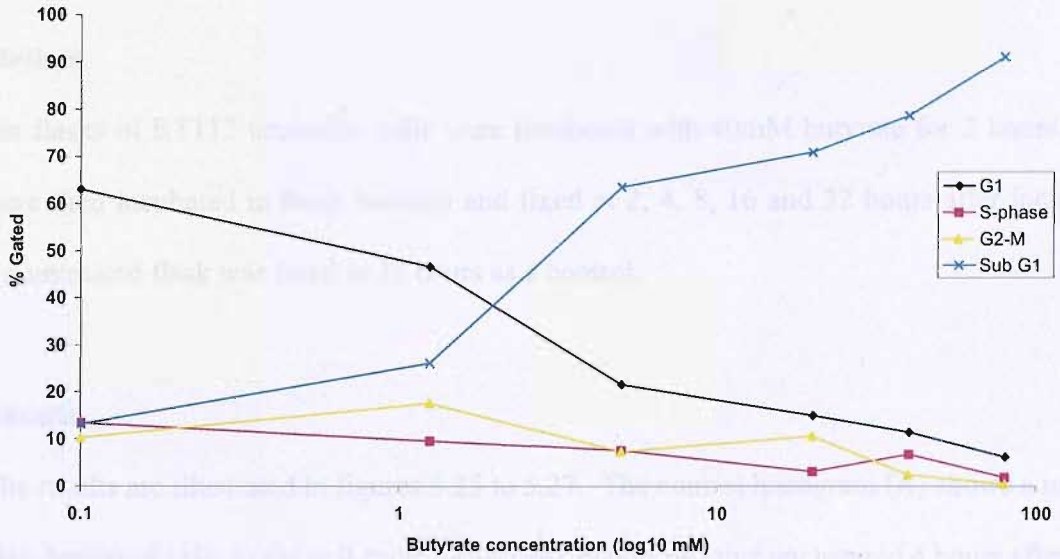


Figure 5.23 RT112 urothelial cells after 48 hours incubation with butyrate.

Figure 5.24 Cell cycle changes in RT112 cells after 48 hours incubation with butyrate



Time course of cell cycle changes in RT112 cells after 2 hours incubation with butyrate

Method

Six flasks of RT112 urothelial cells were incubated with 40mM butyrate for 2 hours. Cells were then incubated in fresh medium and fixed at 2, 4, 8, 16 and 32 hours after incubation. An untreated flask was fixed at 32 hours as a control.

Results

The results are illustrated in figures 5.25 to 5.27. The control histogram (A) shows a normal distribution of cells in the cell cycle. This distribution remains unchanged 4 hours after incubation. Following this period there is a gradual fall in the percentage of cell in the G1 phase. At 8 and 16 hours there is an increase in the early S-phase peak although the total percentage of S-phase cells only increases at 16 hours as illustrated in histograms E and F, figure 5.25. At 32 hours the percentage of cells in S-phase fell below control levels and G2/M population increased. The sub-G1 region does not increase significantly within the first 16 hours, but at 32 hours increased from 10% to 38%. Following the sequence of histograms from 4 hours to 32 hours there is a peak of cells which appear in histogram E at early S-phase and progresses over time through S-phase at 16 hours and G2/M at 32 hours. There is no indication that cells continue back into the G1 peak, but there is an increase in the sub-G1 population.

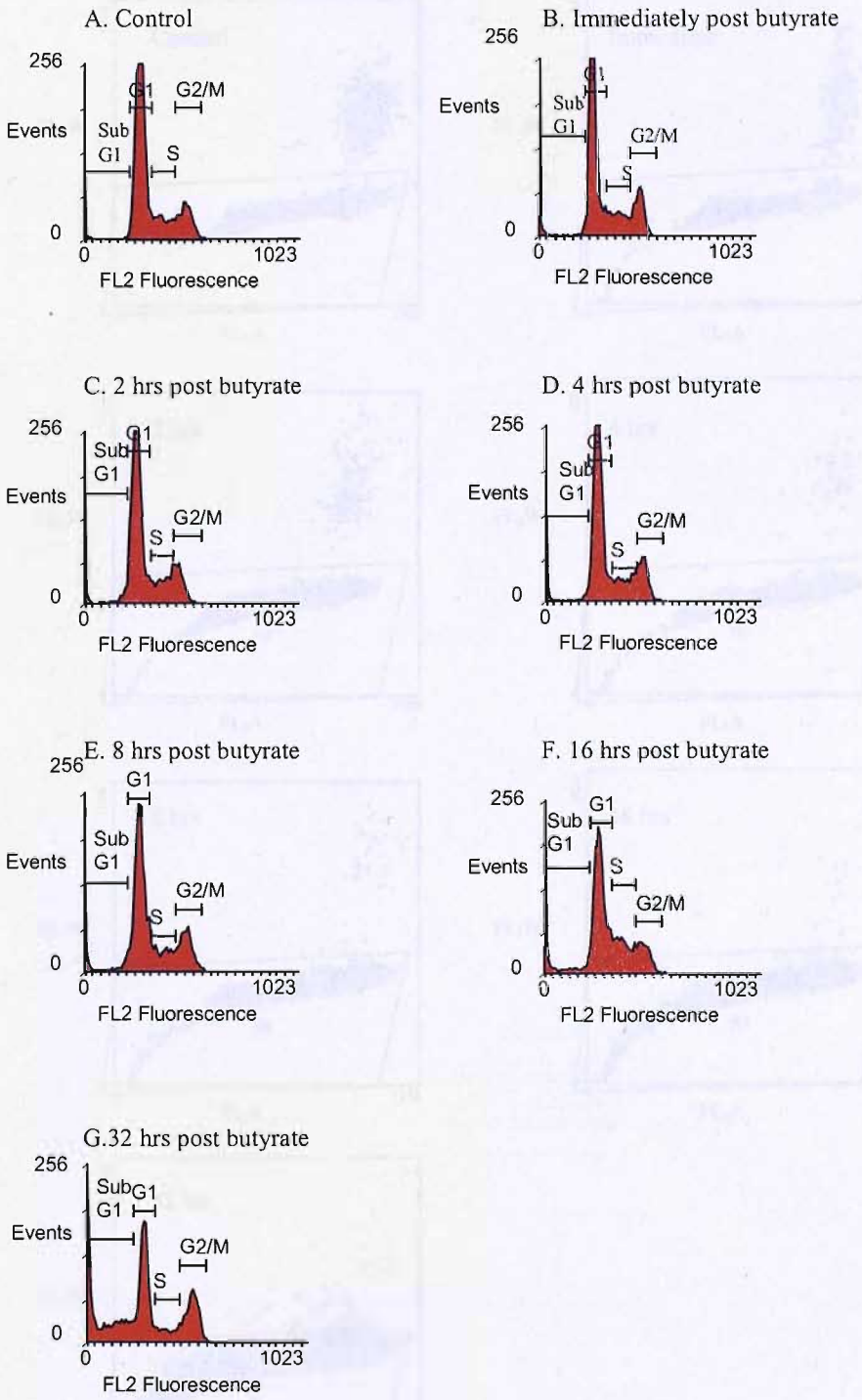


Figure 5.25 Time course of RT112 cells after 2 hour incubation with 40mM butyrate

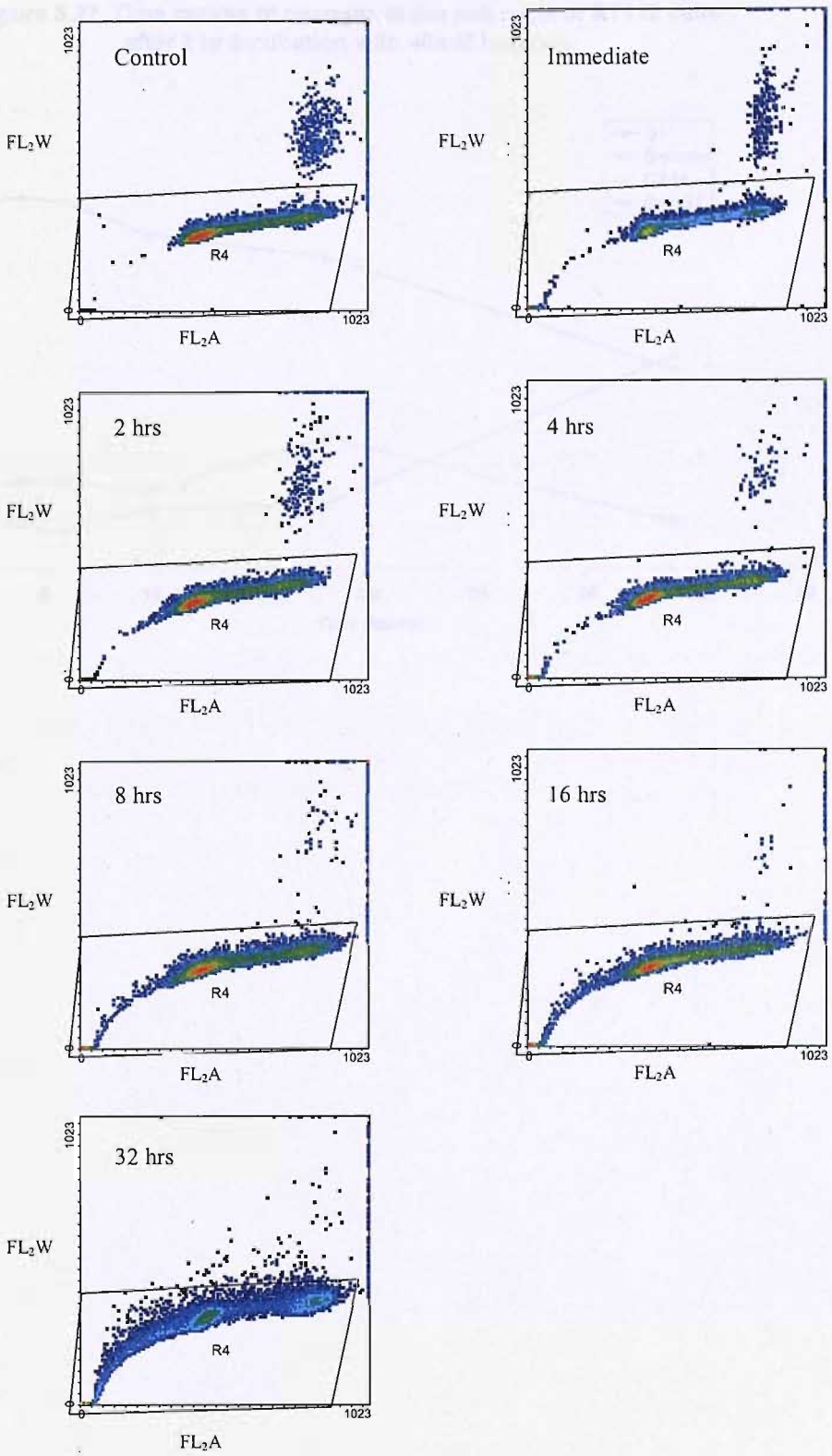
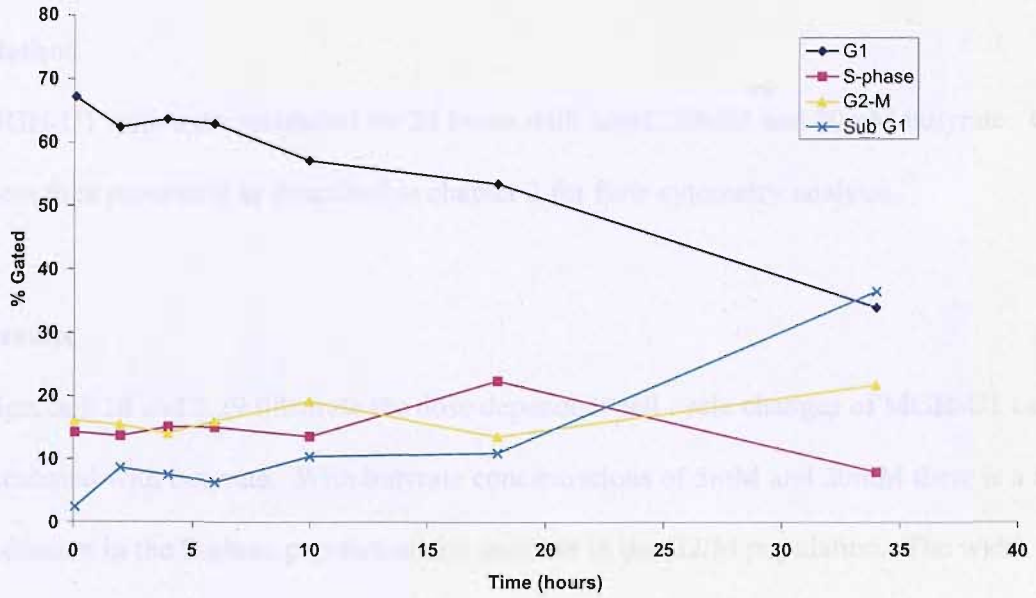


Figure 5.26 Time course of RT112 cells after 2 hour incubation with 40mM butyrate

Figure 5.27 Time course of changes in the cell cycle of RT112 cells after 2 hr incubation with 40mM butyrate



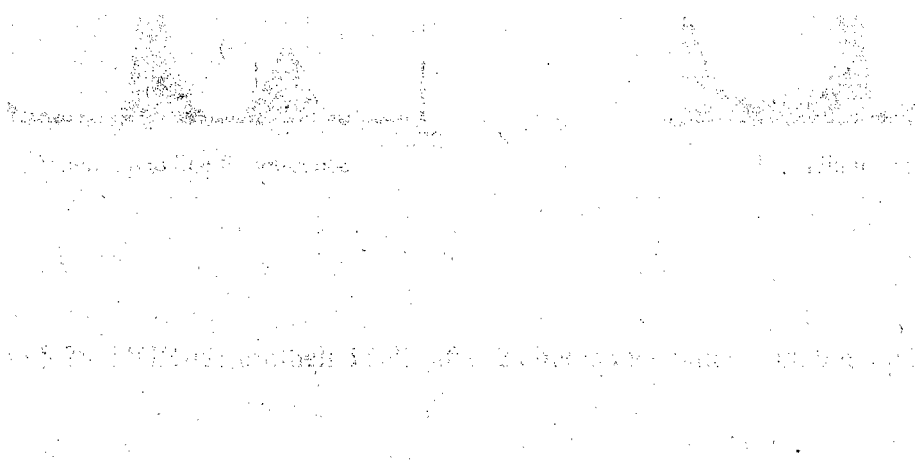
The effect of butyrate on MGH-U1 urothelial cell line

Method

MGH-U1 cells were incubated for 24 hours with 5mM, 20mM and 80mM butyrate. Cells were then processed as described in chapter 2 for flow cytometry analysis.

Results

Figures 5.28 and 5.29 illustrate the dose dependent cell cycle changes of MGH-U1 cells incubated with butyrate. With butyrate concentrations of 5mM and 20mM there is a marked reduction in the S-phase population and increase in the G2/M population. The width of the G1 peak is also increased. At 80mM there is a fall in the height of the G2/M peak, but increase in the width. In all incubations there is a gradual increase in the percentage of cells in the sub-G1 region with increasing butyrate concentration.



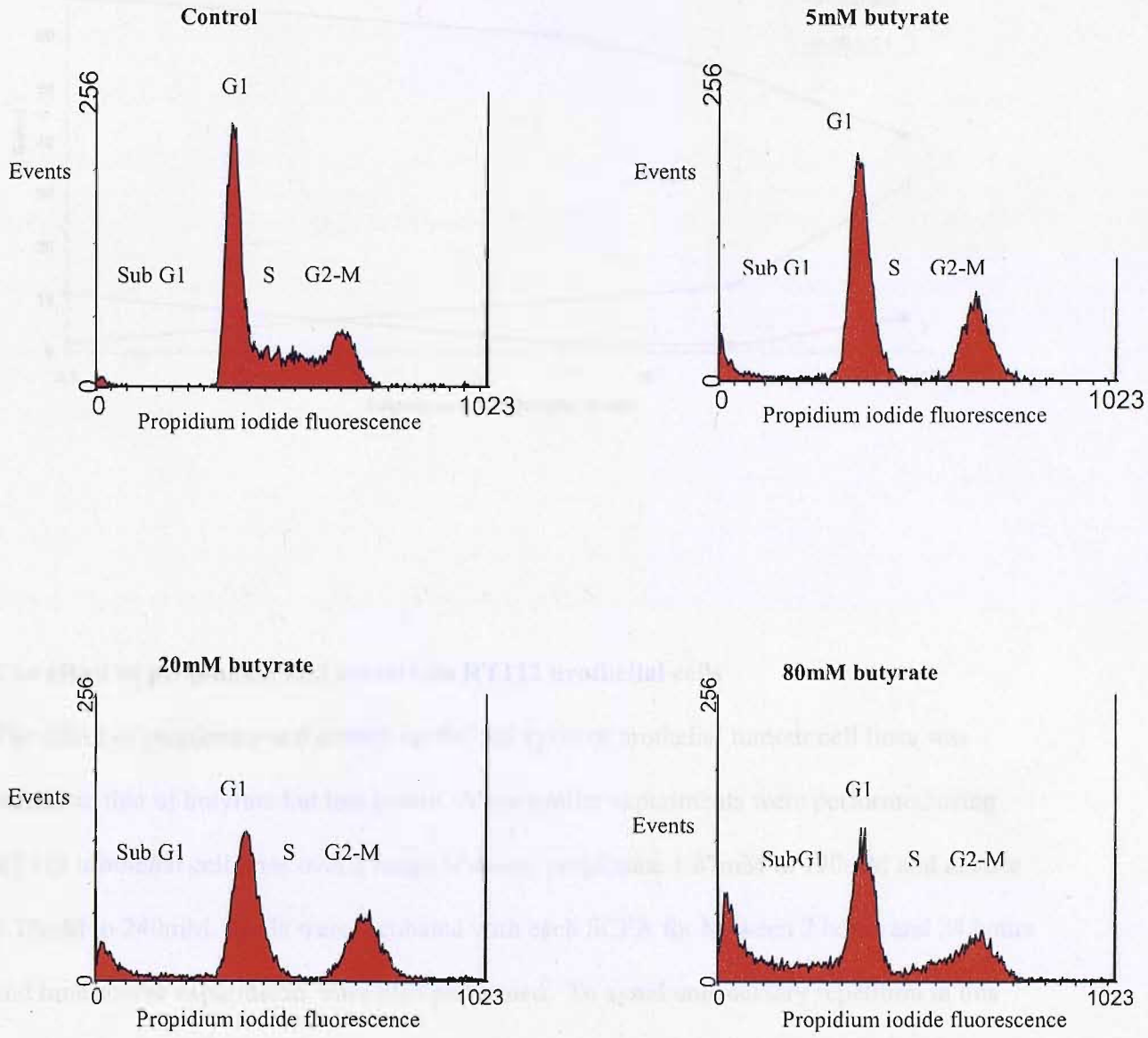
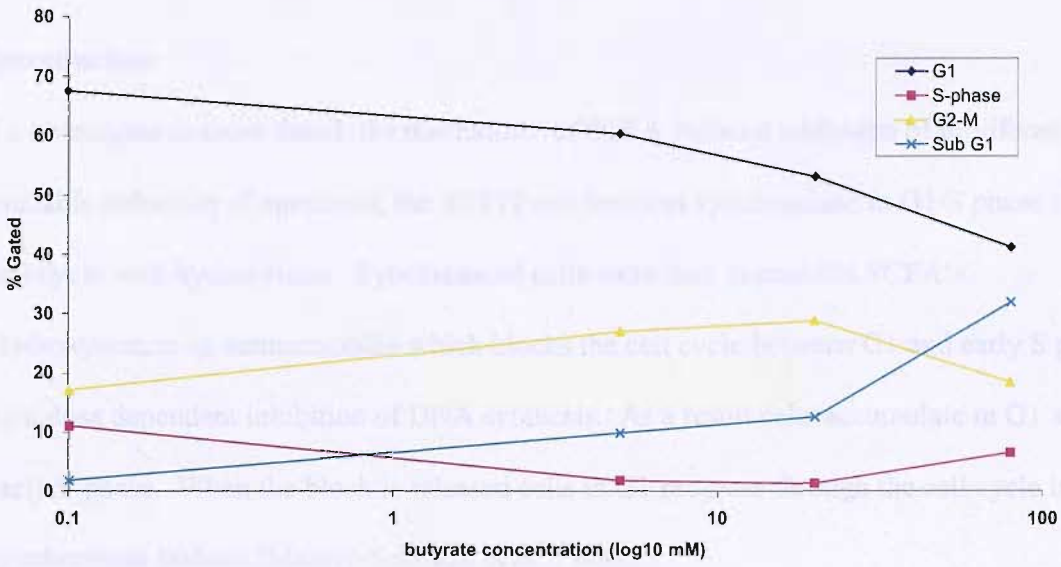


Figure 5.28 MGH-U1 urothelial cells after 24 hours incubation with butyrate

Figure 5.29 Cell cycle changes in MGH-U1 cells after 24 hours incubation with butyrate



The effect of propionate and acetate on RT112 urothelial cells

The effect of propionate and acetate on the cell cycle of urothelial tumour cell lines was similar to that of butyrate but less potent. Very similar experiments were performed using RT112 urothelial cell lines over a range of doses, propionate 1.87mM to 120mM and acetate 3.75mM to 240mM. Cells were incubated with each SCFA for between 2 hours and 24 hours and time course experiments were also performed. To avoid unnecessary repetition in this chapter, details of these experiments and the results (figures 5.30 to 5.49) can be found in appendix 9.

Effect of butyrate on RT112 cells after synchronization with hydroxyurea

Introduction

To investigate in more detail, the mechanism of SCFA induced inhibition of proliferation and probable induction of apoptosis, the RT112 cell line was synchronized in G1/S phase of the cell cycle with hydroxyurea. Synchronised cells were then exposed to SCFA's. Hydroxyurea is an antimetabolite which blocks the cell cycle between G1 and early S phase by a dose dependent inhibition of DNA synthesis. As a result cells accumulate in G1 and early S-phase. When the block is released cells in G1 progress through the cell cycle in a synchronised fashion (Maurer-Schultze et al. 1988).

Method

Eleven flasks of RT112 urothelial cells were incubated with hydroxyurea and one flask with standard medium (as a control) for 20 hours to synchronise cells in G1/S phase. To release the G1/S phase block the incubation medium was changed, removing hydroxyurea from the incubation medium. Six flasks including the control were incubated with standard medium (control group), and five flasks were incubated with medium containing 5mM butyrate (treatment group). One control flask was fixed and labelled with propidium iodide immediately and then one flask from each group was fixed and labelled with propidium iodide at 4, 10, 14, 19, and 24 hours. Finally the control flask was fixed and labeled at 24 hours. Cells were then analysed by flow cytometry.

Results

Twenty hours incubation with hydroxyurea induced effective synchronization of cells in the G1 and S phases of the cell cycle (figure 5.50). Following release of synchronization (figure

5.51) the synchronized population completed a cycle of replication within 10 to 14 hours, returning to a normal pattern of distribution within 24 hours. In contrast cells treated with 5mM butyrate demonstrated similar progression through G1/S to G2/M but failed to return to G1 phase (figure 5.52). There was a degree of arrest at G2/M and an increase in the sub-G1 population of cells within 10 hours of synchronization release and exposure to butyrate. The sub-G1 population continued to increase from 10 to 24 hours and cells failed to progress through the cell cycle from G2/M to G1 compared with untreated cells (figures 5.53 and 5.54).

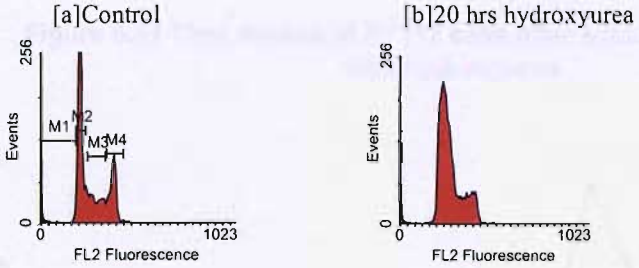


Figure 5.50 RT112 cells. [a] Control [b] After synchronization with hydroxyurea for 20 hours

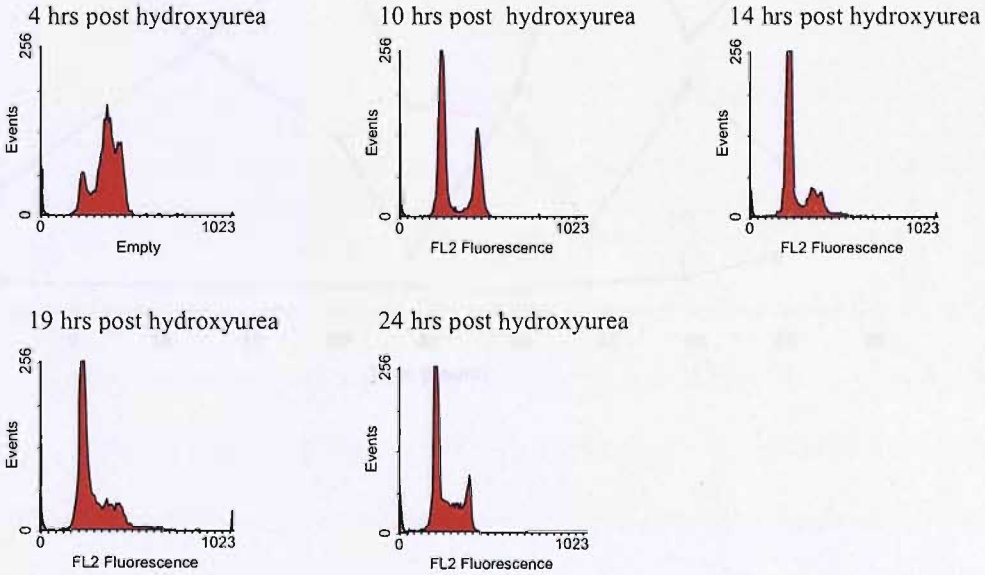


Figure 5.51 Time course of cell cycle changes in RT112 cells after synchronization with hydroxyurea.

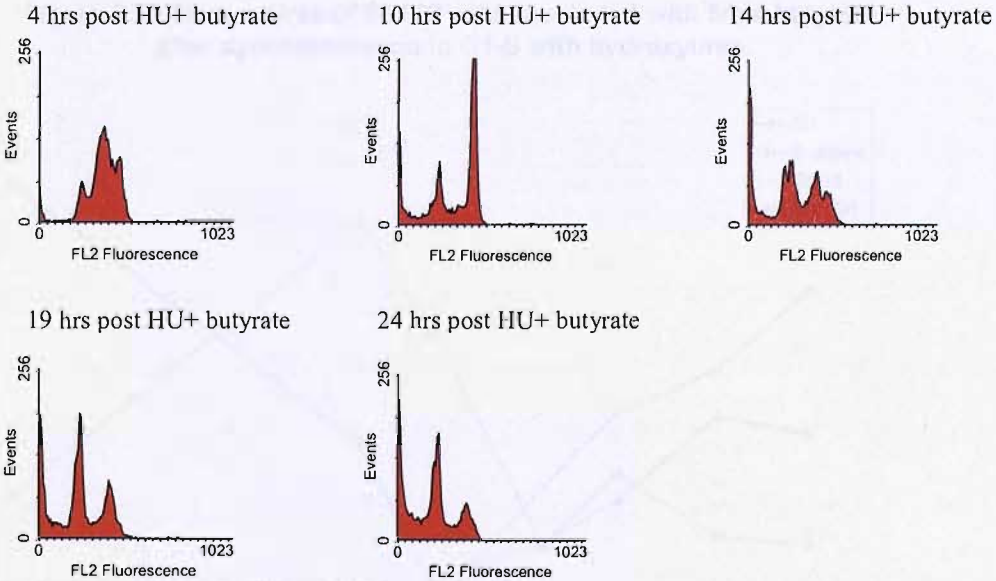


Figure 5.52 Time course of cell cycle changes in RT112 cells incubated with 5mM butyrate after synchronization with hydroxyurea.

Figure 5.53 Time course of RT112 cells after synchronisation in G1-S with hydroxyurea

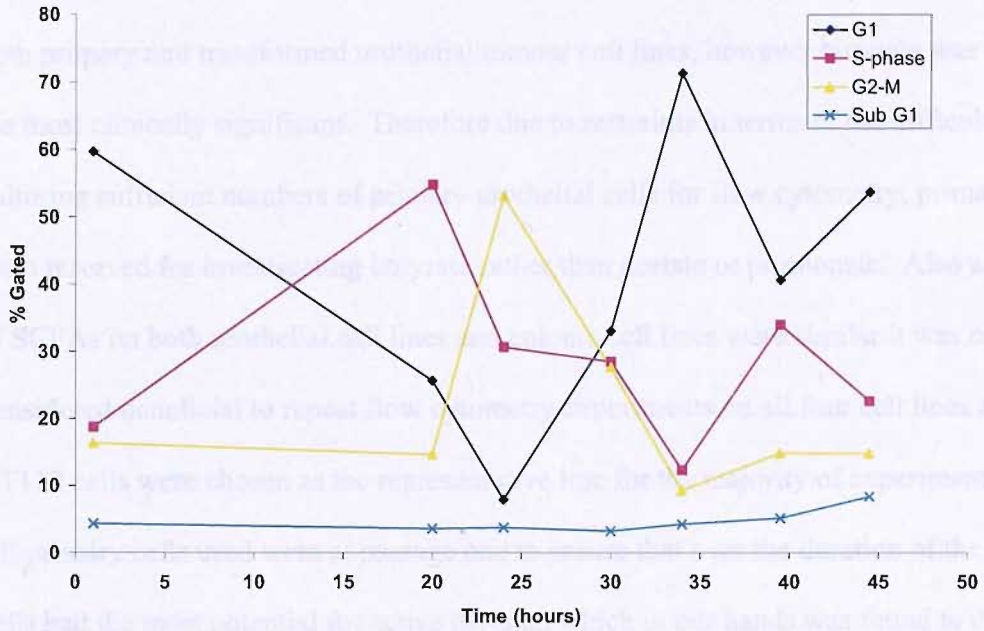
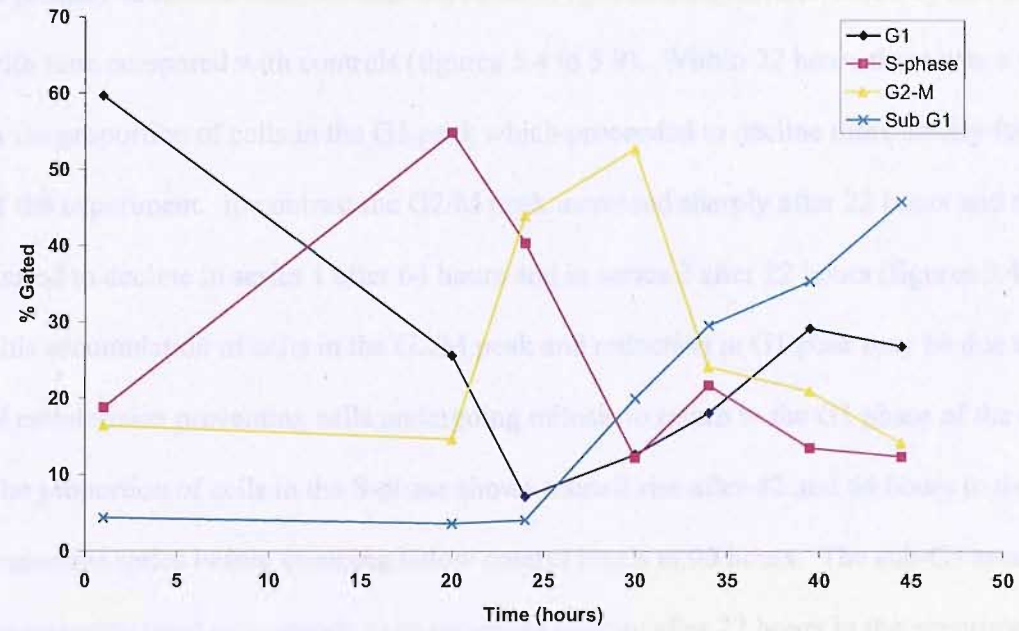


Figure 5.54 Time course of RT112 cells incubated with 5mM butyrate after synchronisation in G1-S with hydroxyurea



Discussion

In chapter four all three short chain fatty acids were shown to have an inhibitory effect in both primary and transformed urothelial tumour cell lines, however butyrate was potentially the most clinically significant. Therefore due to restraints in terms of the difficulty in culturing sufficient numbers of primary urothelial cells for flow cytometry, primary cells were reserved for investigating butyrate rather than acetate or propionate. Also as the action of SCFAs on both urothelial cell lines and colonic cell lines were similar it was not considered beneficial to repeat flow cytometry experiments on all four cell lines and therefore RT112 cells were chosen as the representative line for the majority of experiments.

All primary cells used were at passage one to ensure that over the duration of the experiment cells had the most potential for active division which in our hands was found to decline after 3 or 4 passages. This meant that a relatively large piece of tissue was required to provide enough cells for each experiment. Consequently one piece of tissue, approximately $1cm^2$ provided enough cells for only one experiment and variation in results between experiments due to inter patient variables, age, source and condition of the tissue had to be accepted.

In primary urothelial cells the cocktail of SCFA produced a number of cell cycle alterations with time compared with controls (figures 5.4 to 5.9). Within 22 hours there was a reduction in the proportion of cells in the G1 peak which proceeded to decline more slowly for the rest of the experiment. In contrast the G2/M peak increased sharply after 22 hours and then started to decline in series 1 after 64 hours and in series 2 after 22 hours (figures 5.4 and 5.5). This accumulation of cells in the G2/M peak and reduction in G1 peak may be due to a block of cell division preventing cells undergoing mitosis to return to the G1 phase of the cell cycle. The proportion of cells in the S-phase shows a small rise after 42 and 64 hours in the respective series before dropping below control levels at 90 hours. The sub-G1 area representing dead or apoptotic cells increased sharply after 22 hours in the experimental

flasks compared with the expected small rise in the controls with time. An explanation for the decline in the G1 population cells and increase in sub-G1 population is that cells in G1 undergo apoptosis. The increase in the G2/M population after 22 hours may be due to cell cycle arrest at G2 check point before mitosis as observed in breast and colon cancer cell lines (Lallemand et al. 1999; Siavoshian et al. 2000). This may contribute to the apparent decline in the G1 population, however, with time the G2/M peak declines probably representing release of the block at the G2 check point, and the sub-G1 peak continues to increase. From these experiments the reason for the decline in the G2/M peak is unclear. G2/M cells may progress through the cell cycle into G1, where they undergo apoptosis, or they may be terminally affected by SCFAs, preventing mitosis as demonstrated in breast cell lines which re-replicate DNA rather than divide (Lallemand et al. 1999). Alternatively both G1 and G2 cells may undergo apoptosis as seen in colonic tumour cell lines (Heerdt et al. 1997; Siavoshian et al. 2000). This may explain the appearance of the G2/M peak on the flow cytometry histogram which appears to widen, possibly as a result of DNA condensation and fragmentation during the apoptotic process, creating a sub G2/M population. In contrast to studies in breast, lung, colonic tumour cell lines and kidney epithelial cells, primary urothelial cells did not demonstrate a G1 check point arrest. It is also recognized that colonic epithelial cells undergo apoptosis when incubated with butyrate (Haza et al. 2000; Heerdt et al. 1994), and to a lesser extent with propionate and acetate (Hague et al. 1995). These experiments used physiological concentrations of SCFA, approximately 10 fold less than the concentration of the SCFA cocktail, over longer incubation periods of several days. It appears that a short exposure of 1 to 2 hours at higher concentrations as used in our experiments (to mimic the proposed intravesical treatment protocol) also induces apoptosis in both malignant and primary urothelial cells.

Further experiments undertaking similar short incubations using a range of butyrate concentrations alone demonstrated a time and dose dependent accumulation of cells in G2/M and loss of cells from G1 followed by an increase in sub-G1 population (figures 5.10 to 5.13). Prolonged incubation with physiological and therapeutic concentrations of butyrate (figures 5.14 to 5.16) demonstrated dose dependent loss of cells from G1, S, and G2/M phases of the cell cycle with dose dependent increases in the subG1 component. This suggests that there is a similar mechanism of inhibition at work at both physiological and therapeutic concentrations of butyrate. Also at 90 hours with concentrations in the physiological range 1-5mM there is relative preservation of the G2/M peak compared with the G1 peak suggesting prolonged G2/M block in the cell cycle. At concentrations ≥20mM for this extended incubation period >80% of cells have accumulated in sub-G1 region.

In the RT112 malignant urothelial cell line, butyrate produced a similar time and dose dependent fall in the G1 and rise in sub-G1 peaks. However there was no evidence of accumulation of cells in G2/M. This may represent a paradoxical effect of butyrate between normal and malignant urothelium which would not be unexpected as butyrate is well known to have contrasting effects in normal and malignant colonic epithelial cells which may be due to different states of cell activation (Gibson et al. 1999). On inspection of the histograms there was accumulation of cells at the junction between G1 and early S-phase with butyrate concentration of 5 and 20mM after 24 hours (figures 5.20 to 5.22) and between 8 and 16 hours after a shorter 2 hour incubation with 40mM butyrate (figures 5.25 to 5.27). Despite overall loss of cells from G1 this peak may demonstrate G1/S transition block consistent with findings in other cell lines. An alternative explanation is that as cells die by either apoptosis or necrosis they lose DNA and therefore appear left of the G1 peak, but if cells in G2-M die, loss of DNA fluorescence will cause an accumulation of cells in the S-phase region giving the deceptive appearance of an increase in S-phase. In keeping with the viability experiments

in chapter 4, butyrate had no immediate effect on the distribution of cells within the cell cycle when incubated with butyrate for up to 6 hours over a range of concentrations from 5mM to 80mM (figures 5.17 to 5.19).

Experiments using hydroxyurea (HU) which synchronises cells into the G1/S phase of the cell cycle were employed to clarify the actions of SCFA's on G2/M phase in RT112 cells. After release of the HU induced block control cells proceeded through the cell cycle losing synchronization within 24 hours. There was minimal accumulation in the sub-G1 area. In comparison urothelial tumour cells incubated with 5mM butyrate after HU release demonstrated hold up at G2/M at 10 hours compared with controls. With further incubation to 24 hours cells accumulated in the sub-G1 population with significantly less cells returning to G1 phase. This would suggest that butyrate not only induces G2 check point arrest but G2/M cells then undergo cell death by apoptosis.

Interestingly incubation of a different urothelial cell line MGH U1, a faster growing and less well differentiated cell line demonstrated both G1/S transition block and G2/M block with almost complete loss of S-phase cell population after 24 hours incubation with 5 and 20mM of butyrate. At higher concentrations of butyrate there was reduction of G1 and G2/M peaks and increase in the sub-G1 population. There was also preservation of the S-phase region which in view of the S-phase changes seen with 5 and 20mM butyrate would suggest that this in fact does represent G2/M cells which have lost DNA fluorescence in the process of cell death rather than true preservation of cells in S-phase. Assessment of the other components of the SCFA cocktail, propionate, and acetate demonstrated similar trends in terms of dose dependent reduction in G1 and increase in sub-G1 populations, however, these were less dramatic than with butyrate and occurred at higher concentrations. At physiological concentrations of both propionate and acetate the amplitude of all phases of the cell cycle was maintained similar to control levels. However at therapeutic concentrations (30mM

propionate and 60mM acetate) there was accumulation of cells in the G1/S transition region after 24 hours. At lower concentrations, 7.5mM of propionate and 15mM of acetate there was evidence of G2/M block after 24 hours which was not present at higher concentrations. At the highest concentrations, 4 fold that of the cocktail, propionate and acetate produced loss of both G2/M and S-phase and accumulation in the sub-G1 area, indicating inhibition of cell replication as well as cell death. Over time at the therapeutic concentrations of propionate and acetate, a block at G1/S at 11 hours and G2/M at 18 hours was demonstrated supporting the dose response experiments.

In summary there is evidence that all 3 SCFAs produce G2/M block in both primary and malignant urothelial cells and G1 block in malignant but not primary urothelial cells. All SCFAs induce accumulation of cells in sub-G1 which may represent induced apoptosis in urothelial cells at both physiological and treatment concentrations of butyrate and propionate, and only at treatment concentrations of acetate. Although there was disturbance in the cell cycle induced by propionate and acetate after incubation, the amplitude of the loss of G1 and increase in sub-G1 was much more dramatic with butyrate, confirming that butyrate has potentially the greatest inhibitory effect on the urothelium after intravesical instillation.

Chapter 6

Detection of apoptotic cells by acridine orange fluorescence

Introduction

In previous chapters investigating the effects of SCFAs on cell proliferation and the cell cycle, SCFAs demonstrated inhibition of cell proliferation in a dose and time dependent fashion and cell cycle block at G1/S and G2/M check points. There was also accumulation of cells in the sub-G1 area shown to represent apoptotic cells (Tounekti et al. 1995). To confirm the presence of apoptosis in cells incubated with SCFAs experiments employing acridine orange fluorescence microscopy were conducted to demonstrate the morphological features of apoptosis. Details of the fluorescence microscope, principles of fluorescence and morphology of apoptosis can be found in appendix 10.

Methods

Acridine orange fluorochrome labelling

Primary urothelial cells were cultured in 12 well plates for 36 hours. Cells were then incubated with SCFA cocktail, for 1 hour and 7 hours at concentrations between 1.25mM to 80mM butyrate or 3.75mM to 240mM of propionate and acetate. Immediately after exposure, 1μl of 1μg/ml acridine orange in PBS was added to the culture medium. Cell counting was performed using an inverted fluorescence microscope (Leica DM1RB), and selected digital images were collected (Hamamatasu camera, GMBH) and stored on zip disk. Both adherent and floating apoptotic cells and normal cells were counted and the apoptotic index calculated as the percentage of apoptotic cells against normal cells in one high power field. At least 5 random fields were counted and the average calculated for each well.

Data analysis

Data was tabulated in Microsoft excel and statistical analysis was performed in SPSS software using one way analysis of variance to compare differences between concentrations with post hoc comparisons of means using Bonferroni's correction.

Results

Primary urothelial cells treated with SCFA cocktail with butyrate equivalence ranging from 1.25mM to 80mM for 7 hours demonstrated apoptosis in 17% to 25% of cells per high powered field compared with 12% of cells per high powered field in the control wells ($p=0.017$). The features of apoptosis including chromatin condensation, cell shrinkage, plasma membrane blebbing and apoptotic bodies are illustrated in figure 6.1.

A dose dependent relationship was not demonstrated. In cultures viewed just one hour after exposure to SCFA cocktail there was no difference in the level of apoptosis in the cultures exposed to SCFA cocktail containing 40mM butyrate or less compared with controls. With SCFA cocktail containing 80mM butyrate there was a small increase in the number of apoptotic cells compared with control (figure 6.2).

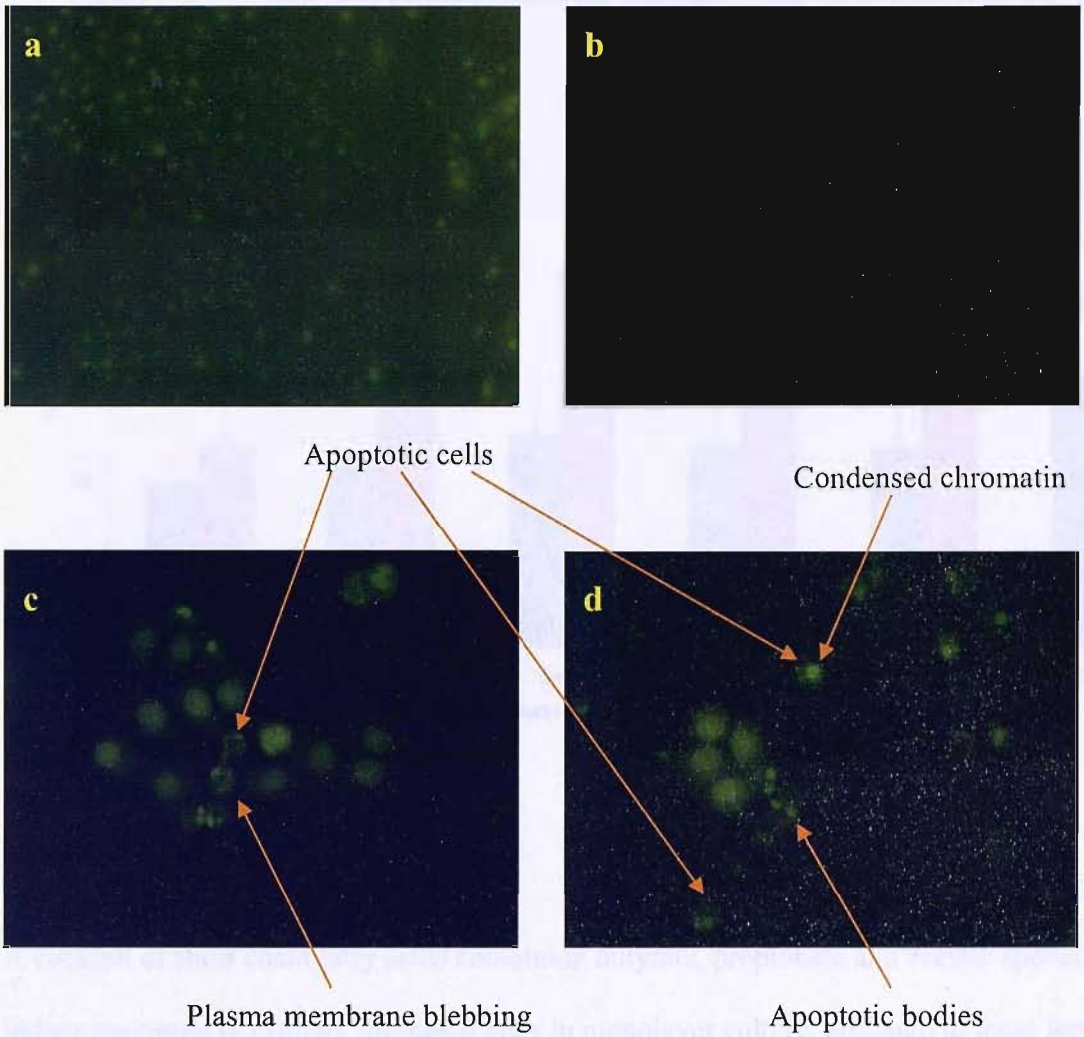
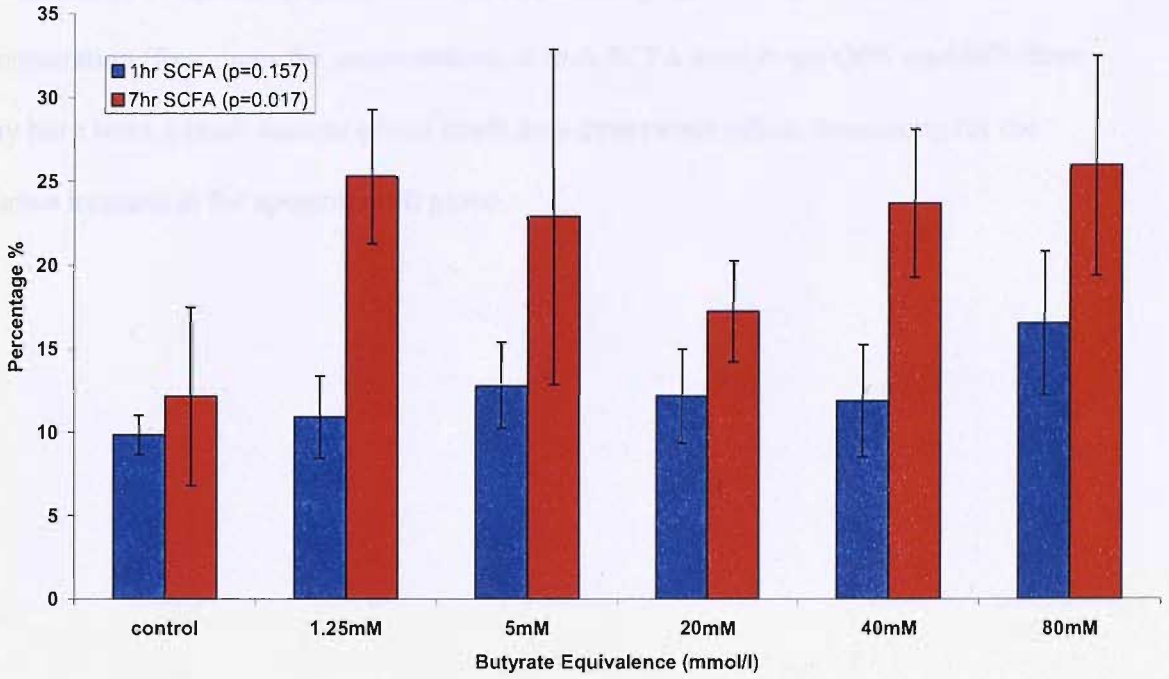


Figure 6.1 Fluorescence microscopy images demonstrating acridine orange fluorescence of primary urothelial cells. (a and b) Under low power apoptotic nuclei are condensed and demonstrate increased fluorescence intensity. (c and d) Apoptotic cells demonstrate cell shrinkage, condensed chromatin, plasma membrane blebbing and apoptotic bodies.

Figure 6.2 Apoptotic primary urothelial cells following incubation with SCFA cocktail.

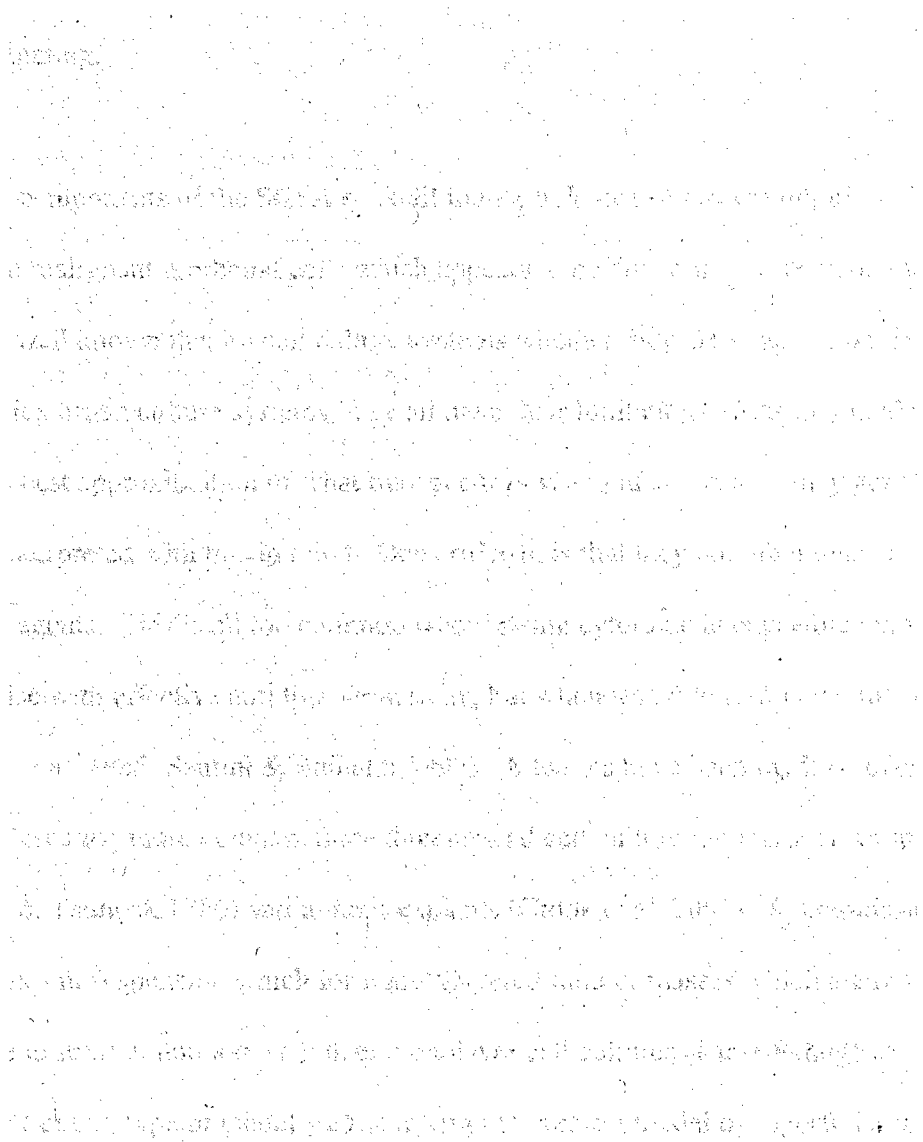


Discussion

A cocktail of short chain fatty acids containing butyrate, propionate and acetate appear to induce apoptosis in primary urothelial cells in monolayer culture, although in these limited experiments a dose dependent effect was not demonstrated. These findings are consistent with the published literature using non urothelial cell lines, and the first time SCFA induced apoptosis has been demonstrated in primary urothelial cell cultures. Although every effort was made to include floating cells (detached apoptotic cells) when performing cell counts this was often difficult and may have resulted in reduced apoptotic counts in wells exposed to higher doses of SCFA. As a result the number of apoptotic cells may have been underestimated hiding any dose dependency.

In chapter 4 SCFAs were shown to have no immediate effect on cell proliferation and similarly in these experiments there was no difference in the number of apoptotic cells after one hours exposure to SCFA cocktail, except in the wells exposed to the highest

concentration of cocktail (80mM of butyrate, 240mM propionate and acetate). At this concentration (four times the concentration of each SCFA used in the GOS cocktail) there may have been a small amount of cell death by a direct toxic effect, accounting for the relative increase in the apoptotic cell group.



Chapter 7

In vivo tolerance of intravesical instillation of short chain fatty acid

Introduction

In this chapter a rodent model was employed to assess the effect of SCFA cocktail on the urothelium *in vivo*. The effect of a single instillation and multiple instillations were investigated. Tissue sections were assessment by standard haematoxylin and eosin histology and Brdu labeling.

In vitro, all components of the SCFA cocktail induce a degree of growth inhibition in both primary and malignant urothelial cells which appears to be due to their effects on the cell cycle. It is well known that all cell culture systems whether they are simple monolayers or more complex organ culture systems, they all have their limitations. Like any model they provide the best approximation of what may occur *in vivo* and any results they generate should be interpreted with this in mind. One criticism is that they are often over sensitive to therapeutic agents. This is all too common when testing cytotoxic agents which in vitro show great promise with effective anti tumour activity, but when tested *in vivo* have little effect (Erlichman et al. 1985; Santini & Rainaldi 1999). Attempts have been made to overcome this problem by creating more complex three dimensional cell culture models such as spheroids (Erlichman & Tannock 1986) and tumour explants (Crook et al. 2002). Spheroids are grown from cell lines in suspension which form multilayered tumour masses which are more comparable to solid tumours *in vivo* than monolayer cell cultures (Kunz-Schughart et al. 1998). The tumour explant model was an attempt to create a model of superficial bladder cancer with cell lines cultured on urothelial basement membrane. The flaws in the monolayer culture system are multifactorial and far more complex than purely a lack of three

dimensional cell contact, and only partly overcome by the use of spheroids which have been shown to be five times more resistant to chemotherapeutic agents than monolayers (Erlichman & Vidgen 1984). A complex interaction exists between the basement membrane, extracellular matrix, various adhesion molecules, angiogenesis factors and other soluble mediators in normal tissues and solid tumours which increase resistance to the effects of therapeutic agents including blockade of drug induced apoptosis (Dalton 1999; Pogany et al. 2001; Sonoda et al. 2003). Therefore any culture system, although useful should only be a guide to possible activity of a therapeutic agent with *in vivo* animal experiments and ultimately human studies remaining the gold standard and true measure of therapeutic activity. For this reason despite the potent anti-proliferative effect of SCFAs, particularly butyrate at therapeutic concentrations *in vitro*, it was important to assess whether SCFAs truly had any adverse effects *in vivo*. Experiments were performed using a rodent model where both single and multiple doses of SCFAs were instilled into the bladder and the bladder examined histologically for any adverse reaction.

Methods

Animal husbandry

Twenty five female Wister rats weighing 170-200grams were obtained for this study. They were kept in the animal house at Southampton General Hospital in plastic cages. Six animals or less were housed in each cage with shredded paper bedding and various cardboard shelters to improve their environment. They had free access to standard diet and water, and were inspected daily.

Anaesthesia

Surgical anaesthesia with good muscle relaxation was achieved for at least 60 minutes with a mixture of one part midazolam, one part hypnorm and 2 parts sterile water administered at 2.7mls/kg via the intraperitoneal route. During anaesthesia the environmental temperature was maintained with a heating lamp.

Intravesical instillation

The anaesthetized rat was placed on its back and catheterized per urethra with an 18 french gauge blunt drawing up needle (Becton-Dickinson). A 1ml syringe containing either 0.5mls of SCFA cocktail (Special Products Ltd) or 0.9% saline was attached to the end of the needle and the cocktail administered by slow injection. Sticky tape was used to secure the syringe to the work bench to prevent the needle and syringe falling out.

After 60 minutes the syringe and needle were removed and the animals were observed until they regained consciousness.

During the experiment urine microscopy was performed on alternate days on samples obtained from the rats by suprapubic stimulation. At the end of the experiment organs including bladder, liver and colon were harvested from the animals. Bladders were inflated with 10% formaldehyde in saline and a ligature applied around the urethra. All specimens were fixed in 10% formaldehyde in saline and processed for histological assessment. Sections were stained using H&E and histological assessment was performed by a single pathologist blinded to the experimental details. Liver was analysed histologically for any adverse reaction to SCFA absorbed into the circulation. Colon was used as a positive control for Bromodeoxyuridine experiments.

Bromodeoxyuridine labelling *in vivo*

5-bromo-2'-deoxyuridine (Brdu) is incorporated into DNA during DNA synthesis which occurs during the S-phase of the cell cycle. It can then be detected by immuno-histochemical techniques. Whilst SCFA is instilled into the bladder, should it induce a chemical injury to the urothelium the normal healing response would result in increased urothelial cell proliferation and thus cell replication and DNA synthesis. In order to detect any increase in cell proliferation as a response to SCFA induced injury 5-bromo-2'-deoxyuridine (Brdu) [Sigma] was administered by the intraperitoneal route 4 hours prior to harvesting specimens at a concentration of 25mg/kg in PBS.

Single instillation of short chain fatty acid cocktail

Method

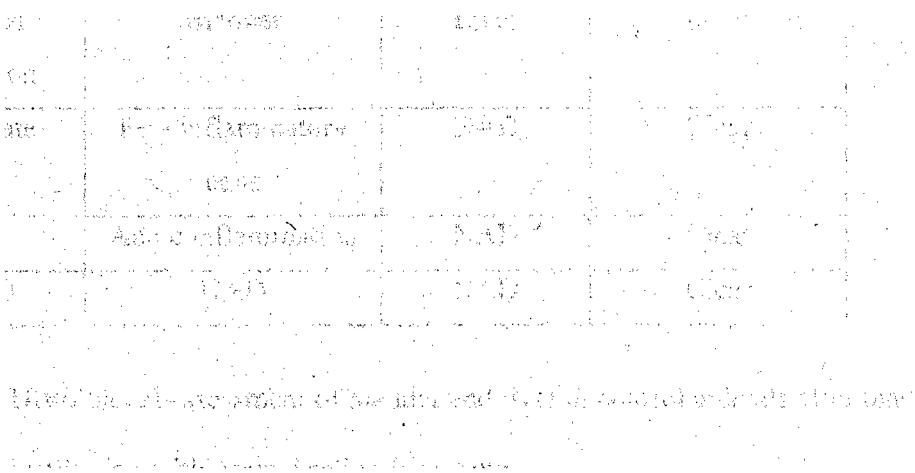
Eighteen female rats weighing between 150-250g were used in this experiment. Fifteen animals were given a 1 hour intravesical instillation of SCFA cocktail. One animal died under anaesthesia. Immediately and at 6 hours after instillation bladder, liver, and colon were retrieved from one animal each. At 1, 3, 5, 7, 10, and 14 days 2 further animals were killed at each time point and their organs harvested. Four hours prior to harvesting 5-bromo-2'-deoxyuridine (Brdu) [Sigma] was administered by the intraperitoneal route. Three control animals received an intravesical instillation of normal saline and were killed immediately, at day 3 and at day 10. Urine cytology was performed on alternate days after intravesical instillation.

Paraffin embedded sections were stained with Haematoxylin and Eosin and histological changes assessed by a single consultant pathologist who was blind to the treatment options. Further sections were stained for Bromodeoxyuridine (appendix 4)

Results

In the one animal that died under the anaesthesia a post mortem examination showed the catheter was correctly sited in a macroscopically normal bladder with no obvious intra-abdominal trauma. All other rats survived the duration of the experiment. All urine microscopy was clear. The results of histological examination are detailed in Table 7.1. Seventeen animals completed the experiment. In the 14 rats which received SCFA cocktail, all had normal livers and 11 had normal bladders on histological assessment. Two rats killed at 1 and 3 days post instillation respectively had acute inflammatory changes in the urothelium and one at 10 days had urothelial thickening and metaplasia. In the three controls one rat killed immediately and at 3 days post instillation had features of mild urothelial inflammation (Table 7.2).

Bromodeoxyuridine staining showed very low levels of cell turnover in both SCFA and normal saline exposed urothelium and no difference between the 2 groups.



Time post instillation	Bladder	Liver	urine
immediate	NAD	NAD	Clear
6 hours	NAD	NAD	Clear
Day 1	Mild acute cystitis	NAD	Clear
Day 1	NAD	NAD	Clear
Day 3	Mild acute cystitis	NAD	Clear
Day 3	NAD	NAD	Clear
Day 5	NAD	NAD	Clear
Day 5	NAD	NAD	Clear
Day 7	NAD	NAD	Clear
Day 7	NAD	NAD	Clear
Day 10	Urothelial metaplasia	NAD	Clear
Day 10	NAD	NAD	Clear
Day 14	NAD	NAD	Clear
Day 14	NAD	NAD	Clear

Table 7.1 Histological assessment of bladder and liver after bladder instillation with SCFA for 1 hour. NAD = No abnormality detected

Time post instillation	Bladder	Liver	urine
Immediate	Few inflammatory cells	NAD	Clear
Day 3	Acute inflammation	NAD	Clear
Day 10	NAD	NAD	Clear

Table 7.2 Histological assessment of bladder and liver in control animals after bladder instillation with normal saline for 1 hour.

Multiple SCFA instillations

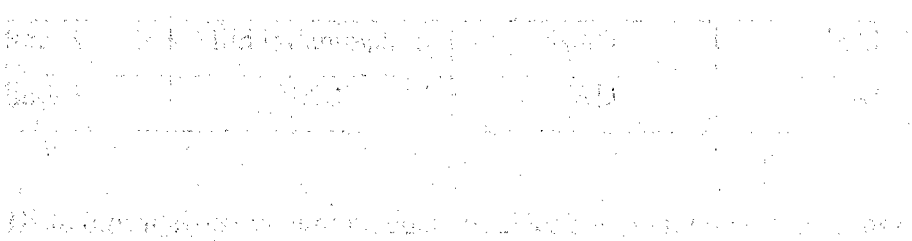
Method

Five female rats received intravesical instillations of SCFA under anaesthesia every 48 hours for a total of 5 instillations. Two further rats received 5 normal saline instillations as controls. Forty eight hours after the last instillations all rats were killed and bladder, liver and colon harvested 2 hours after intraperitoneal administration of Bromodeoxyuridine. Tissue was processed as previously described for histology and Brdu staining.

Results

All seven rats survived the experiment and all urine microscopy was clear prior to each instillation. From the five experimental animals all bladders were considered histologically normal. In the two controls, one bladder was normal and the other had very mild inflammatory changes in the urothelium. All livers were normal except in one of the experimental animals which had an isolated micro abscess in the liver surrounded by normal liver (fig.7.1d). Details of the results are in table 7.3.

Bromodeoxyuridine staining showed very low levels of cell turnover in both multiple SCFA and normal saline exposed urothelium with no difference between the two groups.



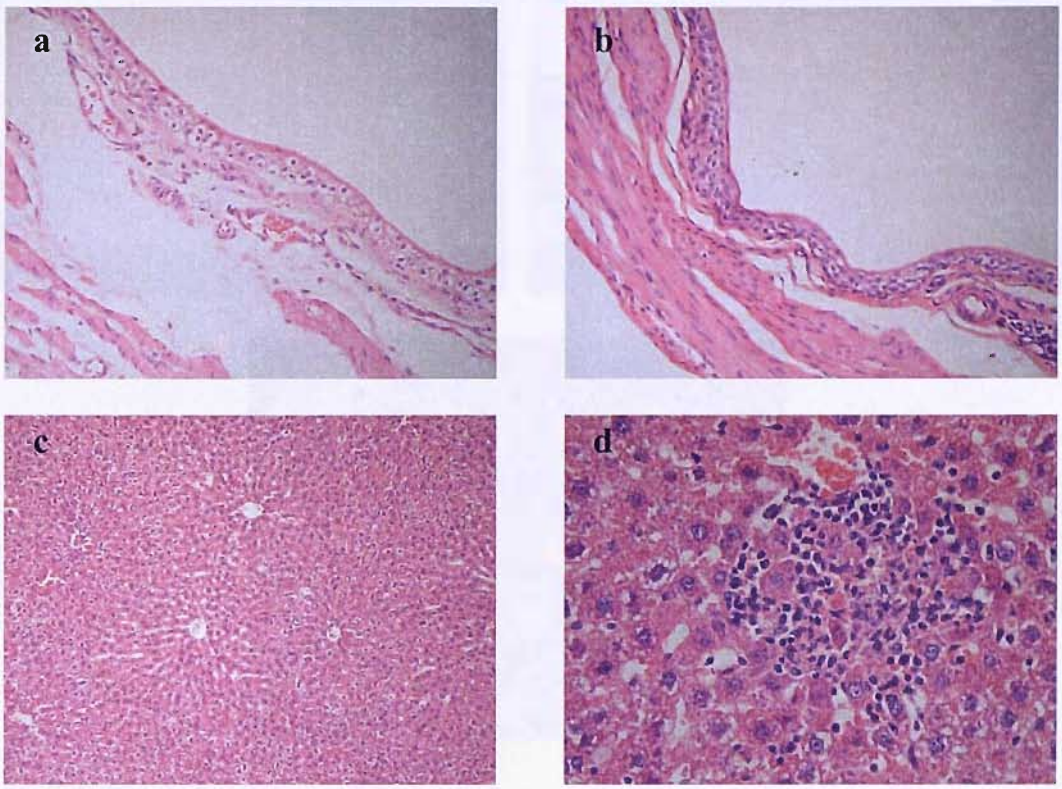


Figure 7.1 H+E histology: (a,b) Bladder after 5 SCFA instillations, objective x20. (c) Liver after 5 SCFA instillations, objective x10. (d) Liver demonstrating micro abscess, objective x40.

Intravesical instillation	Bladder	Liver	Urine microscopy
SCFAx5	NAD	NAD	NAD
SCFAx5	NAD	NAD	NAD
SCFAx5	NAD	1 micro abscess	NAD
SCFAx5	NAD	NAD	NAD
SCFAx5	NAD	NAD	NAD
N/Salinex5	Mild inflammation	NAD	NAD
N/Salinex5	NAD	NAD	NAD

Table 7.3 Histological assessment of bladder and liver after multiple instillations of SCFA cocktail or normal saline.

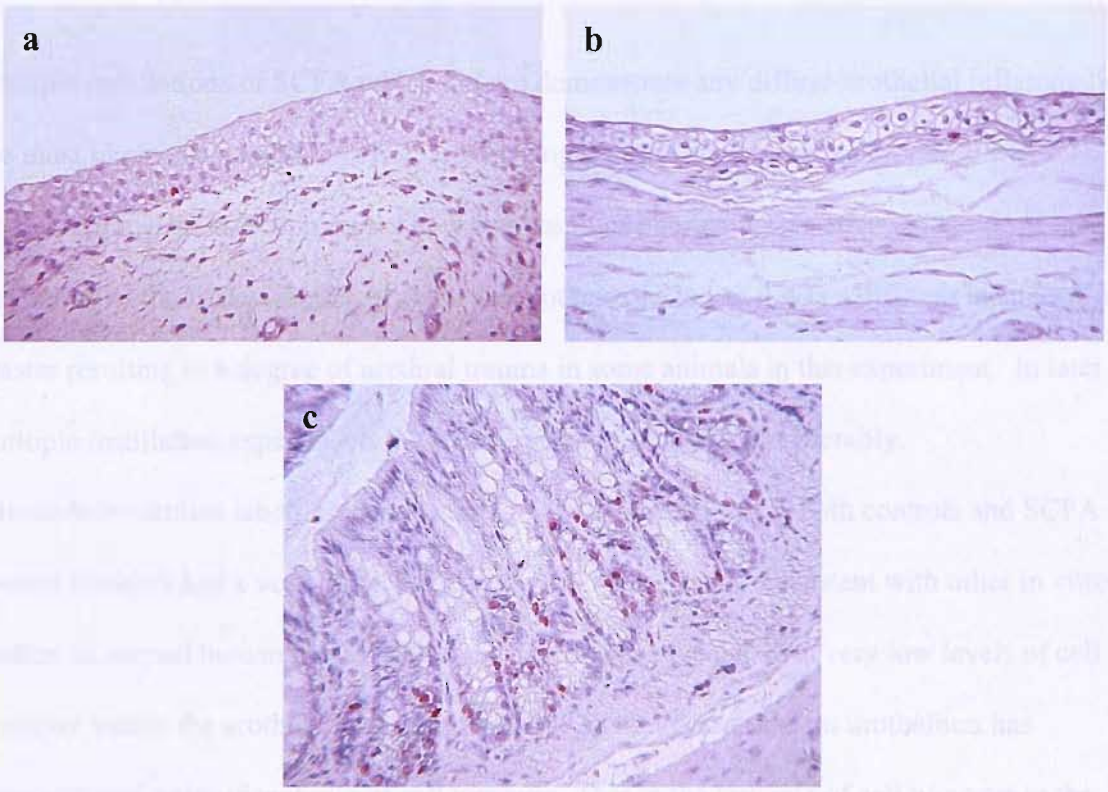


Figure 7.2 BRDU labelling (AEC substrate giving red nuclei, counterstain with Mayers haemalum; objective x20): (a,b) Bladder with BRDU labeling of a solitary cell in the basal layer. (c) Colon with BRDU staining demonstrating greater cell division than in urothelium.

Discussion

These results did not demonstrate any consistent adverse effect of intravesical instillation of SCFA cocktail. In the single instillation group there were features of acute inflammation in the urothelium at 24 and 72 hours after instillation in one of two rats at each time point. This may suggest an acute response in the urothelium as a result of contact with SCFAs. However the metaplastic appearance of the urothelium in one animal at 10 days post instillation was unusual and there is no obvious explanation other than an isolated abnormality pre-existing in that particular bladder. In the control group there was very mild inflammation in the bladder harvested immediately after normal saline instillation. When considering the results of

multiple instillations of SCFA which did not demonstrate any diffuse urothelial inflammation the most likely cause of the inflammatory changes in the single instillation group was probably transient infection most likely as a result of poor catheterization technique at the beginning of the first experiment. This was not unexpected as it was a difficult technique to master resulting in a degree of urethral trauma in some animals in this experiment. In later multiple instillation experiments the technique had improved considerably. Bromodeoxyuridine labelling demonstrated that the urothelium in both controls and SCFA treated bladders had a very low level of cell turn over. This is consistent with other in vitro studies of normal human urothelial biopsy tissue which have shown very low levels of cell turnover within the urothelium (Limas et al. 1993). Only neonatal rat urothelium has demonstrated a significant rate of cell turnover. Due to the low rate of cell turnover in the urothelium there were too few cells in S-phase to enable counting and subsequent calculation of any change in proliferation that may result from exposure to SCFAs. However this lack of activity in the urothelium was reassuring as any SCFA induced damage to the urothelium would have resulted in an increase in cell turnover as part of the repair process. This response was demonstrated in one bladder in the multiple instillation group, which had a focus of urothelial trauma at the dome most likely due to the blunt end of the rigid catheter. In this area there was a marked increase in the number of BRDU stained cells as a result of increased cell turnover in the regenerating urothelium. In conclusion SCFA instillations did not have any adverse effects on the urothelium, and therefore would appear safe as an intravesical agent despite the *in vitro* findings using cultured primary urothelial cells.

Chapter 8

Summary and conclusions of the study

The aim of this thesis was to assess the feasibility of using SCFAs as intravesical agents in augmentation colocystoplasty. There was a large body of work demonstrating the role of SCFAs in the gastrointestinal tract and their efficacy as a treatment for both diversion colitis and other forms of inflammatory bowel disease as well as a role in the prevention of colorectal cancer. Based on our hypothesis that diversion colitis is the underlying mechanism involved in changes seen in the colonic segment of augmentation cystoplasty we proposed that SCFAs may have a role as an intravesical agent in this group of patients. However very little was known about the effects SCFAs have on urothelium and therefore the focus of this study was to investigate the effect they may have both *in vitro* and *in vivo* on urothelium. Butyrate, propionate and acetate are the main SCFAs with therapeutic benefits and form the basis of a cocktail developed by Great Ormond Street Hospital, currently in use as a luminal treatment for diversion colitis.

Initial experiments using urothelial tumour cells and subsequently primary urothelial cell cultures demonstrated that *in vitro* cell proliferation was inhibited in a time and dose dependent manner, and that butyrate was the most potent inhibitor followed by propionate, and then acetate in both normal and malignant cells. A similar degree of inhibition was demonstrated in two colon cancer cell lines. Further experiments demonstrated that the nature of this inhibition was not an immediate cytotoxic effect and that when SCFA was removed from the culture medium, cell growth recovered with concentrations of butyrate less than 20mM. In continuous culture with butyrate adaptation to SCFA occurred with concentrations of butyrate less than 2.5mM.

With the proliferation studies completed the next step was to investigate the nature of the urothelial cell growth inhibition. Other groups had shown that SCFAs inhibit colon cancer cell growth by both G1/S and G2/M cell cycle arrest and therefore it seemed appropriate to focus the next experiments on cell cycle analysis using flow cytometry, as both the equipment and expertise was available within the department. There was a very limited supply of primary urothelial cells and therefore it was only possible to investigate the effects of butyrate and whole cocktail in this cell system. We found that primary urothelial cells incubated with butyrate alone and SCFA cocktail demonstrated G2/M cell cycle arrest and an increased rate of cell death. In urothelial tumour cell lines SCFAs induced G1/S and G2/M cell cycle arrest and increased rates of cell death.

Although it was likely that SCFAs were inducing apoptosis, fluorescence microscopy and acridine orange staining was employed to identify apoptotic cells. This provided qualitative data that apoptosis was present in both primary and malignant urothelial cells exposed to SCFA. This technique was extremely time consuming and therefore only limited work examining the effect of dose escalation on apoptosis was performed using SCFA cocktail in primary cells. This demonstrated apoptosis over a range of physiological and therapeutic concentrations that was independent of dose.

Within a bladder augmentation there is a unique interaction between intestinal epithelial cells and urothelium at the bowel to bladder anastomosis. There is concern and some evidence that this interaction may create instability and promote malignant transformation. It would have been interesting to investigate this interaction in a co-culture system, and preliminary experiments growing colon and urothelial tumour cells together demonstrated that they were able to form morphologically normal monolayers within the same medium, but altered morphology at the junction between the two cell lines on electron microscopy. However for a number of technical reasons including possible incompatibility problems between cells

from different individuals which may overshadow any organ specific interaction, and a lack of normal colonic cell lines or robust techniques for primary colonic cell cultures, this line of work was deferred. Having established that butyrate, propionate and acetate have similar anti proliferative effects on urothelial cells as reported in bowel cell lines, the next phase was to investigate their effects *in vivo*. In a rat model both a single intravesical instillation and multiple intravesical instillations of the Great Ormond Street cocktail containing butyrate (40mM), propionate (30mM) and acetate (60mM) were well tolerated and there were no adverse effects demonstrated on histology both to bladder or liver of treated animals. Cell division measured by bromodeoxyuridine labelling, which is normally extremely low in normal urothelium was unchanged in treated animals compared with controls indicating that SCFAs were having little effect on urothelium in vivo. As anticipated it is likely that although SCFAs adversely affect urothelial cell proliferation *in vitro* causing apoptosis, it is unlikely that *in vivo* (where cells are more resilient), there will be any adverse effect on urothelium.

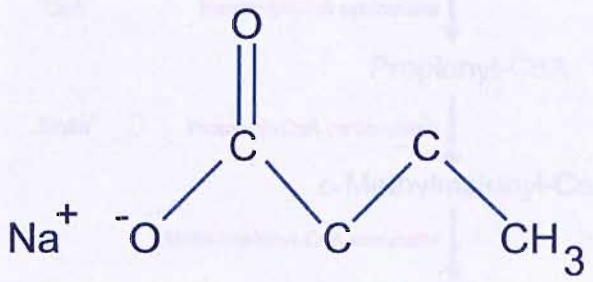
In conclusion, this study has demonstrated that SCFAs have similar anti proliferative and pro-apoptotic activity against primary and malignant urothelial cells *in vitro*, but they are likely to be a safe intravesical agent. This sets the scene for a randomized controlled trial in patients with enterocystoplasty. This would seem most appropriate in the paediatric population with colocystoplasty or colonic neobladders. Initial assessment for histological changes consistent with diversion colitis would be required prior to recruitment to a trial of intravesical SCFA therapy. Alternatively a rodent model of colocystoplasty to assess both diversion colitis and pre-malignant change initially, followed by investigation of the effect of intravesical SCFA would be an interesting project. In addition, SCFAs may in the same way that they promote differentiation and reverse the pre-malignant phenotype in intestinal epithelium, have similar anti neoplastic activity in pre-malignant urothelium. This area of research offers a number of

challenging projects for the future, including development of co-culture models of urothelial and colonic cell lines to investigate alterations in markers of differentiation and neoplasia. Also further work with markers of apoptosis using flow cytometry would compliment the work in this thesis.

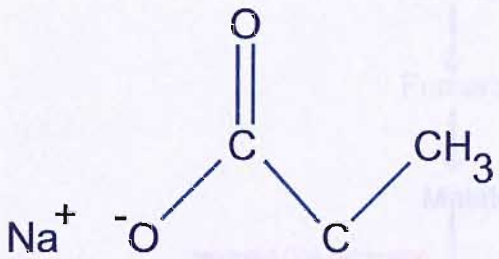
Appendix 1

Chemical structure of short chain fatty acids

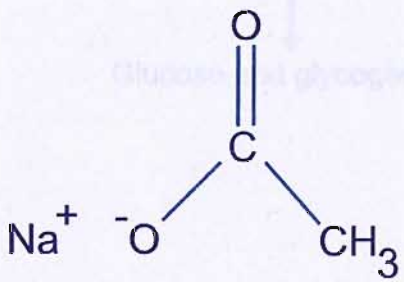
Sodium Butyrate



Sodium Propionate

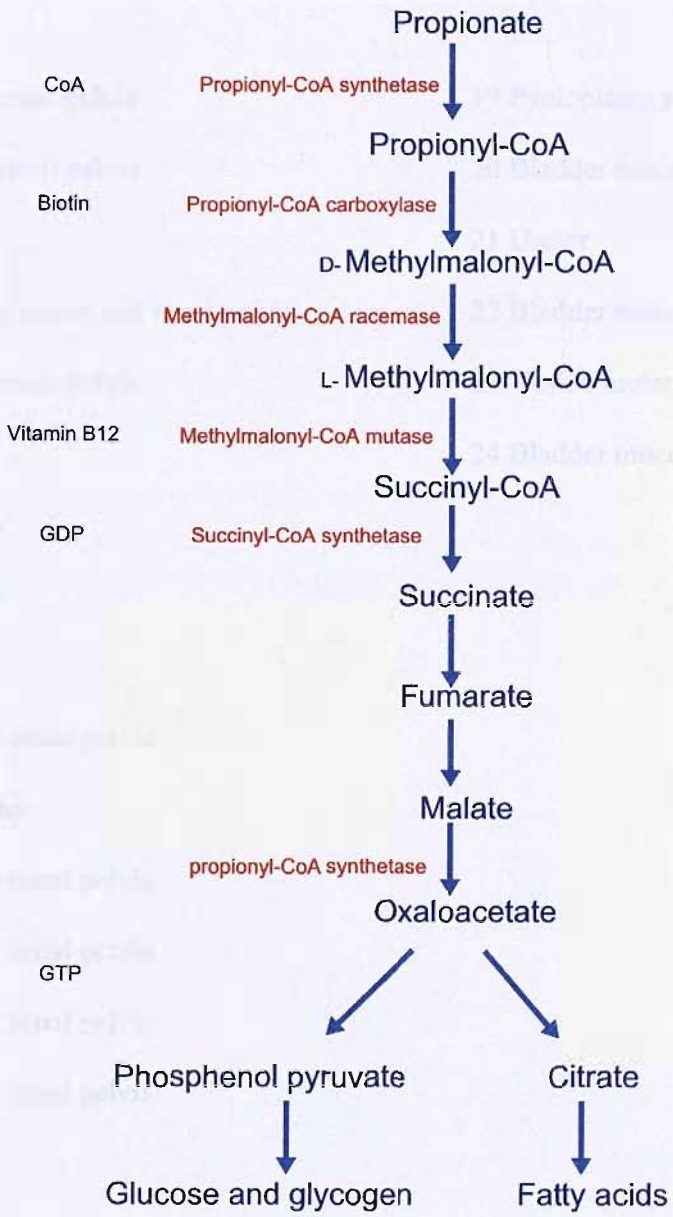


Sodium Acetate



Appendix 2

Propionate metabolism in the liver



Appendix 3

Origin of paediatric urothelial tissue specimens

24 paediatric specimens were collected with ethical approval and parental consent. Mean age 5.7yrs

Origin

1 pyeloplasty renal pelvis

2 Pyeloplasty renal pelvis

3 Ureter

4 Nephrectomy ureter and renal pelvis

5 Pyeloplasty renal pelvis

6 Ureter

7 Bladder neck

8 Ureterocoele

9 Megaureter

10 Pyeloplasty renal pelvis

11 Duplex ureter

12 Pyeloplasty renal pelvis

13 Pyeloplasty renal pelvis

14 Pyeloplasty renal pelvis

15 Pyeloplasty renal pelvis

16 Ureter

17 Nephrectomy ureter

18 Pyeloplasty renal pelvis

19 Pyeloplasty renal pelvis

20 Bladder mucosa

21 Ureter

22 Bladder mucosa

23 Vesico ureteric junction

24 Bladder mucosa

Patient Information Sheet

During your child's operation tissue may be removed routinely as part of the surgical procedure. This is always sent to the pathologist for more detailed examination under the microscope.

At Southampton General Hospital we are carrying out research into conditions affecting the bladder and the effect of nutrients called short chain fatty acid on the cells that line the bladder. Part of this research involves using small pieces of tissue provided at operation.

You have been asked by your doctor to help with this research, by allowing a small piece of tissue from the surgical specimen, removed during your child's operation to be used in this way.

The provision of the tissue sample will not affect the performance of the surgery in any way and no extra tissue will be removed. The care you and your child receive before, during and after surgery will also remain unchanged.

Your participation is voluntary and you are free to change your mind at any time.

CONSENT FORM

Consent for part of the surgical specimen to be used for research.

Patient details:

Please initial box

1. I confirm that I have read and understand the information sheet for the above study. ☐
2. I confirm that I have had the opportunity to ask questions, and have received satisfactory answers to all my questions. ☐
3. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason and withdrawal will not affect the future medical care of my child. ☐
4. I agree to take part in the above research. ☐

_____ _____

Signed Date

_____

Name of Parent or Guardian

_____ _____

Signed (researcher) Date

Appendix 4

Principles of immunohistochemistry

The demonstration of antigen in tissues and cells by immunohistochemistry is a two step process involving first, the binding of an antibody to the antigen of interest, and then, the detection and visualization of bound antibody by one of a variety of direct or indirect enzyme chromogenic systems.

With the direct method, the enzyme is conjugated directly to the primary antibody whereas, in the indirect method, the enzyme is conjugated or bound to a secondary reagent (link antibody), providing additional steps of amplification of the antigen binding event, thus offering improved sensitivity over the direct methods (Illustration 3.1).

The most common indirect methods are the Peroxidase-Anti-Peroxidase (PAP), the Avidin-Biotin Complex (ABC) and Biotin-Streptavidin Amplified (B-SA) systems.

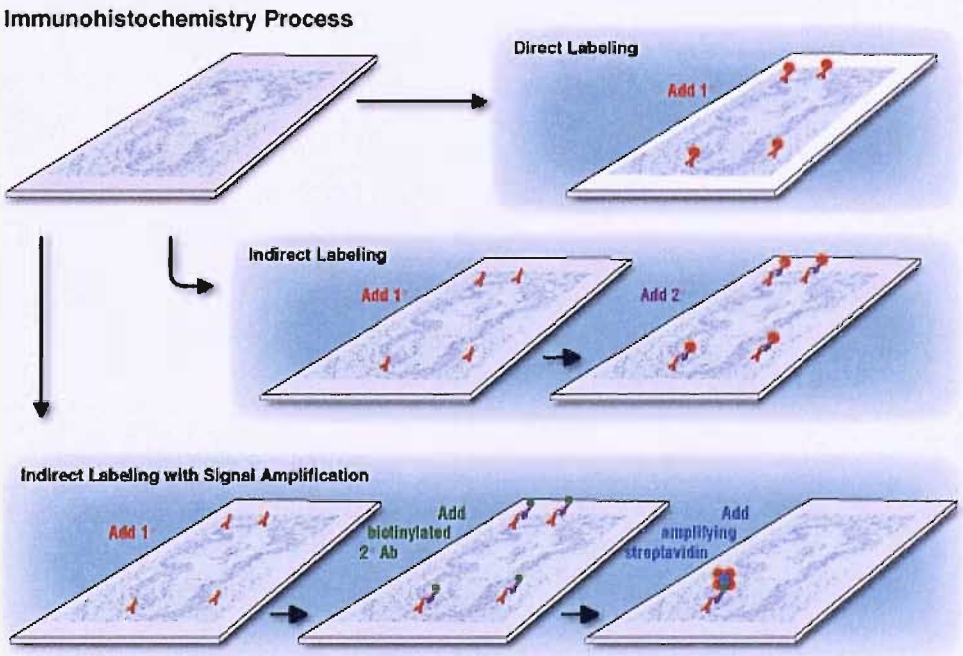


Illustration 3.1 Schematic of direct and indirect immunohistochemistry.

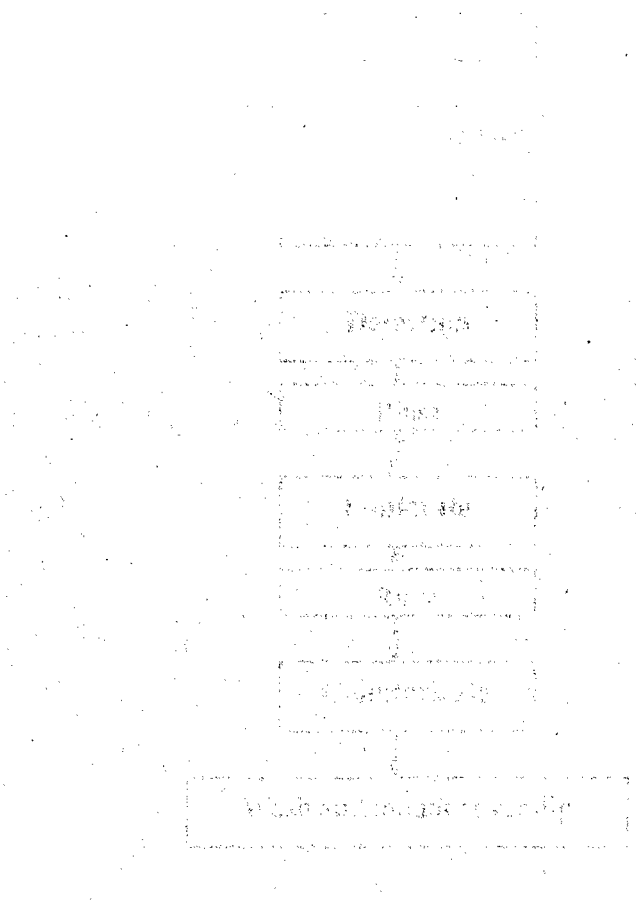
The key steps in the procedure can be summarized as follows:

1) Primary antibody binds to specific antigen;

2) Antibody-antigen complex is bound by a secondary, enzyme-conjugated, antibody;

3) In the presence of substrate and chromogen the enzyme forms a coloured deposit at the sites of antibody-antigen binding which can be viewed with a microscope.

All the steps involved are illustrated in the following flow chart, and details applicable to each kit used can be found in appendix 5.



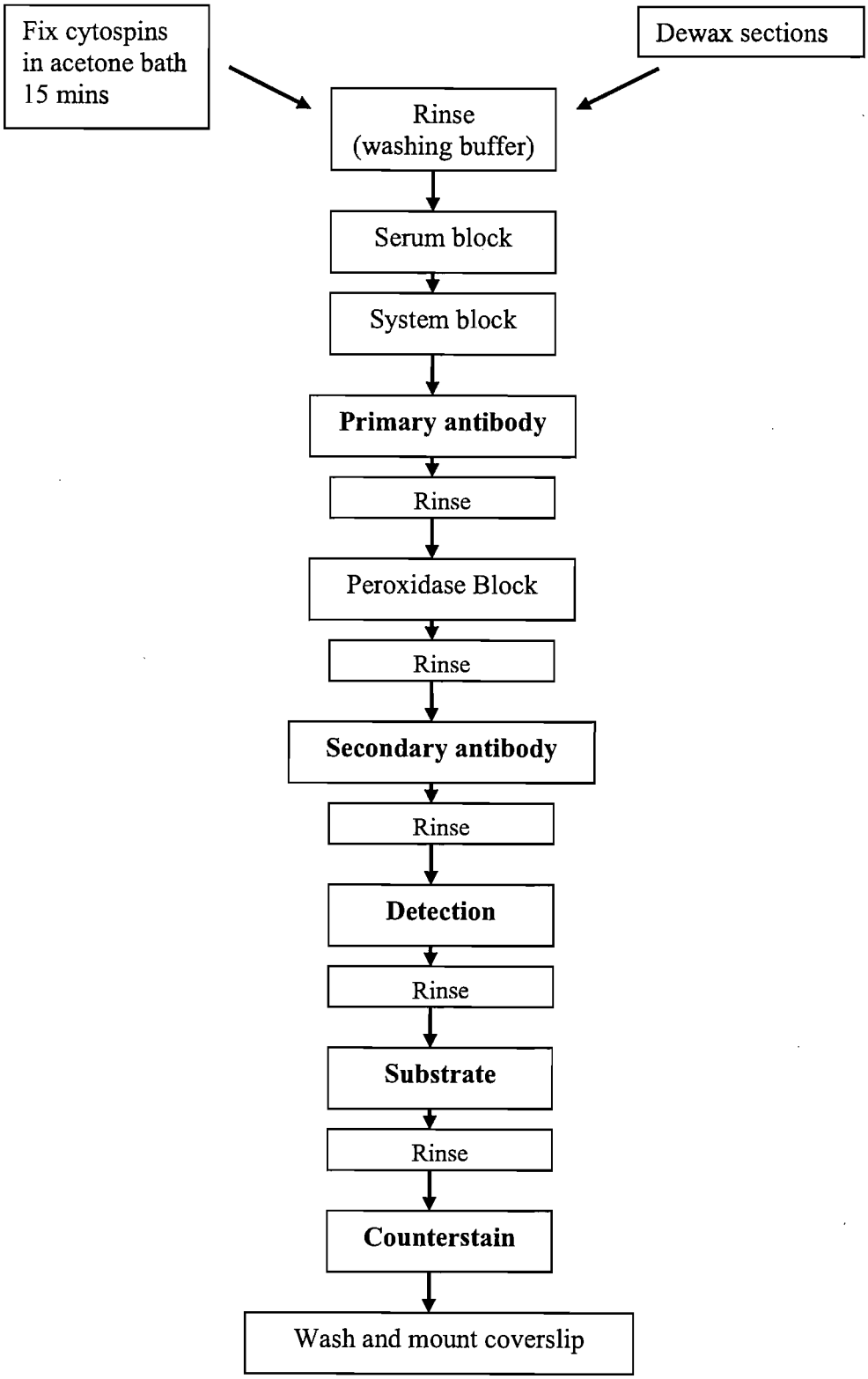


Illustration 3.2 Flow chart of histochemical staining techniques

Appendix 5

Immunohistochemistry techniques

Dewax of paraffin sections

Sections cut from paraffin embedded tissue samples were mounted on glass slides. Slides were dewaxed and rehydrated using a standard technique. Wax was melted by gently warming the slide on a hot plate then slides were transferred through a series of baths as follows:

1. Xylene 10minutes
2. Xylene 10minutes
3. 100% ethanol 10 minutes
4. 100% ethanol 10 minutes
5. 90% ethanol 10 minutes
6. Distilled water

The rehydrated slides were then stained using standard immunohistochical technique

Fixation of cytospins and chamber slides

Cells were fixed before immunohistochemical staining as follows:

1. 3 washes in PBS for 3-5 minutes each to remove free Brdu labeling reagent.
2. Fixation in 70% ethanol in glycine buffer or acetone at –20 °C for 20 minutes
3. 3 washes in PBS for 3-5 minutes each

Immunohistochemical staining procedure

Both DAB and AEC Horseradish peroxidase kits (BioGenex) were used which utilized the Biotin-Streptavidin (B-SA) antibody detection system

All steps were performed in a humidified atmosphere and between each step the edges of the slide was dabbed dry with tissue. The tissue sections or cells were not allowed to dry out between steps.

1. Peroxide block applied at room temperature for 10 minutes
2. Wash with 50 mM glycine buffer pH 2
3. Rinse in PBS bath for 5 minutes
4. Power block (Biogenex) applied at room temperature for 20 minutes
5. Power block tipped off
6. Primary antibody applied
    a. mouse anti-Brdu (Roche kit) diluted 1 in 10 with incubation buffer applied and incubated at 37 °C for 30 minutes
    b. CK20
    c. CK17
    d. Cam 5.2
    e. HB 95
    f. α SMA
    g. Mouse irrelevant
    h. Alkaline phosphatase
7. Rinse in PBS bath for 15 minutes
8. Link reagent (biotinylated multilink) applied at room temperature for 20 minutes

9. Rinse in PBS bath for 5 minutes
10. Label reagent (strepavidin peroxidase label) applied at room temperature for 20 minutes
11. Rinse in PBS bath for 5 minutes

For AEC substrate (alcohol soluble- red/brown stain)

12. AEC substrate (1 drop of AEC chromogen added to 2.5mls hydrogen peroxide substrate buffer) applied for <1 minute at room temperature
13. Rinse with PBS
14. Counter stain with Mayer's Haematoxylin for 10 minutes.
15. Apply aqueous mounting medium (warm gelatin) and cover slip.

For DAB substrate (insoluble permanent brown stain):

16. DAB (3,3'diaminobenzidine) substrate (2 drops of DAB Chromogen to 2.5ml substrate buffer then add 1 drop hydrogen peroxide substrate solution and mix.) applied for 5-40 minutes at room temperature until acceptable colour intensity is reached.
17. Rinse with PBS.
18. Counterstain with Mayer's Haematoxylin for 10 minutes.
19. Apply permanent mounting medium and cover slip.

Appendix 6

Bromodeoxyuridine immunohistochemical staining

Fixation and staining of paraffin embedded sections

Sections were cut from paraffin embedded bladder and colon specimens and mounted on glass slides. Slides were dewaxed and rehydrated using a standard technique. Wax was melted by gently warming the slide on a hot plate then slides were transferred through a series of baths as follows:

1. Xylene 10minutes
2. Xylene 10minutes
3. 100% ethanol 10 minutes
4. 100% ethanol 10 minutes
5. 90% ethanol 10 minutes
6. Distilled water

The rehydrated slides were then stained using an a biogenex kit. The following steps were performed in a humidified atmosphere. Between each step the edges of the slide was dabbed dry with tissue. The tissue sections were not allowed to dry between each step.

1. Peroxide block applied at room temperature for 10 minutes
2. Wash with 50 mM glycine buffer pH 2
3. Rinse in PBS bath for 5 minutes
4. Power block (Biogenex) applied at room temperature for 20 minutes
5. Power block tipped off

6. Primary antibody (mouse anti-Brdu)[Roche kit] diluted 1 in 10 with incubation buffer applied and incubated at 37 °C for 30 minutes
7. Rinse in PBS bath for 15 minutes
8. Link reagent (biotinylated multilink) applied at room temperature for 20 minutes
9. Rinse in PBS bath for 5 minutes
10. Label reagent (strepavidin peroxidase label) applied at room temperature for 20 minutes
11. Rinse in PBS bath for 5 minutes
12. AEC substrate (1 drop of AEC chromogen added to 2.5mls hydrogen peroxide substrate buffer) applied for <1 minute at room temperature
13. Rinse with PBS
14. Counter stain with Mayer's Haematoxylin for 10 minutes
15. Warmed gelatin mounting medium and cover slip applied

Appendix 7

The cell cycle

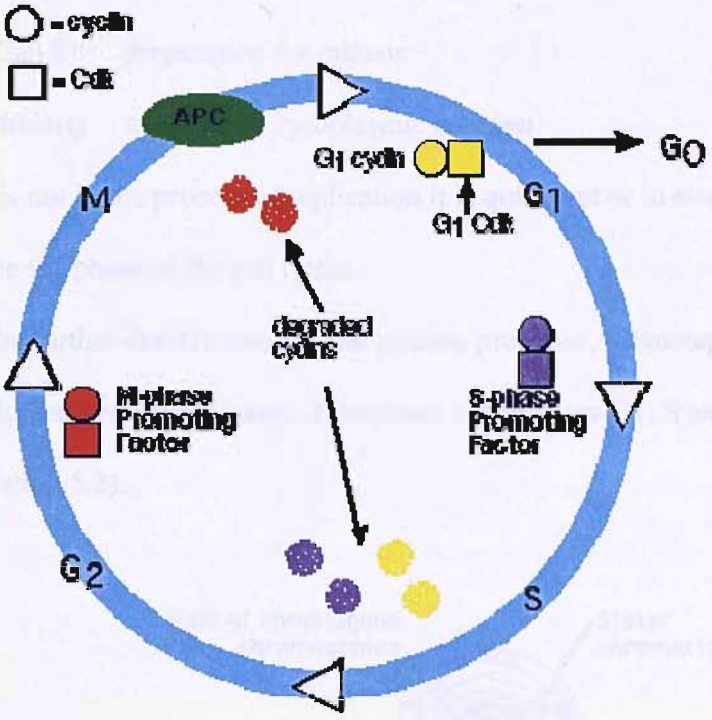


Illustration 5.1 Schematic of the cell cycle phases and controls

Eukaryotic cells replicate by process of copying their DNA followed by division of the genome. This process is termed the cell cycle and consists of an ordered set of events culminating in cell growth and division into two daughter cells. Illustration 5.1 demonstrates the 4 phases of the cell cycle and key areas of control.

Phases of the cell cycle

G1 (Gap 1): growth phase and preparation of the chromosome for replication.

S (Synthesis): synthesis of centrosome and DNA replication

G2 (Gap 2): preparation for mitosis

M (Mitosis): nuclear and cytoplasmic division

When a cell is not in the process of replication it is quiescent or in stage G0 which the cells enter from the G1 phase of the cell cycle.

Mitosis can be further divided into several phases, prophase, prometaphase, metaphase, anaphase, telophase and cytokinesis. Interphase encompasses G1, S and G2 phases of the cell cycle (illustration 5.2).

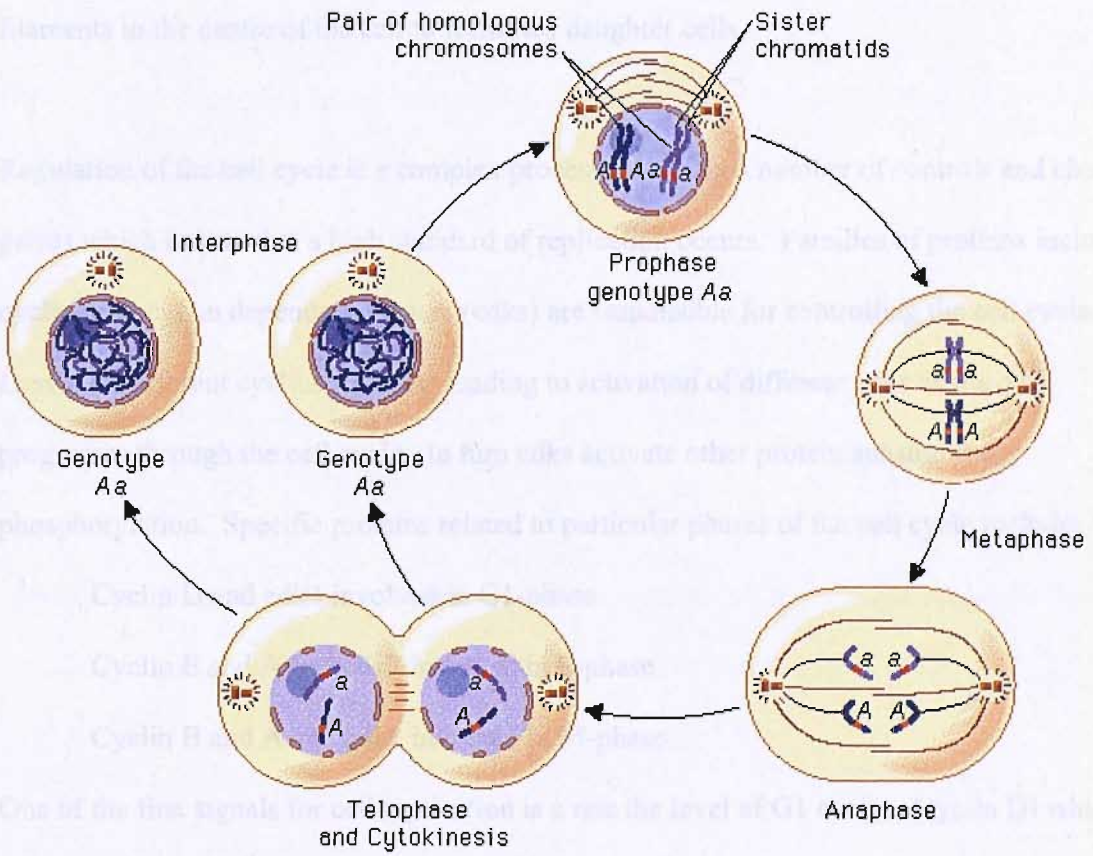


Illustration 5.2 Schematic of the phases of mitosis

Prophase: Chromatin condenses, becoming visible under the light microscope. The nucleolus disappears, centrioles move to opposite ends of the cell and mitotic spindles form.

Prometaphase: Nuclear membrane dissolves, proteins attach to the centromeres forming the kinetochore and the chromosomes begin to align.

Metaphase: Chromosomes are aligned in the middle of the cell, at the metaphase plate by the spindle apparatus

Anaphase: Paired chromosomes separate at the kinetochores and move to opposite sides of the cell along spindle microtubules.

Telophase: Chromatids collect at opposite poles of the cell, where they are enveloped by membrane forming daughter nuclei. The chromosomes and spindle apparatus disperse and partitioning of the cytoplasm (cytokinesis) occurs by a process involving contraction of actin filaments in the centre of the cell to form two daughter cells.

Regulation of the cell cycle is a complex process involving a number of controls and check points which ensure that a high standard of replication occurs. Families of proteins including cyclins and cyclin dependent kinases (cdks) are responsible for controlling the cell cycle. Levels of different cyclins fluctuate leading to activation of different cdks as the cell progresses through the cell cycle. In turn cdks activate other protein substrates by phosphorylation. Specific proteins related to particular phases of the cell cycle include:

Cyclin D and cdk4 involved in G1-phase

Cyclin E and A and cdk2 involved in S-phase

Cyclin B and A and cdk1 involved in M-phase

One of the first signals for cell replication is a rise the level of G1 cyclins (cyclin D) which binds cdks causing the cell to prepare for replication. Rising levels of cyclin A and E as well as other S phase promoting factors initiates DNA replication in S-phase.

As S phase cyclins are destroyed and M phase promoting factors (cyclins and cdks) rise, the mitotic spindle forms, nuclear envelope disintegrates and the chromosomes condense. Other proteins are activated preventing further replication of DNA. The anaphase promoting complex (APC) is activated, and the bonds between duplicated chromatids are broken allowing separation along the spindle apparatus completing mitosis. The APC activates G1 cyclins and degrades S phase inhibitory proteins in preparation for the next round of replication.

Check points exist in the cell cycle to allow the process to be aborted should something go wrong. If the damage is irreparable then apoptosis is triggered. Check points include:

1. At completion of S phase, the cell cannot proceed unless all Okazaki fragments have disappeared.
2. Check points which detect DNA damage occur at the G1 check point, before the cell enters S-phase, during S-phase and at the G2 check point, after DNA replication.
3. Check point, which detect abnormalities in the spindle apparatus (spindle checkpoints) occur at the M check point including correct attachment to the kinetochores and spindle alignment.

The most studied check point protein is p53, which can arrest cell cycle progression until damaged DNA is repaired or when damage is too severe trigger apoptosis. Another protein p27 binds cyclin and cdk preventing entry into S-phase.

Appendix 8

Flow cytometry principles and methods

Introduction

Flow cytometry is a technique based on the measurement of light scattered by single cells within a fluid stream, ideal for quantifying the characteristics of each cell within a population. A single cell suspension is passed in a narrow stream of fluid across a beam of light. The scatter of light from each cell is recorded and measurements from thousands of cells can be acquired within seconds. Basic information on cell size and granularity are recorded, but the attachment of fluorescent labels allows study of nuclear, cytoplasmic and membrane bound elements of the cell.

Principles

Fluidics

The flow cytometer contains a flow chamber designed to produce a hydrodynamically focused core of cells within a column of sheath fluid. Utilising the principles of laminar flow with viscous drag, hydrodynamic focusing and turbulent boundary drag each cell is delivered to a point of intersection with the beam of light. Sheath fluid is delivered from a reservoir and the cell suspension is injected directly into the center of the flow chamber.

Laminar flow is the phenomenon of non turbulent motion in which parallel layers within the fluid stream have different velocities. Within the flow chamber fluid velocity is adjusted to ensure cells flow in a central core of fluid. The effect of viscous drag from the walls of the chamber slows the outer fluid layers creating a velocity differential across the column, with the central core of fluid containing cells traveling fastest.

Hydrodynamic focusing occurs as a consequence of the velocity gradient of laminar flow. Cells are drawn into the center of the fluid column where they remain as long as laminar flow is maintained. A focused core of cells is formed at a distance from the chamber inlet of 50 times the chamber diameter, known as the inlet length.

Turbulent boundary flow is caused by back pressure created by tapering the flow chamber to a narrow exit and combined with viscous drag from the boundary of the chamber creates a velocity parabola in a shorter distance than required for laminar flow. The end result is that cells are focused at the centre of a narrow fluid column.

Laser light source

The Becton-Dickinson FACSCalibur utilizes an argon-ion laser (light amplification by stimulated emission of radiation) to create an intense, coherent, plane polarized beam of light at a specific wavelength. The laser consists of a resonator containing a gas filled plasma tube and mirrors at either end. Electrons of the gas atoms are stimulated by current applied to the plasma tube and as they decay from the stimulated state they emit light at a specific wavelength. These photons of light stimulate further gas atoms to emit photons as they are reflected back and fourth within the plasma tube. Some of the resulting amplified light is allowed through a window at the end of the tube which is set at a specific angle (Brewster's angle) which polarizes the emitted light. The wavelength of emitted laser light can be altered using different mirror coatings or prisms placed in front of the reflecting mirrors.

Optical collection system

Light in the flow cytometer is either scattered by the cell in its path or absorbed and re-emitted as fluorescence. This light is collected in two ways either by a forward collection lens which gathers light deflected 1-20 degrees of the axis of the laser beam or by side

collection lens which gathers light deflected at right angles to the laser beam. Side scattered light is further processed by a sequence of lenses and filters designed to split the light into different wavelengths to measure different types of fluorescence (illustration 5.3).

Detection and processing systems

The Becton-Dickinson FACSCalibur uses a photomultiplier tube to convert light impulses into electrical signals which are converted into a smooth voltage pulse of between 0-10 V by a pre-amplifier. The amplitude of this pulse is proportional to the number of photons reaching the photodetector, and the shape of the pulse is determined by the size and speed of the particle and the width of the illuminating beam and distribution of the fluorochrome within the particle. Background noise from the pre-amplifier output is filtered out prior to further processing of the signal. A preset input voltage is set as a threshold for further processing to take place, usually on a single parameter. Forward scatter is most often set as the system trigger pulse, as use of fluorescence as a trigger may result in negative cells going undetected.

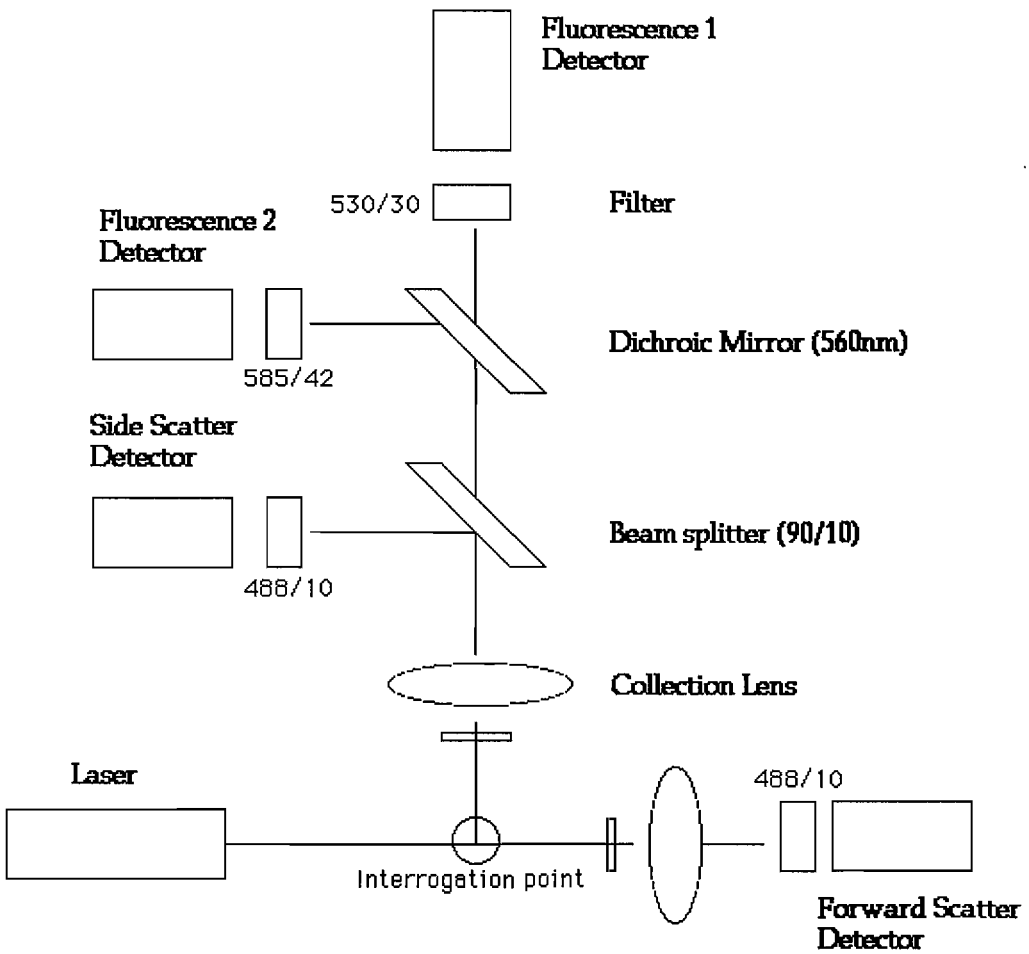


Illustration 5.3 Simplified layout of a typical analytical flow cytometer

Flow cytometry methods

Flow cytometry is a technique capable of making individual measurements on each cell within a population and it was employed to study the mechanism of short chain fatty acid induced inhibition of primary and transformed urothelial cells in particular changes in the distribution of cells within the cell cycle. Measurement of fluorescence intensity, size and granularity of whole cells or nuclei is performed, and with DNA labeling the position of each

cell within the cell cycle can be identified by the difference in DNA content that exists across the cell cycle. Subsequently the changes in distribution of cells within the cell cycle can be calculated for a population and an agent's site of action identified.

The flow cytometer used in this study was the Becton-Dickinson FACSCalibur (fluorescence activated cell sorting device) linked to an Apple MacIntosh computer with Cell Quest Software and the property of Clinical Oncology Research Group, Southampton General Hospital (Illustration 5.4). The instrument has a single 15mW air cooled argon-ion laser emitting light at 488nm and photodetectors which collect forward scattered light (FSC), side scattered light (SSC), emitted green fluorescence (FL1, 530^{+}/- 15nm), orange fluorescence (FL2, 585 $^{+}$/- 21nm), and red fluorescence (FL3,>620nm). Each particle measured by the flow cytometer is called an event. For each event the FSC is related to its size and SSC is related to the internal granularity of the particle. The FL channels detect emitted fluorescence across their respective ranges. Propidium iodide fluorochrome label emits fluorescence at a wavelength of 600nm detected best in the orange/red range in FL2. FITC used to label bromodeoxyuridine incorporation emits fluorescence detected in the green range in FL1.

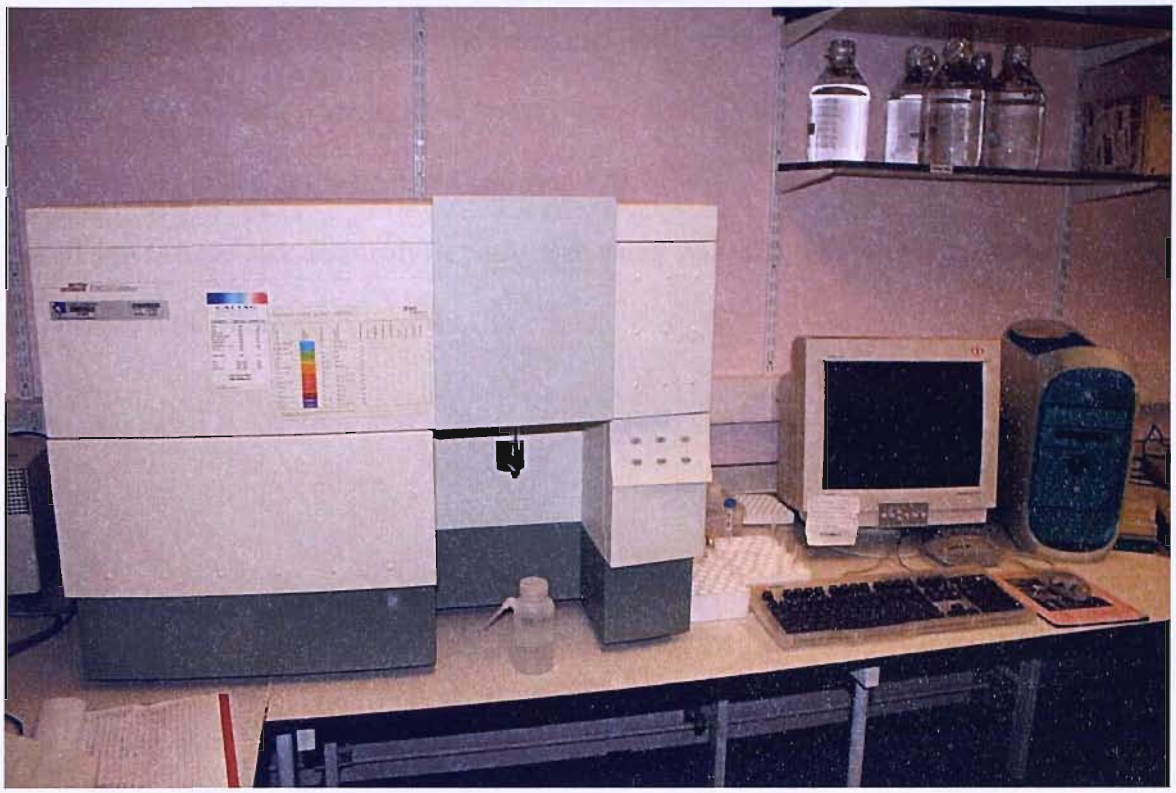


Illustration 5.4 Becton-Dickinson FACSCalibur linked to an Apple MacIntosh computer.

Propidium iodide labelling

Cells were labeled with propidium iodide prior to analysis by flow cytometry using the following protocol. A 12.5 cm^2 flask of adherent cells was incubated with SCFA. Cells were disaggregated as previously described, centrifuged at 1000rpm for 4 minutes, fixed in ethanol 70% and stored at 4 °C. For analysis cells were centrifuged at 1000rpm, supernatant was carefully removed with a Pasteur pipette and the cell pellet resuspended in phosphate buffered saline containing propidium iodide (20ug/ml), and RNAase (1ug/ml)(Sigma) for 30minutes at room temperature in standard facs tubes. They were then analysed on the flow cytometer at a rate of 100-400 cells/sec. Every 10 minutes and immediately prior to analysis each facs tube was gently agitated to prevent cell clumping at the base of the tube. For each

analysis at least 10,000 events were recorded and displayed as a histogram and dot plot. A printout of the analysis was produced and the data stored on the hard drive of an Apple MacIntosh PC linked to the Becton-Dickinson FACSCalibur machine. All data was transferred to a zip disk and analysis performed using WinMDI facs analysis software compatible with Microsoft Windows.

Appendix 9

Chapter 5 cell cycle analysis experiments using propionate and acetate.

Within this section are details of the methods and results of flow cytometry experiments investigating the effect of propionate and acetate on the cell cycle in the RT112 urothelial cell line. To avoid repetition within chapter 5 these experiments are included in this appendix, but they are discussed in detail with the rest of the work involving flow cytometry within the discussion section at the end of chapter 5.

The effect of propionate on RT112 urothelial cells

Dose response to propionate

Method

RT112 urothelial cells were incubated for 6, and 24 hours with 1.87mM, 7.5mM, 30mM, and 120mM propionate. Cells were then processed as described in chapter 2 for flow cytometry analysis.

Results

There was no significant alteration in the distribution of cells in the cell cycle after 6 hours incubation with propionate over a range of concentrations from 7.5mM to 30mM, however at the highest concentration there was a fall in the percentage of cells in G1 peak and a rise in the percentage of cells in the sub-G1 region, as illustrated in Figure 5.30 and 5.33.
Over the longer incubation of 24 hours there was a dose dependent reduction in the percentage of cells in the G1 population and a similar dose dependent increase in the Sub-G1

region (figures 5.34 to 5.39). With 30mM of propionate there was an increase in the early S-phase region, but with 120mM both the S-phase and G2/M population were reduced. At concentrations below 30mM there was no consistent change in the S-phase or G2/M populations after 24 hours incubation.

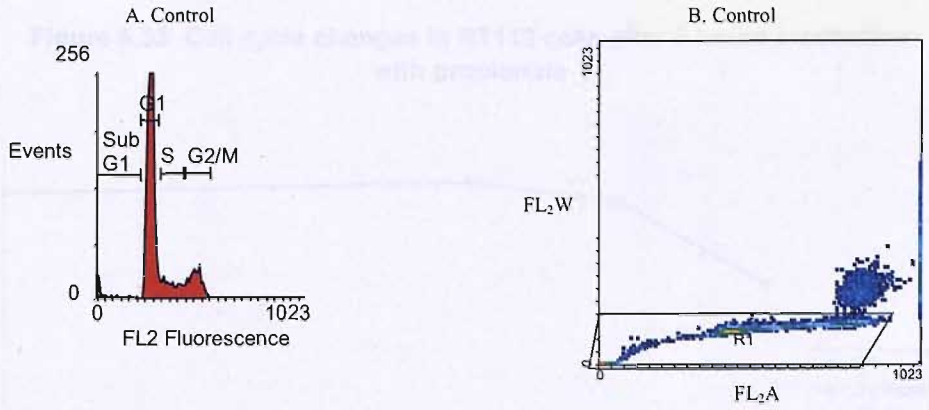


Figure 5.30 A. Propidium iodide fluorescence histogram of untreated RT112 urothelial cancer cells.
B. Doublet discrimination plot.

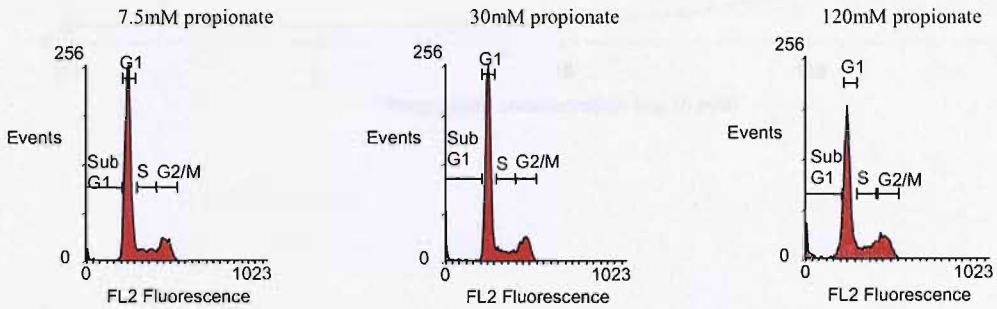


Figure 5.31 RT112 cells after 6 hours incubation with propionate

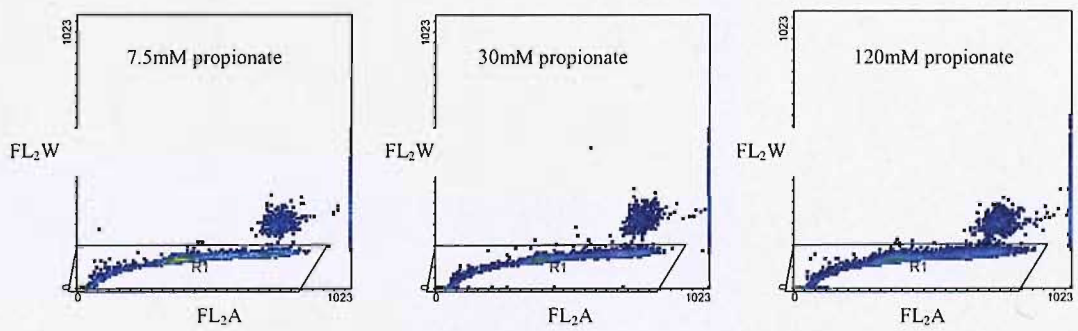
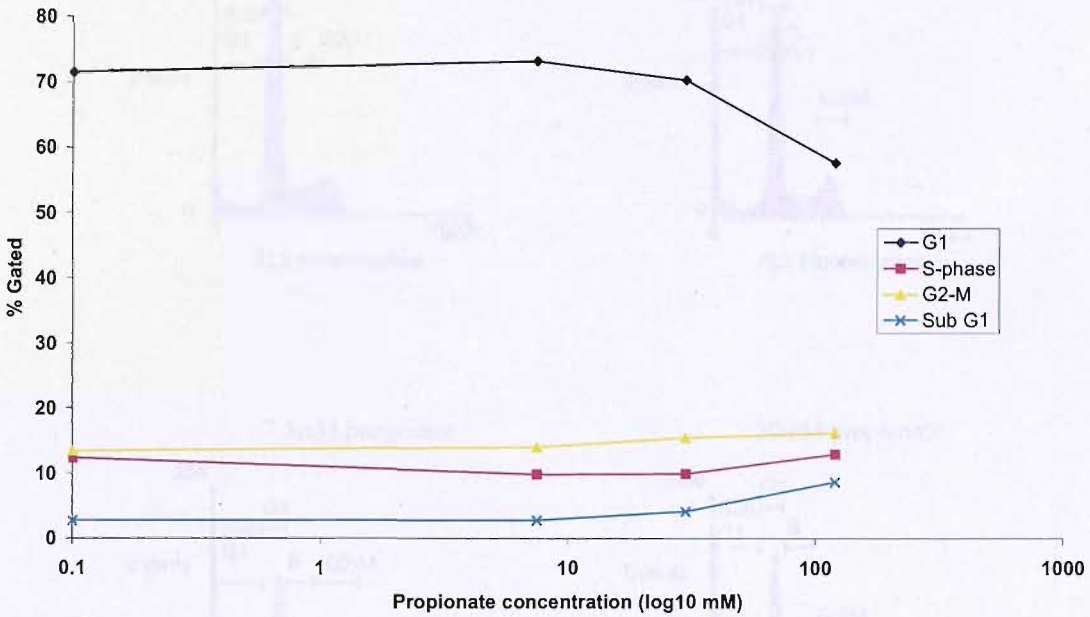


Figure 5.32 RT112 cells after 6 hours incubation with propionate (doublet discrimination)

Figure 5.33 Cell cycle changes in RT112 cells after 6 hours incubation with propionate



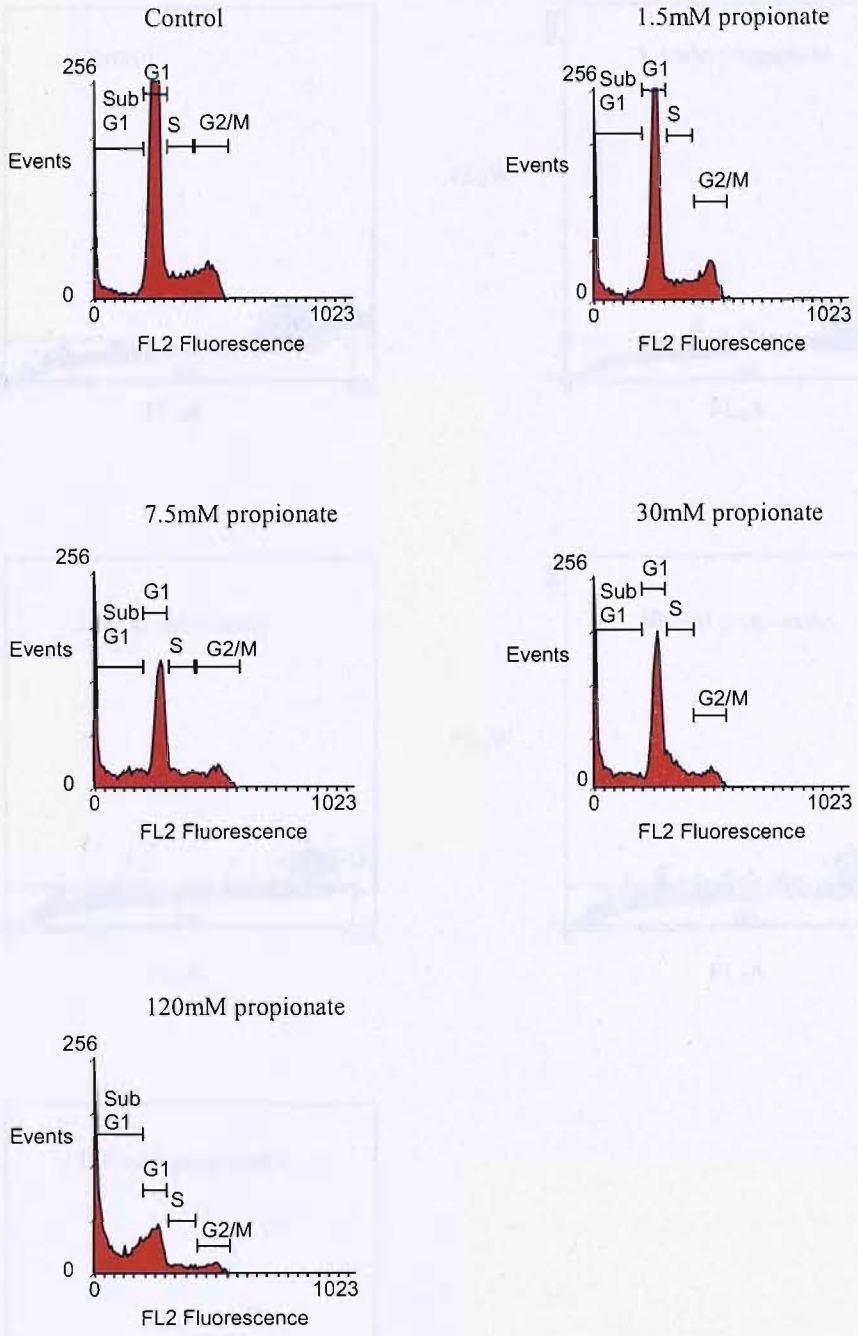


Figure 5.34 RT112 cells after 24 hours incubation with propionate.

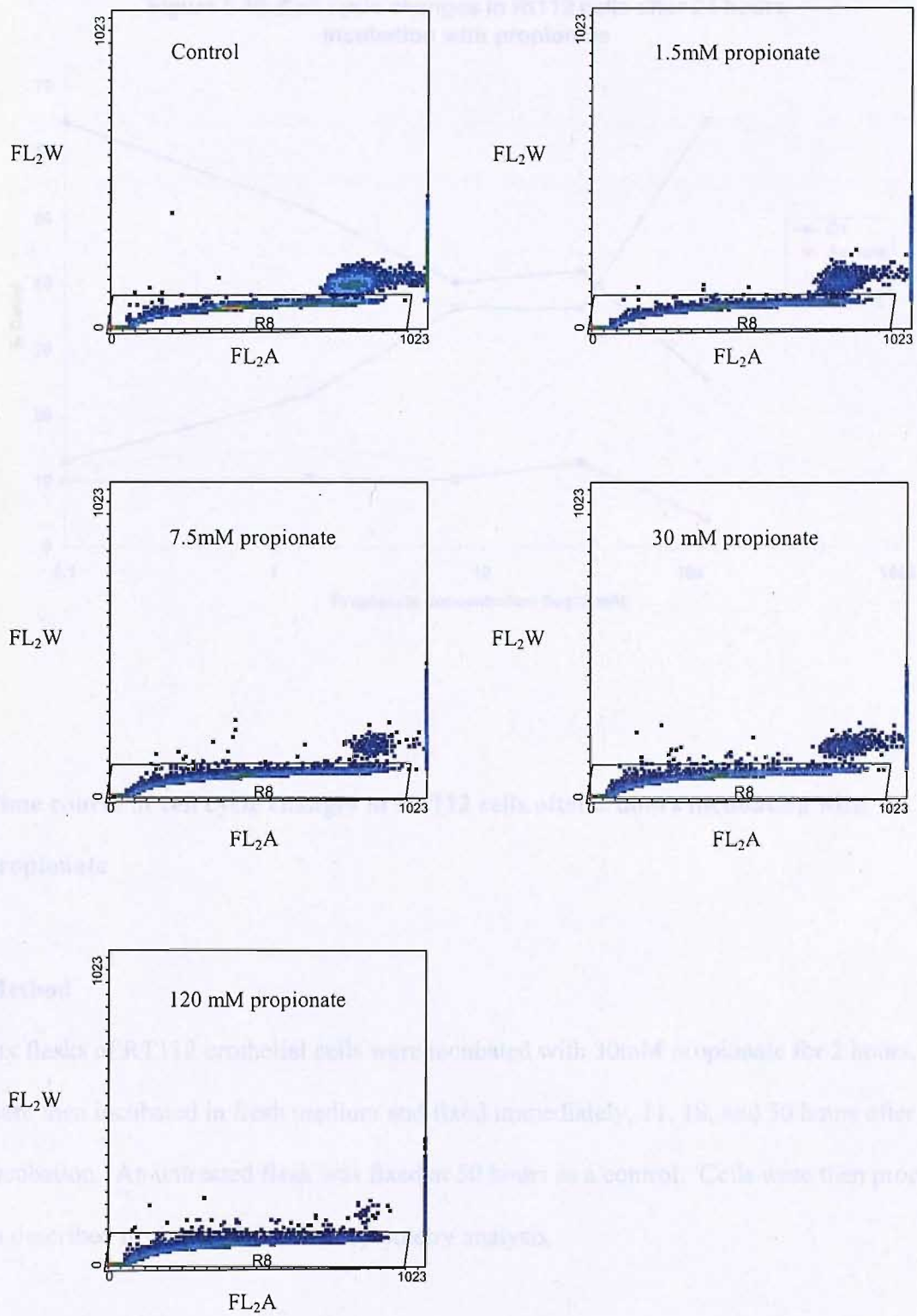
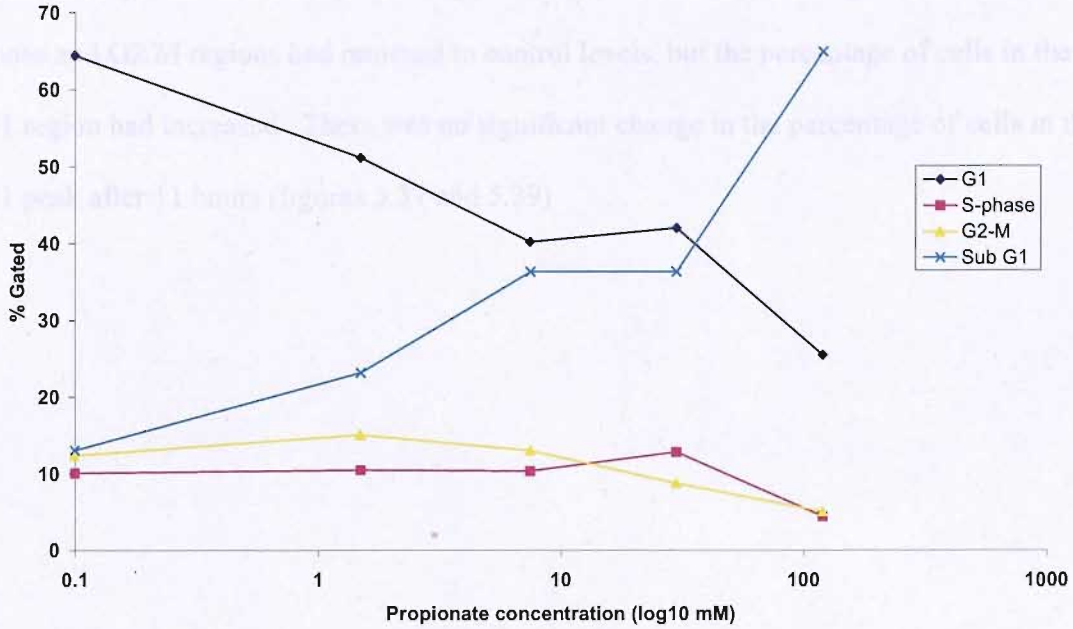


Figure 5.35 RT112 cells after 24 hours incubation with propionate (doublet discrimination)

Figure 5.36 Cell cycle changes in Rt112 cells after 24 hours incubation with propionate



Time course of cell cycle changes in RT112 cells after 2 hours incubation with propionate

Method

Six flasks of RT112 urothelial cells were incubated with 30mM propionate for 2 hours. Cells were then incubated in fresh medium and fixed immediately, 11, 18, and 50 hours after incubation. An untreated flask was fixed at 50 hours as a control. Cells were then processed as described in chapter 2 for flow cytometry analysis.

Results

Immediately after incubation there was no difference between treated and untreated DNA histograms. At 11 hours post incubation there was a fall in the G1 population and an increase

in the early S-phase region and G2/M peak. At 18 hours the S-phase peak had returned to control levels and the G2/M peak increased. At 50 hours the percentage of cells in both S-phase and G2/M regions had returned to control levels, but the percentage of cells in the sub-G1 region had increased. There was no significant change in the percentage of cells in the G1 peak after 11 hours (figures 5.37 and 5.39)

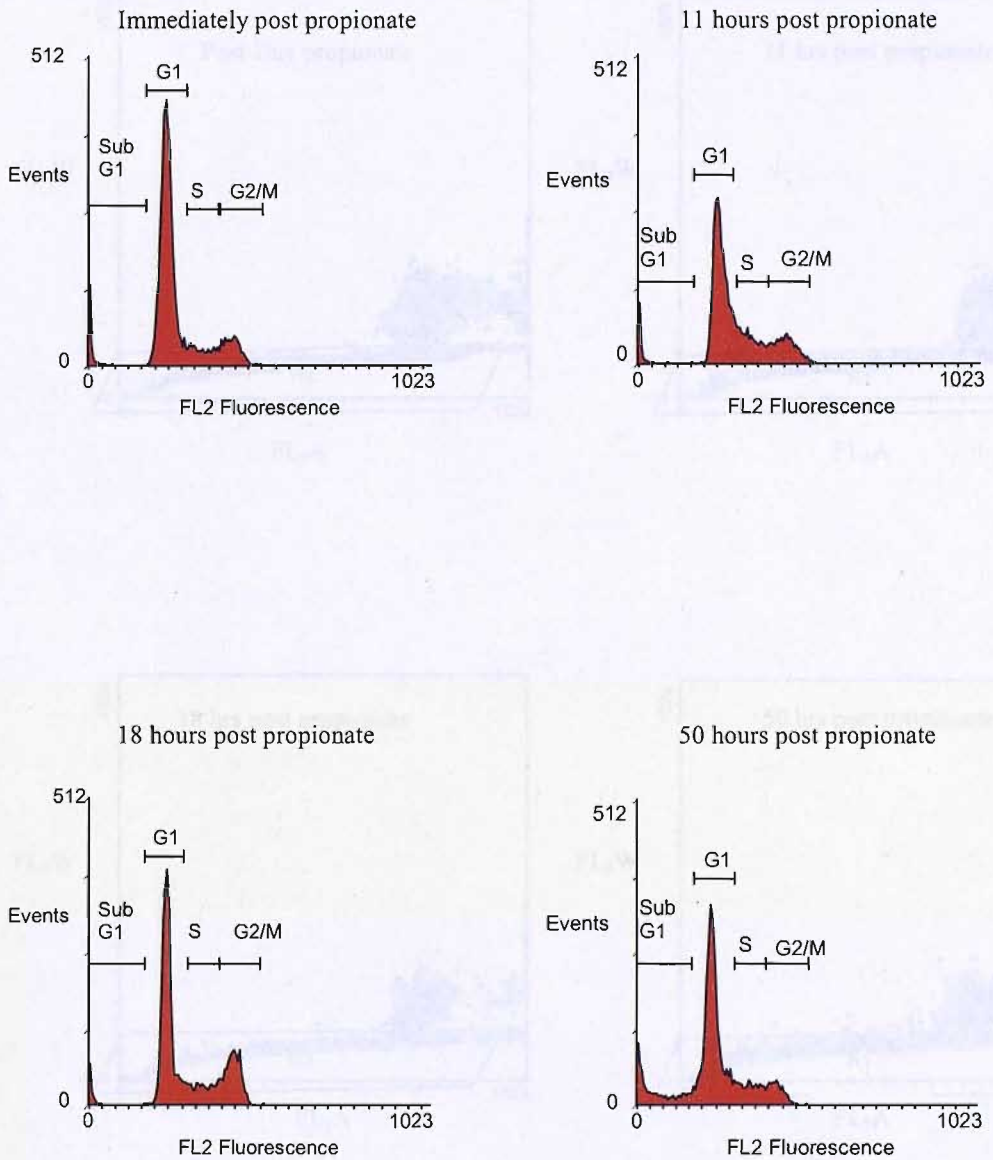


Figure 5.37 Time course of cell cycle changes in RT112 cells after 2 hours incubation with 30mM propionate.

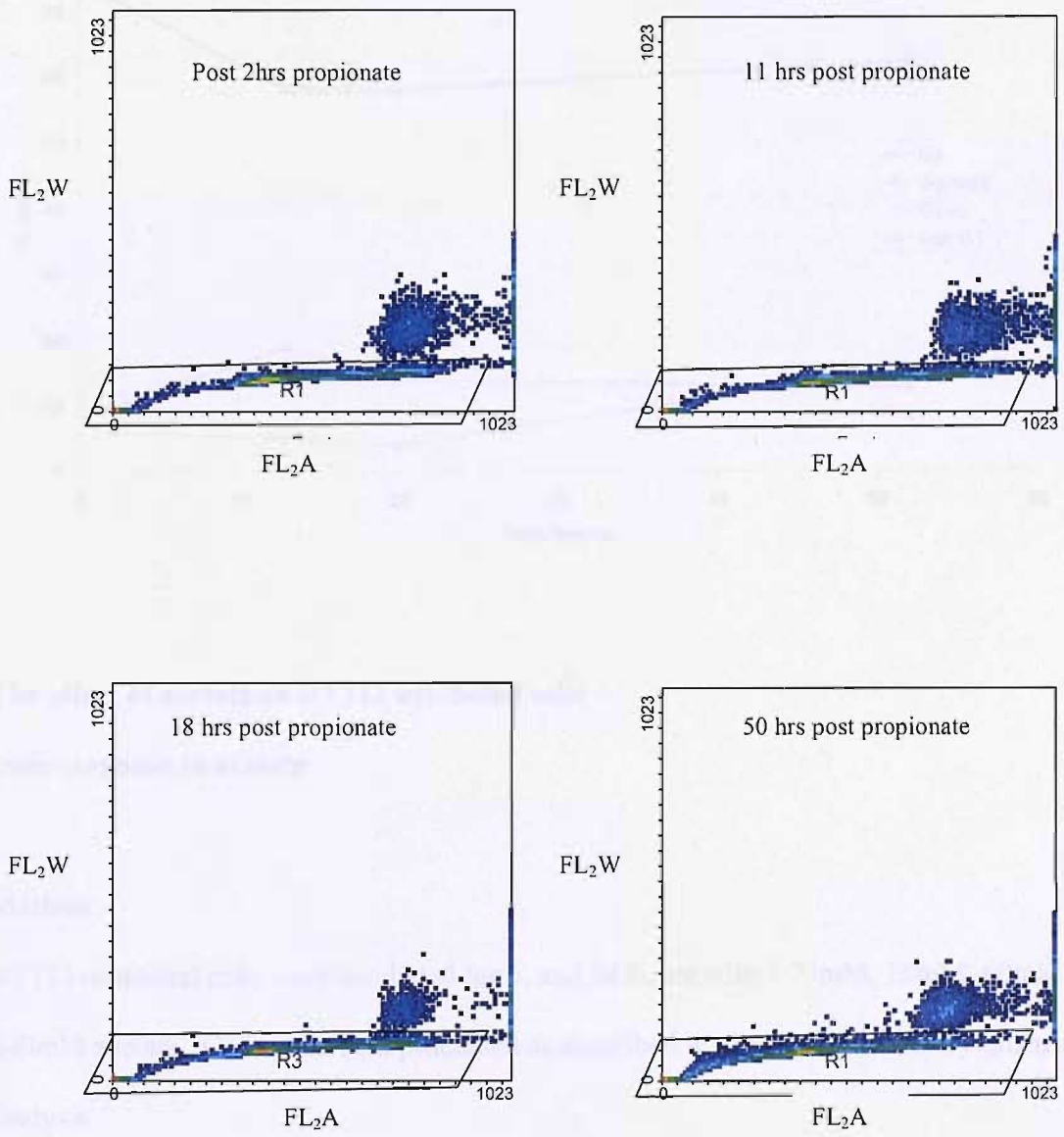
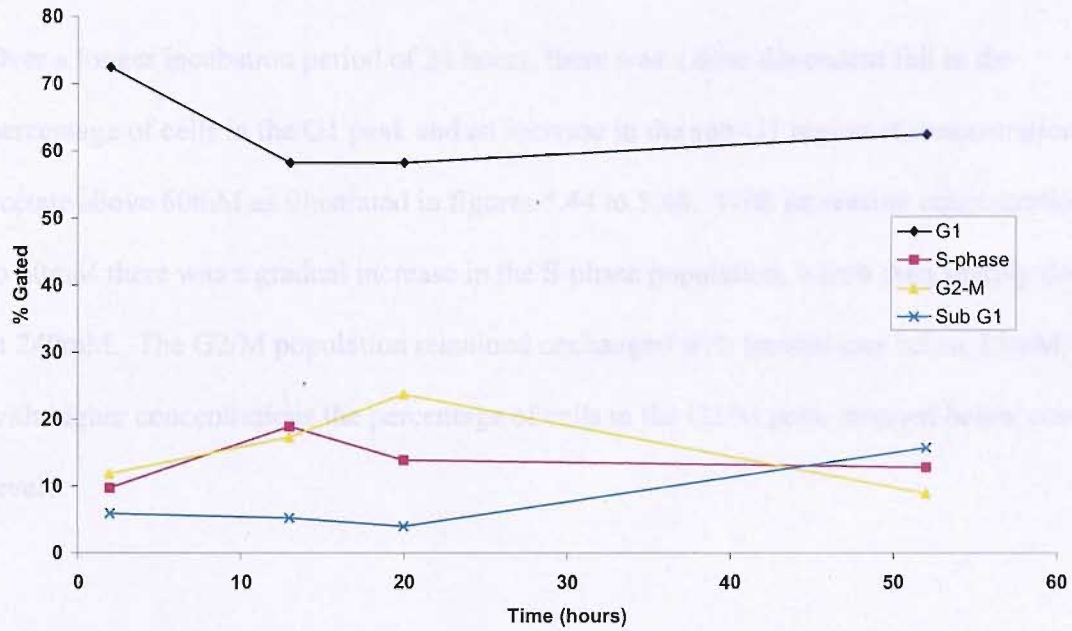


Figure 5.38 Time course of RT112 cells after 2 hours incubation with 30mM propionate (doublet discrimination)

Figure 5.39 Time course of cell cycle changes in RT112 cells after 2 hours incubation with 30mM propionate



The effect of acetate on RT112 urothelial cells

Dose response to acetate

Method

RT112 urothelial cells were incubated for 6, and 24 hours with 3.75mM, 15mM, 60mM, and 240mM acetate. Cells were then processed as described in chapter 2 for flow cytometry analysis.

Results

There was no significant alteration in the distribution of cells in the cell cycle after 6 hours incubation with acetate at a concentration of 15mM, however from 60mM to 240mM there was a dose dependent fall in the percentage of cells in the G1 peak and a rise in the percentage of cells in the sub-G1 region, as illustrated in Figure 5.40 and 5.43. There was no

significant change in the percentage of cells in the S-phase or G2/M populations with 15mM to 240mM concentrations of acetate.

Over a longer incubation period of 24 hours, there was a dose dependent fall in the percentage of cells in the G1 peak and an increase in the sub-G1 region at concentrations of acetate above 60mM as illustrated in figures 5.44 to 5.46. With increasing concentration up to 60mM there was a gradual increase in the S-phase population, which then sharply declined at 240mM. The G2/M population remained unchanged with incubations below 15mM, but with higher concentrations the percentage of cells in the G2/M peak dropped below control levels.

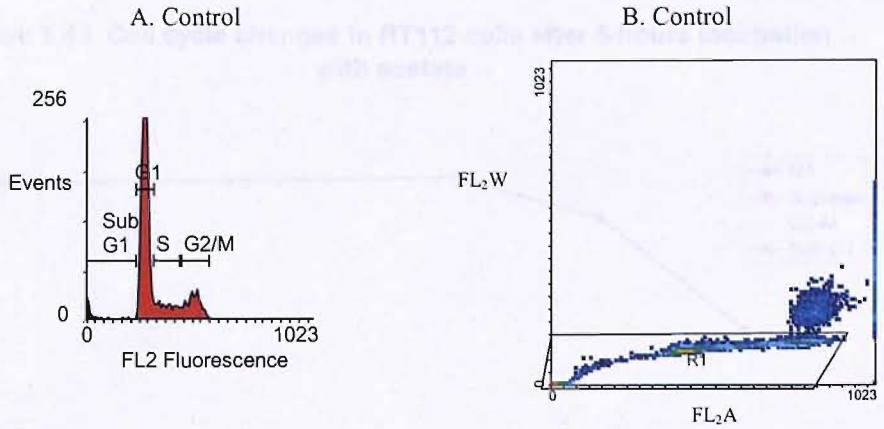


Figure 5.40 A. Propidium iodide fluorescence histogram of untreated RT112 urothelial cancer cells.
B. Doublet discrimination plot.

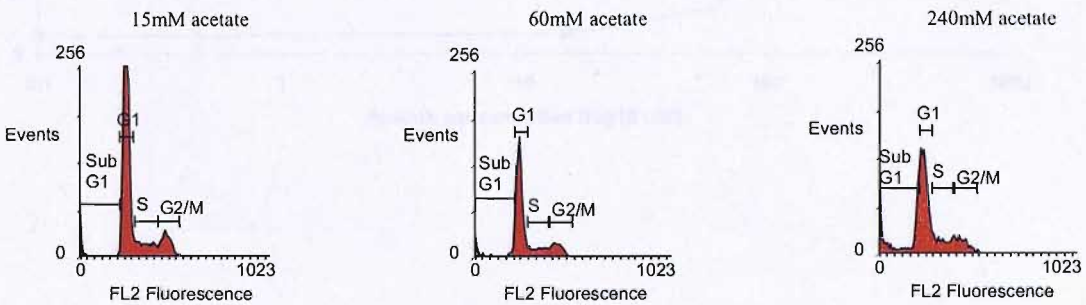


Figure 5.41 RT112 cells after 6 hours incubation with acetate

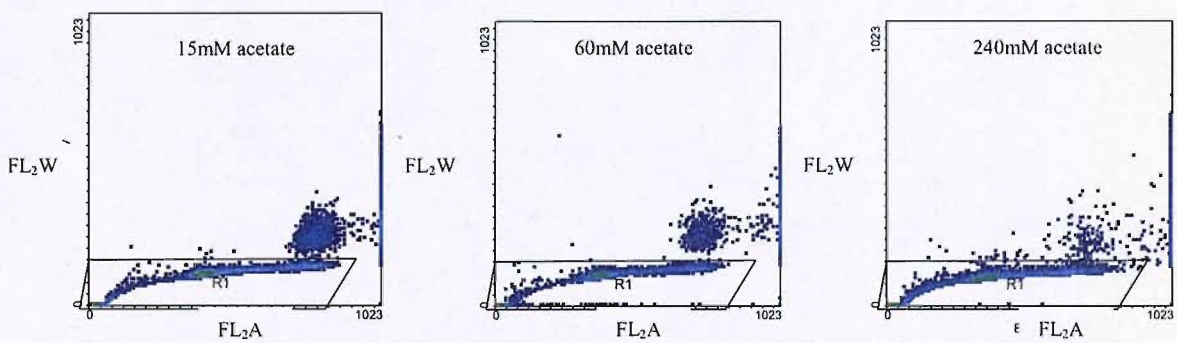
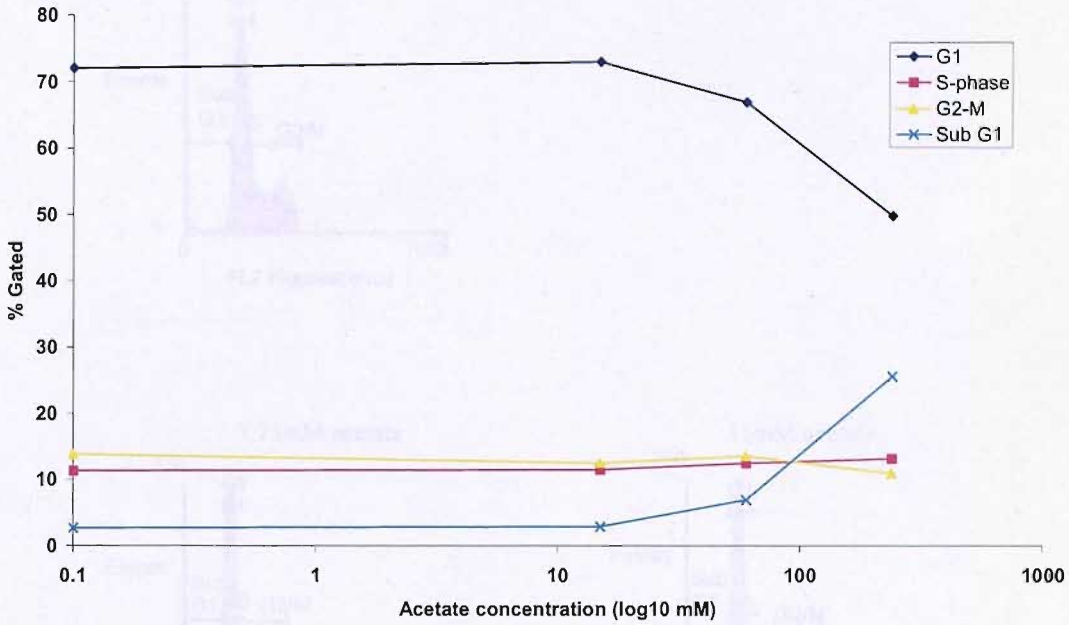


Figure 5.42 RT112 cells after 6 hours incubation with acetate (doublet discrimination)

Figure 5.43 Cell cycle changes in RT112 cells after 6 hours incubation with acetate



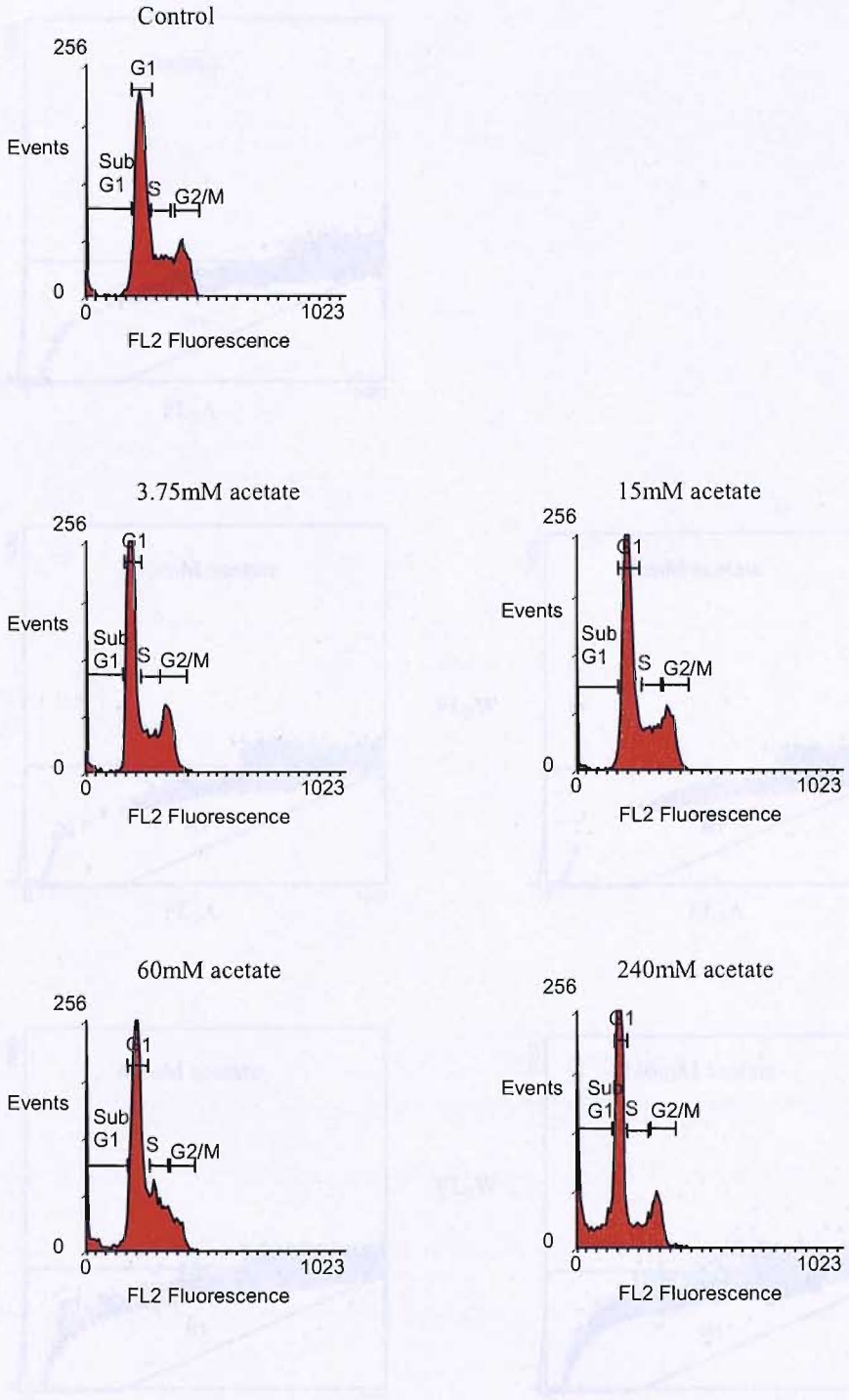


Figure 5.44 Cell cycle changes in RT112 cells after 24 hours incubation with acetate

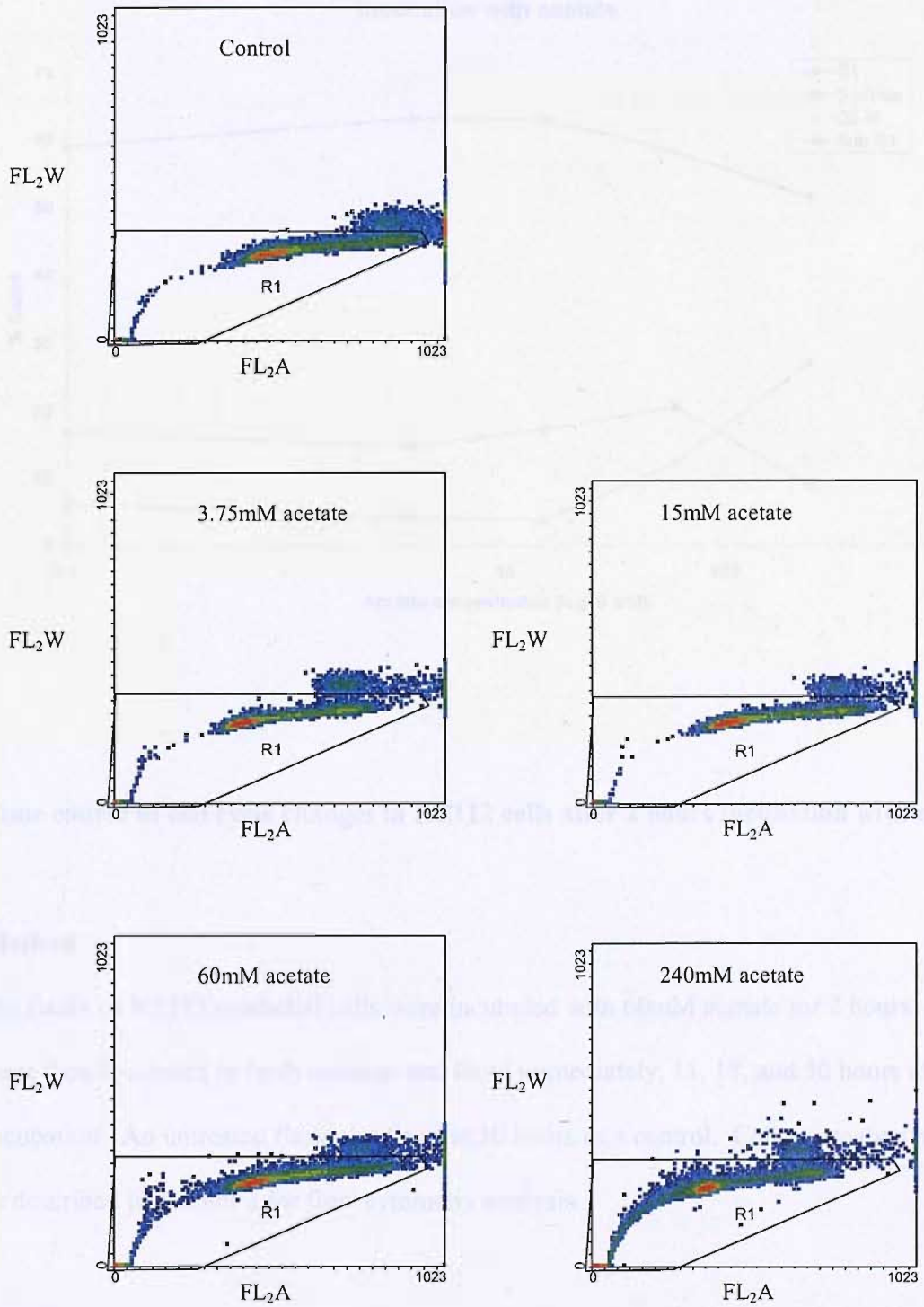
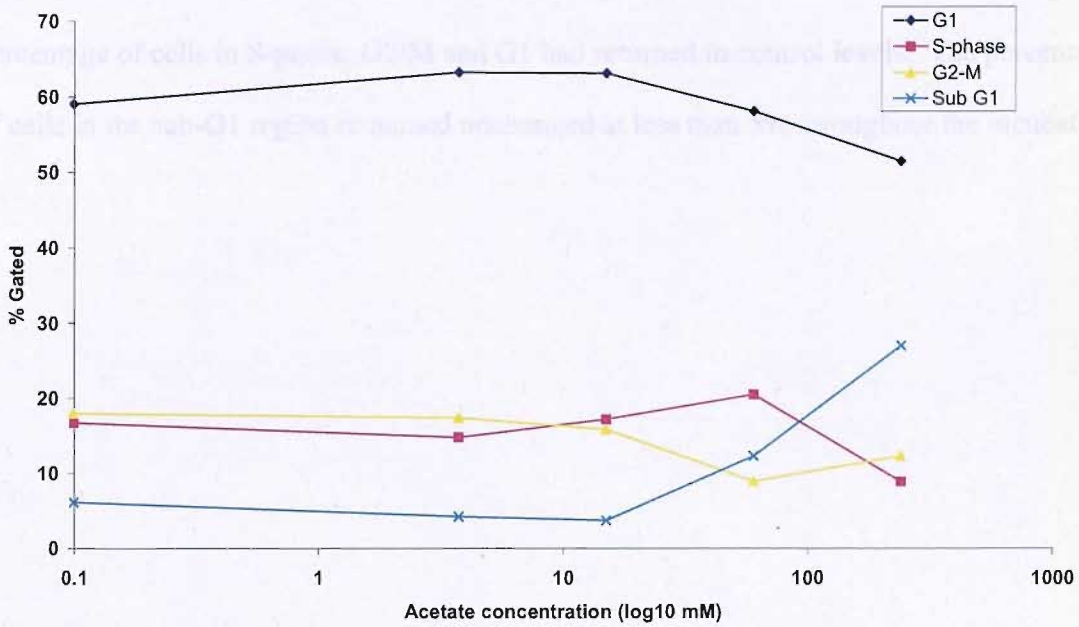


Figure 5.45 RT112 cells after 24 hours incubation with acetate (doublet discrimination plots)

Figure 5.46 Cell cycle changes in RT112 cells after 24 hours incubation with acetate



Time course of cell cycle changes in RT112 cells after 2 hours incubation with acetate.

Method

Six flasks of RT112 urothelial cells were incubated with 60mM acetate for 2 hours. Cells were then incubated in fresh medium and fixed immediately, 11, 18, and 50 hours after incubation. An untreated flask was fixed at 50 hours as a control. Cells were then processed as described in chapter 2 for flow cytometry analysis.

Results

Figures 5.47 and 5.49 illustrate the cell cycle changes over a 50 hour period following 2 hours incubation with 60mM acetate. Immediately after incubation there was no difference between treated and untreated DNA histograms. At 11 hour post incubation there was a fall

of 7% in the G1 population and an increase in the early S-phase region. At 18 hours the S-phase peak had returned to control levels and the G2/M peak increased. At 50 hours the percentage of cells in S-phase, G2/M and G1 had returned to control levels. The percentage of cells in the sub-G1 region remained unchanged at less than 5% throughout the incubation.

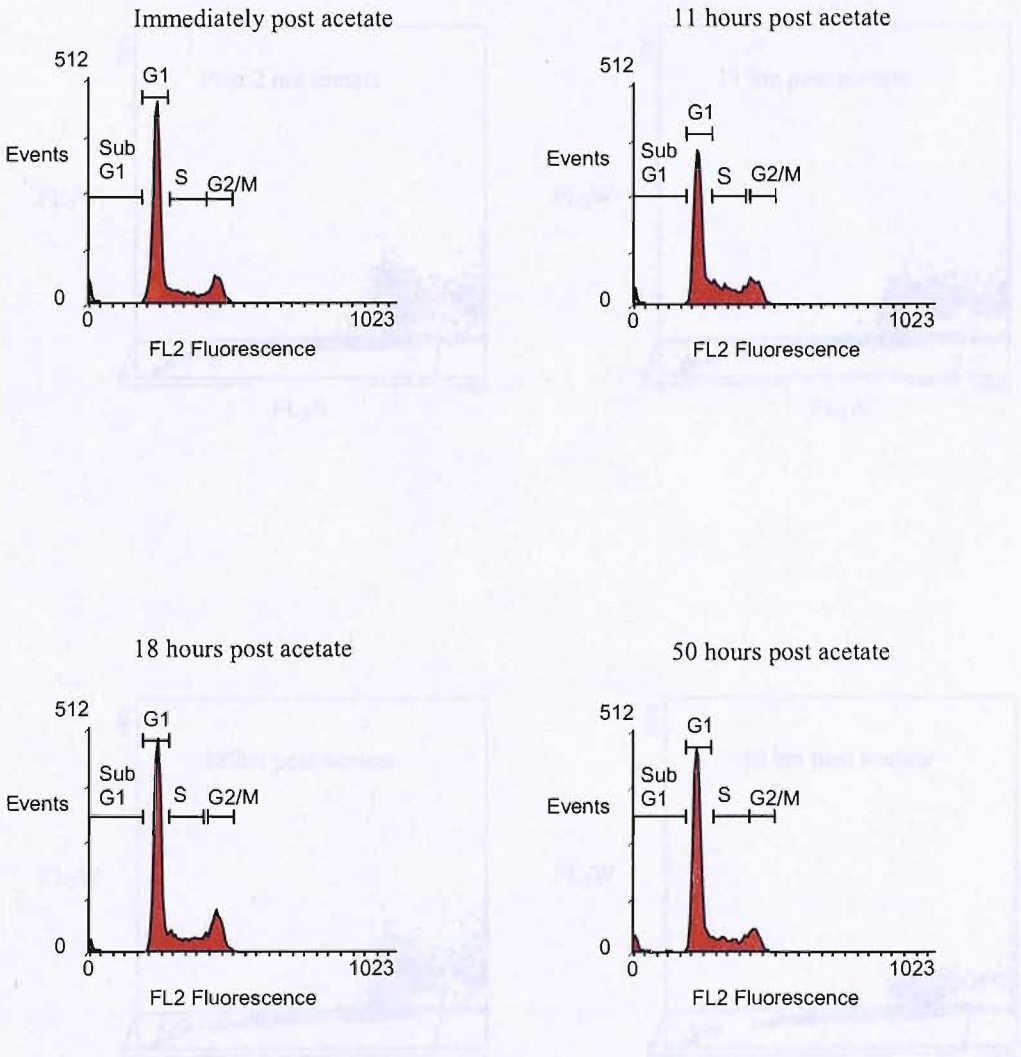


Figure 5.47 Time course of cell cycle changes in RT112 cells after 2 hours incubation with 60mM acetate

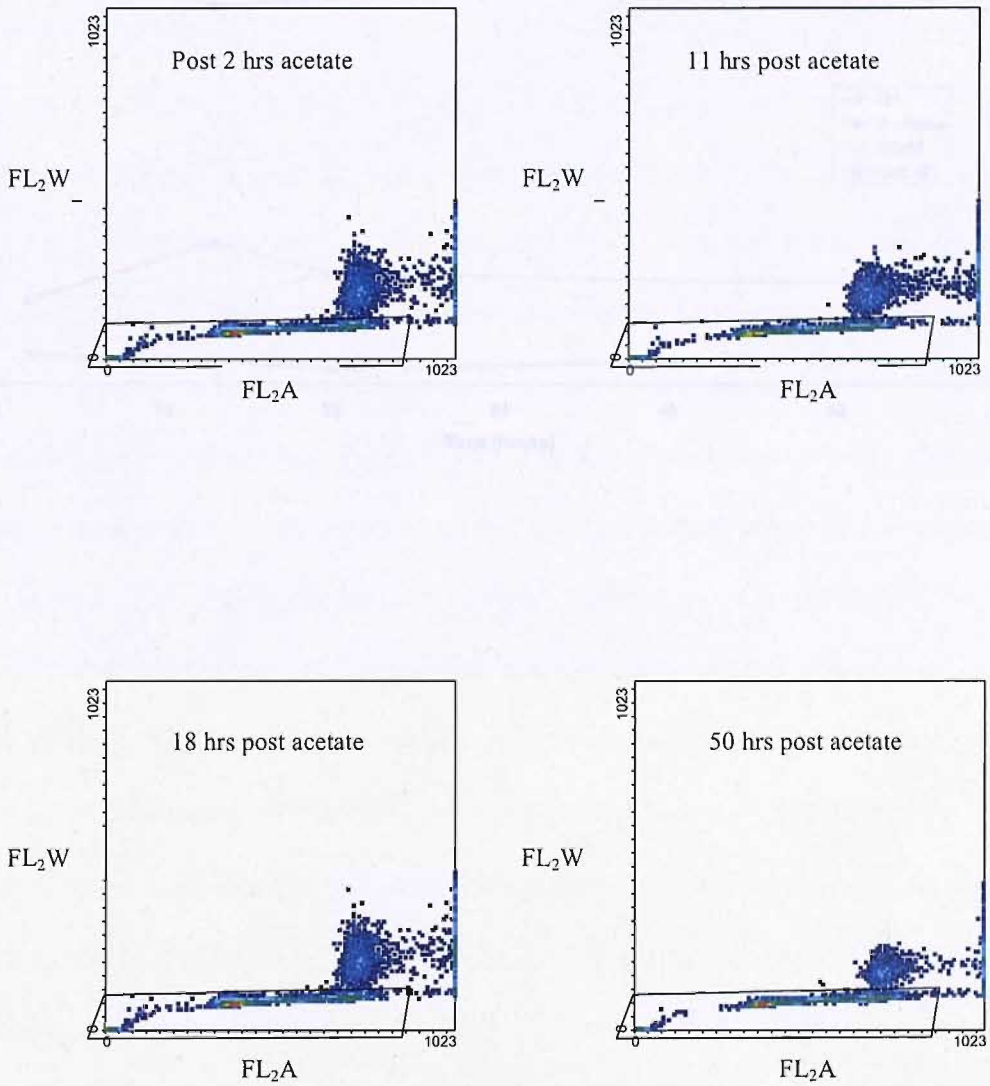
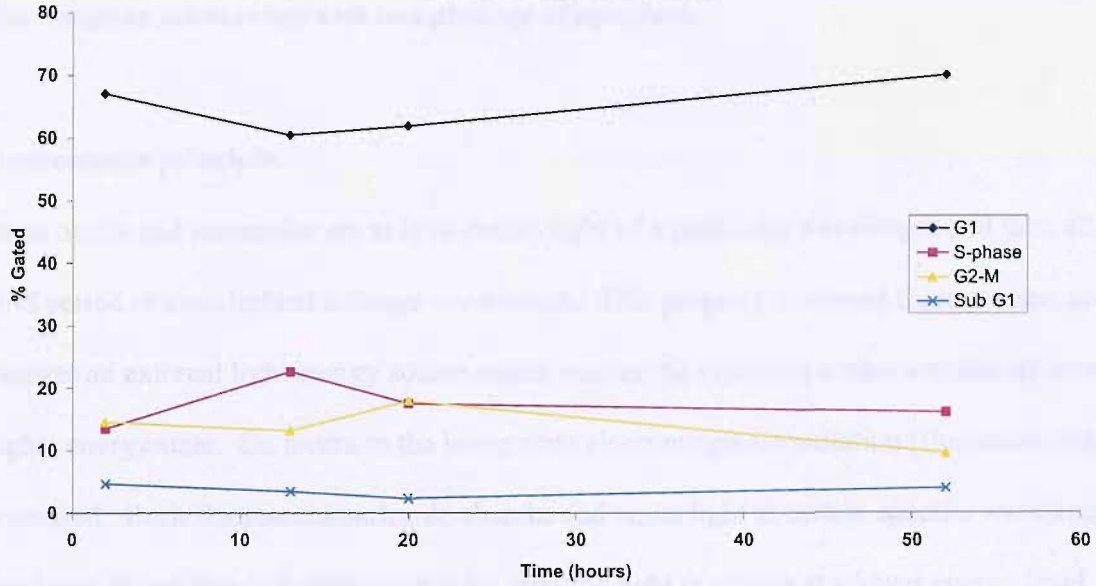


Figure 5.48 Time course of RT112 cells after 2 hours incubation with 60mM acetate (doublet discrimination plot)

Figure 5.49 Time course of cell cycle changes in RT112 cells after 2 hours incubation with 60mM acetate



Appendix 10

Fluorescence microscopy and morphology of apoptosis

Fluorescence principle

Some atoms and molecules are able to absorb light of a particular wavelength and then after a brief period re-emit light at a longer wavelength. This property is termed fluorescence, and requires an external light energy source which excites the electrons within a molecule into a higher energy state. On return to the lower state electromagnetic radiation (fluorescent light) is emitted. Each fluorescent molecule absorbs and emits light at certain specific wavelengths, known as absorption and emission bands. Emitted light is always at a lower energy level (longer wavelength) than the absorption band due to losses during molecular interactions with the environment. This shift to a longer wavelength is referred to as 'stokes shift'. The emission efficiency or the ratio of photons absorbed to photons emitted is known as the quantum yield (Q). The amount of time that a molecule remains in the excited state before returning to the ground state is known as the fluorescence lifetime (τ) and determines the duration of fluorescence decay after a pulse of excitation. Alternative processes for return of the excited state electron to the ground state exist including internal conversion, phosphorescence, and quenching. The fluorescence is often influenced by the solvent molecules surrounding the fluorophore. The interaction can diminish (red shift) or increase (blue shift) the energy separation between ground and excited energy states resulting in a shift in the emitted fluorescence. Temperature, pH and viscosity of the solvent also affect the fluorophore-solvent interaction.

Components of the fluorescence microscope

Excitation light source

Light sources include tungsten or halogen lamps for transmitted or incident illumination and mercury, xenon (eg Hg, Xe or Hg/Xe combination) or metal halide arc lamps or laser for epi-illumination. Selection of fluorescence light sources depends on the fluorescent probes used. Mercury, xenon lamps or lasers are the most popular light sources as most available fluorophores require excitation in the blue/green area of the visible spectrum. The mercury vapour lamp emits light at several discrete wavelengths making it a suitable energy source for several common fluorophores (Hoescht, DAPI, fluorescein and rhodamine based fluorophores)

Wavelength selection devices

The wavelengths of light visible to the human eye falls between 400-700nm. The visible spectrum is divided as follows.

400-430nm	Violet
430-500nm	Blue
500-560nm	Green
560-620nm	Yellow-orange
620-700nm	Orange-red

The fluorescence microscope houses a number of wavelength selection devices which select the excitation and emission wavelengths of interest (Illustration 6.1). These include tunable lasers, monochromators, individual and multiple band-pass filters, acousto-optical tunable filters (AOTF) and liquid crystal tunable filters (LCTF). Band-pass filters (1) are the most

widely used, selecting a specific wavelength of excitation light which is reflected onto the specimen by a dichromatic beam splitting mirror (2). The emitted fluorescence is viewed through a barrier filter (3) which cuts out unwanted fluorescence

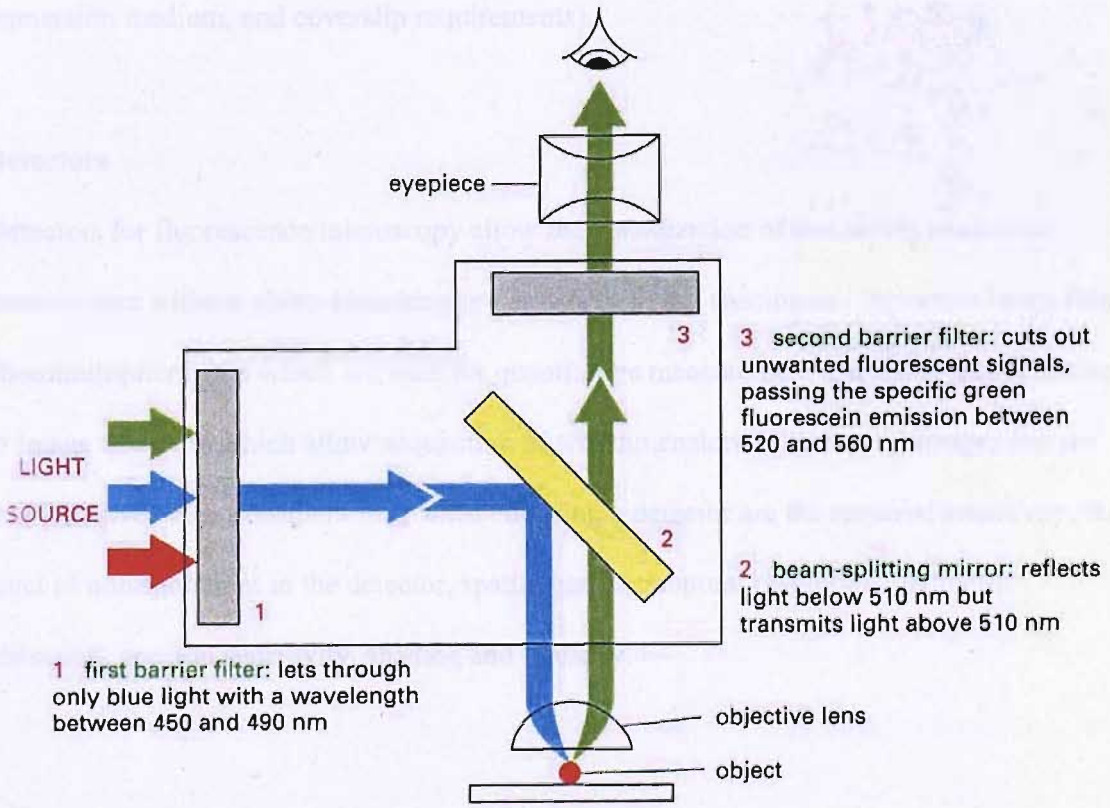


Illustration 6.1 Typical fluorescence microscope.

Objectives

Objectives for fluorescence microscopy are classified as transmitted light (designed for use with coverslips) or reflected light versions (have antireflective coating). More recently infinity focused objectives have been developed which pass light in parallel between the

objective and the eye piece making them insensitive to other components placed in the path of the beam such as filters, analysers and compensators. Multi-immersion objectives allow imaging of living cells and tissues within medium. The choice of objective depends on the transmitted wavelength, the type of application (phase contrast, photomicroscopy) and the specific characteristics of each objective (magnification, numerical aperture, tube length, immersion medium, and coverslip requirements).

Detectors

Detectors for fluorescence microscopy allow the visualization of low levels of emitted fluorescence without photo-bleaching or damage to living specimens. Detectors range from photomultiplier tubes which are used for quantitative measurement and are highly sensitive, to image detectors which allow acquisition of two dimensional fluorescent images but are less sensitive. Key considerations when choosing a detector are the required sensitivity, the level of noise inherent in the detector, spatial versus temporal resolution, geometric distortion, spectral sensitivity, shading and linearity.

The morphology of apoptosis

Cell death is an integral part of development and maintenance of a complex organism and was first described in the toad species by Vogt in 1842. Since then a number of terms have been used to describe cell death including degeneration, necrosis, autolysis, chromatolysis, and cell suicide to name but a few. Today the most commonly used terms are necrosis, apoptosis and programmed cell death. Necrosis refers to a passive form of cell death characterised by a slow disintegration without any features of active cell death. Apoptosis on the other hand refers to an active dismantling and disposal of the cell and the contents in an

organised fashion, applied both to the morphological features and the underlying mechanisms involved.

Cells dying by apoptosis, regardless of the physiological situation or the mechanism of induction, display a very similar pattern of morphological changes because the process is probably implemented by the same intracellular pathway (Kerr et al. 1972). This pathway involves a class of cysteine proteases encoded by the ced-3 gene known as caspases which are conserved in evolutionarily widely separated organisms. They are responsible for digestion of other proteins resulting in the morphological changes seen in apoptosis. The term programmed cell death is used in the context of cell death as part of normal development, where apoptosis occurs as a result of some intracellular program.

The morphological features of apoptosis can be demonstrated by the light microscope or the electron microscope aided by various specific dyes including the DNA intercalating dyes, acridine orange and Hoechst dyes which help visualise the nucleus. Early morphological features of apoptosis include condensation of nuclear chromatin along the perimeter of the nucleus. Cells then separate from surrounding cells and extracellular attachments, adherent cells appear to round up. Protrusions from the plasma membrane appear, referred to as blebs and the nucleus condenses completely and segregates into several fragments (Illustration 6.2).

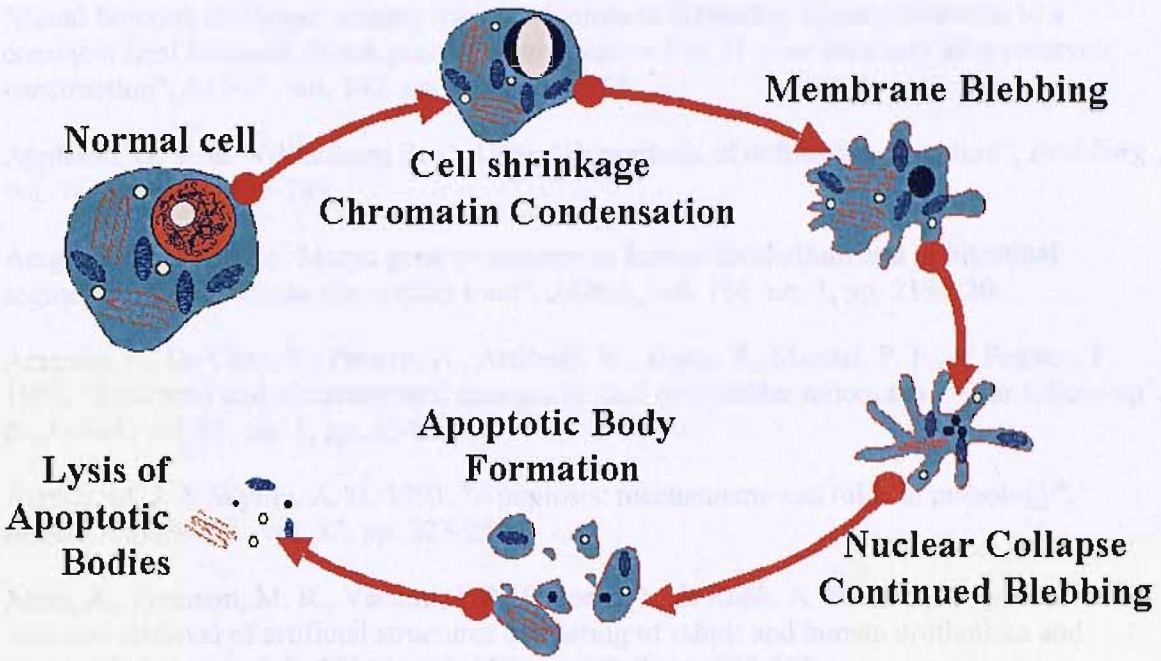


Illustration 6.2 Morphological changes characteristic of apoptosis.

These features can be seen microscopically using acridine orange fluorescence. In fluorescent blue light acridine orange produces a bright fluorescent green emission when bound to double stranded DNA. By virtue of the increased density of condensed chromatin, a brighter green fluorescence is emitted from apoptotic nuclei, than from uncondensed DNA in normal nuclei, enabling apoptotic cells to be distinguished easily from normal cells (Dive et al. 1992).

As apoptosis continues the entire cell is divided into apoptotic bodies, containing intact organelles and fragments of the nucleus. Changes in cytoplasmic organelles such as mitochondria and the endoplasmic reticulum, occur late in apoptosis and can be seen using scanning electron microscopy (Hacker 2000).

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