αvβ6 Integrin Upregulates Matrix Metalloproteinase 9 and Promotes Migration of Normal Oral Keratinocytes

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The integrin αvβ6 is a fibronectin receptor that is undetectable on normal keratinocytes *in situ*, but is increased significantly in wound healing and in culture-established keratinocytes, suggesting that it may promote changes associated with cell motility. Using normal human oral keratinocytes we have shown that cultured cells express relatively high levels of αvβ6 and this integrin has a functional role in both cell adhesion and migration towards fibronectin. We provide experimental evidence that the increased expression of αvβ6 by normal human oral keratinocytes results in coordinate changes, which promote a more migratory phenotype. Thus increased expression of αvβ6 results in a fibronectin-dependent increase in pro-matrix metalloproteinase 9, matrix metalloproteinase 9 activity increases normal human oral keratinocyte migration, and this may be further dependent on plasmin activation. The results suggest a key role for αvβ6 in these processes and indicate a coordinated link between αvβ6 expression and upregulation of matrix metalloproteinase 9. It appears that αvβ6 may function in normal human oral keratinocyte migration through matrix-metalloproteinase-9-dependent and -independent mechanisms. ***Key words: integrins/ keratinocytes/ metalloproteinases/ migration. J Invest Dermatol 116:898–904, 2001***

Migration of epithelial cells into the underlying connective tissue is a fundamental feature of many processes including development and wound healing. During migration epithelial cells alter their cell–cell and cell–extracellular matrix interactions and gain the ability to degrade connective tissue, either by secreting proteolytic enzymes themselves or by utilizing enzymes produced by other cells. Matrix metalloproteinases (MMPs) are one such group of proteolytic enzymes that are capable of degrading different substrates within the extracellular matrix. In fact, secretion of MMPs, accompanied by altered cell adhesion molecules, is critical for epithelial migration.

Integrins are a family of heterodimeric, cation-dependent, cell membrane receptors expressed by a variety of cells. Integrins mediate cell–extracellular matrix and cell–cell interactions and facilitate many cell processes including adhesion, proliferation, migration, and differentiation (Hynes, 1992). These functions are modulated substantially by signals from the extracellular matrix (Damsky and Werb, 1992).

Successful epithelial wound healing is associated with a specific sequence of changes in the process of cell–matrix adhesion and extracellular matrix degradation. *De novo* expression of the αvβ6 integrin by keratinocytes in both cutaneous and oral wounds has been demonstrated in several studies (Breuss *et al*, 1993; Clark *et al*, 1996; Haapasalmi *et al*, 1996) and there is some evidence to suggest that re-epithelialization of epidermal wounds is associated with a switch from αvβ5 expression to αvβ6 in the keratinocyte population (Clark *et al*, 1996). Similarly, during wound healing the type IV collagenase MMP-9 is upregulated by keratinocytes whereas MMP-2 remains localized to the stroma (Salo *et al*, 1994). Type IV collagenases degrade basement membrane, a critical step in the detachment and migration of keratinocytes. Intriguingly, many of these changes are similar to those seen in squamous cell carcinoma where, in addition to changes in proteolytic activity, there is loss of αvβ5 and *de novo* expression of αvβ6 (Breuss *et al*, 1994; Jones *et al*, 1997). Thus squamous cell carcinoma may represent a process of uncontrolled tissue remodeling.

The upregulation of αvβ6 by wound keratinocytes suggests that this integrin heterodimer could have an active role in cell movement. Huang *et al* (1998) reported greatly reduced migration on fibronectin and vitronectin by keratinocytes from β6 knockout mice. Restoration of αvβ5 to a malignant keratinocyte cell line resulted in a reversal of the malignant phenotype with suppression of anchorage-independent growth and increased capacity for terminal differentiation (Jones *et al*, 1996) whereas retrovirally mediated transduction of β6 cDNA into this cell line significantly increased fibronectin-dependent migration and was associated with increased expression of MMP-9 (Thomas *et al*, 2001). Activation of MMP-9 significantly enhanced cell migration and invasion in this system (Thomas *et al*, 2001).

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Abbreviations: KGM, keratinocyte growth medium; MMP, matrix metalloproteinase; NHK, normal human oral keratinocytes; uPA, urokinase-type plasminogen activator.

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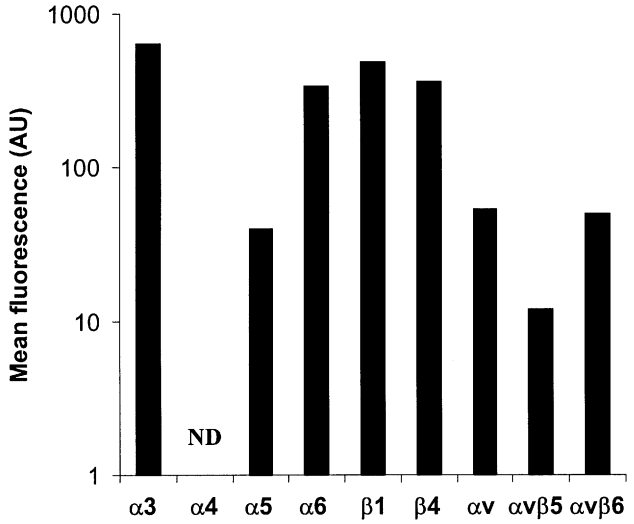

Figure 1. Flow cytometric analysis of integrin expression by NHK. The geometric mean fluorescence (arbitary units, log scale) as measured by flow cytometry of cells labeled with anti-integrin antibodies is shown. Negative control had secondary antibody only and has been subtracted from the results. The figure shows a representative experiment (ND, not detected). Flow cytometry confirmed relatively high αvβ6 expression by NHK and demonstrated that NHK also express the α5β1 fibronectin receptor and show high expression of α6, β4, α3, and β1 integrin subunits.

In this study we have examined the functional significance of αvβ6 expression by primary oral keratinocyes established in tissue culture. Our results show that such cultured keratinocytes express high levels of functional αvβ6 and that MMP-9 upregulation by keratinocytes plated on fibronectin is αvβ6 dependent. Increased expression of αvβ6 resulted in a fibronectin-dependent increase in pro-MMP-9; this MMP-9 activity increased migration and migration was shown to be further dependent on plasmin activation. The results suggest a key role for αvβ6 in these processes and indicate a coordinated link between αvβ6 expression and upregulation of MMP-9.

MATERIALS AND METHODS

Antibodies and reagents Eight monoclonal antibodies (all of murine origin unless stated) were used in this study. L230 (antihuman αv) was prepared in our laboratory from hybridoma cells obtained from the American Type Culture Collection (Rockville, MD) (Weinacker *et al*, 1994). The anti-αvβ6 (E7P6 and R6G9) (Weinacker *et al*, 1994), anti-αvβ6 (10D5; Huang *et al*, 1998), anti-α5β1 (P1D6), and anti-β1 (P4C10) antibodies were purchased from Chemicon International (Harrow, U.K.). Anti-αvβ5 (P1F6) was obtained from Life Technologies, Paisley, U.K. Fluorescein isothiocyanate (FITC) and horseradish peroxidase conjugated rabbit antimouse antibodies were purchased from Dako (High Wycombe, U.K.). Plasma fibronectin and bovine serum albumin (BSA) were purchased from Sigma Chemical (Poole, Dorset, U.K.). Anti-MMP-9 (66-b), rabbit anti-MMP-9, TIMP-1, and MMP inhibitor 1 were obtained from Calbiochem (Nottingham, U.K.).

Cell culture The cells used in this study were normal human oral keratinocytes (NHK) prepared as outgrowths from normal oral mucosa obtained with permission during minor oral surgery procedures. The preparation of cells from explant cultures was modified from the method described by Freshney (1987). Briefly the tissue was finely chopped into small pieces, placed in 25 cm^2 flasks in keratinocyte growth medium (KGM) as described. KGM comprised α-modified Eagle's medium (α-MEM) containing 10% fetal bovine serum (Globepharm, Surrey, U.K.) supplemented with 100 IU per liter penicillin, 100 μg per liter streptomycin, and 2.5 μg per liter amphotericin B (Gibco BRL), 1.8×10^{-4} M adenine, 5 μg per ml insulin, 1×10^{-10} M cholera toxin, 0.5 μg per ml hydrocortisone, and 10 ng per ml epidermal growth factor (Sigma). After 1 wk, outgrowth of cells was observed and the medium was changed at this time. Cultures were fed every 48 h and were ready for passage at about day 18. NHK in this study were used up to passage 2.

Flow cytometry Subconfluent cells were washed with phosphate-buffered saline (PBS) and harvested by trypsin/ethylenediamine tetraacetic acid (0.25% wt/vol, 5 mM). Cells were incubated with primary antibody for 40 min at 4°C and washed twice with PBS. FITC-conjugated secondary antibody was applied to the cells for 30 min at 4°C. Briefly, cells were washed twice with PBS and resuspended in 0.5 ml PBS with 10% fetal bovine serum. Labelled cells were scanned on a FACSCalibur cytometer (Becton Dickinson) and analyzed using Cellquest software, acquiring 1×10^4 events.

Adhesion assays Ninety-six well plates (Falcon 3912; Becton Dickinson) were coated with plasma fibronectin (Sigma). A 50 μl fibronectin solution at a concentration of 10 μg per ml was added to the wells and incubated at 37°C for 1 h. After incubation wells were washed with PBS and then blocked with 0.5% BSA at 37°C for 30 min. Control wells were incubated with 0.5% BSA. Cells were chromium [^{51}Cr] labeled (Brunner *et al*, 1976), washed, and resuspended in α-MEM (1.5×10^4 cells per well). For blocking experiments, cells were incubated with specific antibodies (as described in *Results* in each well) for 10 min on ice. Plates were incubated at 37°C for 1 h. Non-adherent cells were removed by flooding plates with PBS (supplemented with 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$). After two washes, the plates were cut into individual wells and the radioactivity associated with each well was determined in a gamma counter (1261 Multigamma; LKB Wallac, Bromma, Sweden). The percent adhesion was expressed as the adherent cell radioactivity as a proportion of the total cell input. The nonspecific adhesion (attachment to wells coated with BSA) was subtracted. Experiments were repeated on three occasions, in quadruplicate, with similar results.

Immunolocalization of MMP-9 and αv Two $\times 10^4$ cells were plated onto 13 mm glass coverslips coated with fibronectin (10 μg per ml) and blocked with 0.5% BSA in PBS. Coverslips were incubated for 24 h at 37°C in 5% CO_2. For MMP-9 analysis cells were incubated for 24 h in serum-free medium containing 1 μM monensin (Sigma) to block protein secretion. Cells were rinsed twice in PBS, fixed for 15 min in 10% formalin, and permeabilized with 0.1% Triton X-100 for 10 min, followed by incubation for 60 min in wash buffer. L230 (anti-αv; 18 μg per ml) or 66-b (anti-MMP-9; 1:100) diluted in wash buffer was added for 60 min at 4°C. Bound antibody was detected with FITC-conjugated rabbit antimouse secondary antibody (Dako; 1:40). Actin was visualized with TRITC-conjugated phalloidin (Sigma: 5 ng per ml). Coverslips were washed three times for 5 min in wash buffer, mounted with MOWIOL 4-88 (Novabiochem, Nottingham, U.K.: 0.1 g per ml of Citifluor mounting medium), and viewed with a confocal laser scanning microscope (Zeiss LSM510; Welwyn Garden City).

Preparation of cell supernatants for MMP determination Twenty-four well plates were coated with 200 μl of fibronectin at 10 μg per ml. After incubation wells were washed with PBS and blocked with 0.1% BSA for 30 min. 10^5 cells were seeded in additive-free α-MEM into uncoated and fibronectin-coated wells. For blocking experiments, cells were incubated with specific antibodies (as described in *Results*) for 30 min at 4°C and plated in medium containing an excess of antibody. Supernatant was sampled after 24 h at which time a cell count was carried out. Conditioned medium was cleared of cells and debris by centrifugation at 4000 rpm for 10 min followed by protein estimation using the BCA protein assay reagent (Pierce Warriner, Chester, U.K.).

Zymography MMP-9 and MMP-2 activity were analyzed using sodium dodecyl sulfate (SDS) polyacrylamide substrate gels. Gelatin (bloom 300, Sigma) was added to a 12% acrylamide separating gel at a final concentration of 1 mg per ml. To each gel, samples containing equal protein (as determined by BCA protein assay; Pierce Warriner) were mixed with nonreducing sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.1% bromophenol blue) and added to the gel without boiling. Molecular weight markers and MMP standards were run on each gel. Following electrophoresis, gels were washed twice in 2.5% Triton X-100 for 30 min at 37°C to remove the SDS. Gels were incubated at 37°C overnight in developing buffer containing 50 mM Tris-HCl, 0.2 M NaCl, 5 mM $CaCl_2$, and 0.02% Triton X-100. Gels were stained with 0.5% Coomassie blue G250 in 30% methanol, 10% glacial acetic acid for 30 min and destained in the same solution without Coomassie blue. Gelatin-degrading enzymes were identified as

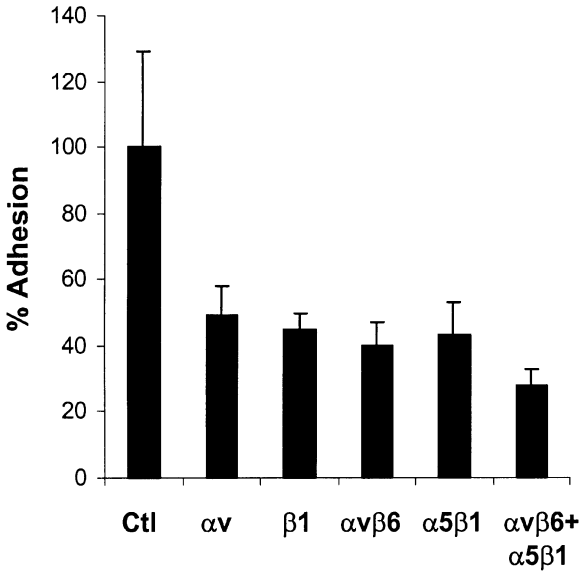

Figure 2 NHK adhere to fibronectin using both αvβ6 and α5β1 integrins. Adhesion to fibronectin of NHK in the presence of control antibody was 24% of the plated cells. Background binding to BSA-coated wells was 5% and has been subtracted from the results. Results are expressed relative to control antibody (= 100%). Adhesion of NHK was blocked by antibodies to both αvβ6 (60% inhibition) and α5β1 (57% inhibition), as well as against the αv and β1 subunits (51% and 55% inhibition, respectively). Maximal inhibition of adhesion was obtained using a combination of anti-αvβ6 and anti-α5β1 (72% inhibition). The adhesion of cells (28%) in the presence of combined antibodies indicates that other fibronectin receptors may also be expressed by NHK. The figure shows a representative experiment performed in quadruplicate. Error bars represent standard deviation.

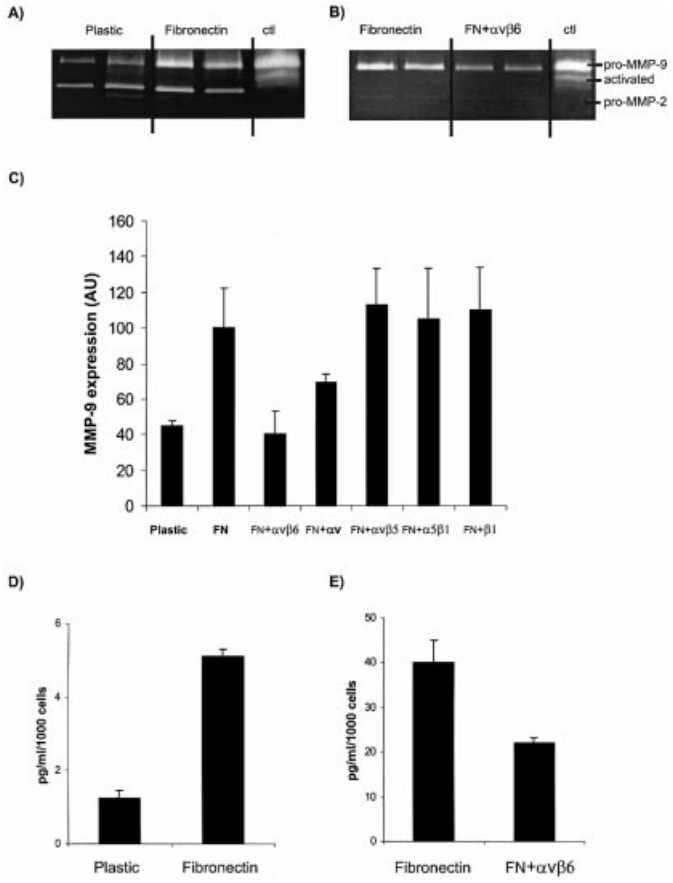

Figure 3. Upregulation of MMP-9 by NHK on fibronectin is αvβ6 dependent. Cell supernatant samples were analyzed by zymography and ELISA. (*A*) Zymogram showing the upregulation of MMP-9 by NHK when plated on fibronectin (10 μg per ml). Control is recombinant MMP-9 (Amersham). Note that under serum-free conditions both MMP-9 and MMP-2 are present in pro-enzyme form. (*B*) Zymogram showing inhibition of MMP-9 expression on fibronectin by anti-αvβ6 antibody. Control is recombinant MMP-9 (Amersham). (*C*) Densitometric analysis of zymograms from representative experiments showing MMP-9 expression by NHK cells on fibronectin and following blockade with anti-integrin antibodies. Results are expressed relative to MMP-9 expression by NHK cells on fibronectin (= 100). Error bars represent standard deviation. (*D*) ELISA showing upregulation of MMP-9 by NHK on fibronectin. Error bars represent standard deviation. (*E*) ELISA showing inhibition of MMP-9 on fibronectin by NHK by anti-αvβ6 antibody. Error bars represent standard deviation.

clear bands against the blue background of the stained gel. Images of stained gels were captured under illumination using the UVP Imagestore 5000 (Ultra-Violet Products, U.K.) and exported for use on a PC using the Scion image program (Scion; based on the Macintosh NIH Image program developed at the National Institutes of Health, MD). The intensity of the bands was measured by densitometric analysis and comparisons were made within each gel to determine relative changes in MMP activity. Data for each zymogram were expressed as relative changes in MMP activity and these relative changes were compared with repeat experiments. Direct comparison between separate gels was not made, as the intensity of background staining was variable. Experiments were repeated a minimum of three times, each time in triplicate, and are discussed only if consistent observations were made.

Enzyme-linked immunosorbent assay (ELISA) Commercial ELISA kits for MMP-9 were purchased from Amersham Life Science, Buckinghamshire, U.K. Cell supernatants were prepared as for zymography. A hundred microliters of concentrated sample was added to each well and MMP-9 was detected with peroxidase labeled F(ab) fragment of anti-MMP-9 antibody. The reaction was stopped by addition of 50 μl 1 M H_2SO_4 and the resultant color change was read at 450 nm on a spectrophotometer (SLT Labinstruments, Austria). Concentration was determined by interpolation from a standard curve using known concentrations of MMP standards as supplied.

Migration assays Haptotactic cell migration assays were performed using matrix-coated polycarbonate filters (8 μm pore size, Transwell®, Becton Dickinson). The membrane undersurface was coated with fibronectin (10 μg per ml) in PBS for 1 h at 37°C and blocked with migration buffer (0.5% BSA in α-MEM) for 30 min at 37°C. To assess integrin and MMP-9 specificity of migration, blocking antibodies or chemical inhibitors, together with plasminogen (to activate MMP-9) (as described in *Results*), were added for 30 min at 4°C prior to plating. The lower chamber was filled with 500 μl of migration buffer, following which cells were plated in the upper chamber of triplicate wells, at a density of 4×10^4 in 100 μl of migration buffer, and incubated at 37°C for 20 h. After 20 h, the cells in the lower chamber (including those attached to the undersurface of the membrane) were trypsinized and counted on a Casy 1 counter (Sharfe System, Germany).

Statistical analysis Data are expressed as the mean ± SD of a given number of observations. Where appropriate, one-way analysis of variance was used to compare multiple groups. Comparisons between groups were by Fisher's PLSD (set at 5% significance). A p-value of less than 0.05 was considered to be significant.

RESULTS

Cultured keratinocytes show relatively high αvβ6 expression Levels of surface expression of integrins were determined by flow cytometry. NHK express low levels of αvβ5 (mean fluorescence 12) but much greater levels of αvβ6 (mean fluorescence 50) (**Fig 1**). Flow cytometry demonstrated that NHK also express the α5β1 fibronectin receptor and show high expression of α6, β4, α3, and β1 integrin subunits.

Adhesion of NHK to fibronectin is mediated by αvβ6 and α5β1 NHK were plated onto fibronectin in the presence or

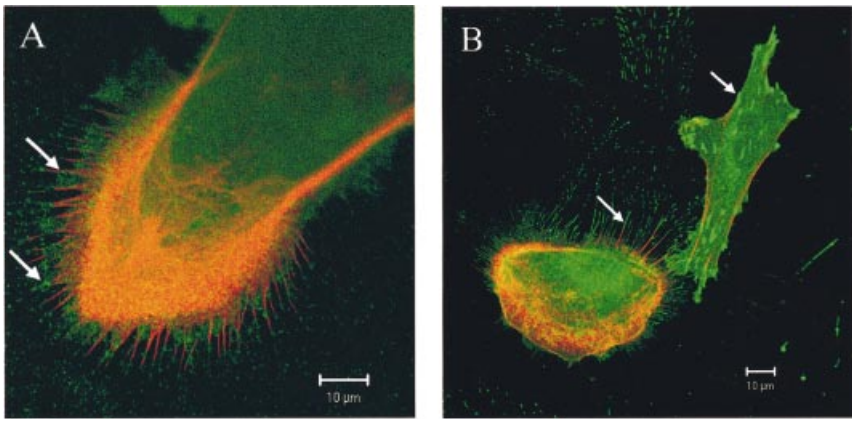

Figure 4. MMP-9 localizes to the terminal aspects of αv-positive filopodia. Cells were examined on a confocal microscope for MMP-9 and αv expression. The figure shows combined red (actin) and green (*A*, MMP-9; *B*, αv) images. Magnification bar: 10 μm. Image *A* shows localization of MMP-9 to the terminal aspects of filopodia (*A*, *arrows*). The filopodia in NHK are strongly αv positive (*B*, *lower arrow*). αv also localizes to focal adhesions (*B*, *upper arrow*).

absence of blocking antibodies against the αv subunit, αvβ6, α5β1, β1, or an irrelevant antibody (W6/32) against class I major histocompatibility complex (**Fig 2**). Combinations of these antibodies also were used. Antibodies directed against αvβ6 (60% inhibition) and α5β1 (57% inhibition) significantly inhibited adhesion of NHK to fibronectin (as did antibodies against the αv and β1 subunits; 51% and 55%, respectively). Maximal inhibition of NHK adhesion to fibronectin, however, required a combination of antibodies against αvβ6 *and* α5β1 (72% inhibition). This demonstrates a functional role for αvβ6 in NHK adhesion to fibronectin and that NHK may adhere through both α5β1 and αvβ6. The adhesion of a percentage of NHK in the presence of the combined antibodies indicates that other fibronectin receptors may also be involved.

NHK upregulate secretion of MMP-9 on fibronectin in an αvβ6-dependent manner Expression of MMP-9 and MMP-2 by NHK on uncoated and fibronectin-coated tissue culture plastic was examined by zymography and ELISA. Both approaches gave qualitatively and quantitatively similar results. Zymography on supernatant samples from cells grown in serum-free medium showed that the cells produced MMP-9 and MMP-2, and that the enzymes were present in proenzyme form. In agreement with previous studies the level of MMP-9 expression increased and the level of MMP-2 decreased with time in culture (Kobayashi *et al*, 1997, 1998). When plated on fibronectin, the NHK showed significant upregulation of MMP-9 ($p < 0.001$) (**Fig 3*A***).

To test the integrin dependence of the MMP-9 upregulation on fibronectin NHK were incubated with specific integrin-blocking antibodies against αv, αvβ5, αvβ6, α5β1, or β1 prior to plating. A significant reduction in MMP-9 levels was observed when αv or αvβ6 was inhibited ($p = 0.03$, $p < 0.001$, respectively; **Fig 3*B***). In contrast, expression of MMP-9 was not affected by the other integrin-blocking antibodies (αvβ5, α5β1, or β1; **Fig 3*C***), confirming the specificity of αvβ6 in mediating these effects. These data confirm that increased synthesis of MMP-9 by NHK in response to cell adhesion to fibronectin is mediated by the αvβ6 integrin.

Expression of MMP-9 was also quantitated by ELISA (**Fig 3*D***, ***E***). This confirmed the αvβ6-dependent increase in expression of MMP-9 and MMP-2 even though the ELISA assay was less sensitive than zymography. This method also confirmed that NHK produced increased amounts of MMP-9 with time in culture (Kobayashi *et al*, 1997, 1998).

Subcellular distribution of MMP-9 and αv We examined the subcellular distribution of MMP-9 in NHK. Indirect immunochemistry on NHK revealed that MMP-9 located to the terminal aspects of filopodia in monensin-treated cells (**Fig 4*A***, *arrow*) or in larger submembranous vesicles (data not shown) suggesting that alteration in the cell morphology affects MMP-9 distribution. The αv subunit was detected in focal adhesions (**Fig 4*B***, *upper arrow*) and, interestingly, was also expressed strongly on filopodia (**Fig 4*B***, *lower arrow*).

αvβ6 functions in NHK migration towards fibronectin To determine whether αvβ6 and MMP-9 are involved in keratinocyte migration, haptotactic migration assays were performed using fibronectin-coated Transwell filters. In serum-free conditions the MMP-9 produced by NHK is in proenzyme form. As plasmin can activate pro-MMP-9 (Ramos-DeSimone *et al*, 1999), we added the plasmin precursor, plasminogen, to some migration assays. This substrate could be cleaved by the urokinase-type plasminogen activator (uPA), which is present on NHK cells (data not shown). **Figure 5(*A*)** shows activation of MMP-9 on addition of plasminogen. Specific MMP-9 inhibitors were also included. Migration towards fibronectin was reduced significantly by anti-αvβ6 antibody (39% inhibition; $p = 0.014$) (**Fig 5*B***). Interestingly, inhibition of α5β1 increased migration (30% increase; $p = 0.003$) (**Fig 5*B***), suggesting that this integrin may function in a purely adhesive capacity and may be inhibitory to cell migration. Migration was also reduced by anti-β1 antibodies (28% inhibition; $p = 0.023$) suggesting that integrins of the β1 family may also be involved in keratinocyte migration towards fibronectin. This is further suggested by the observation that the greatest degree of inhibition was produced by a combination of anti-αv and anti-β1 antibodies (71% inhibition; $p = 0.001$). In plasminogen-free assays, neither MMP-9 nor uPA inhibitors inhibited cell migration indicating that the cell movement is MMP-9 and uPA independent (**Fig 5*B***).

Activation of MMP-9 increases NHK migration towards fibronectin In the presence of plasminogen, a significant increase in migration towards fibronectin was observed (158%) (**Fig 5*C***). Thus relative to the plasminogen-free migration of cells treated with the control antibody W6/32, there was a 58% increase in migration ($p = 0.001$) (**Fig 5*C***). This increase in migration could be abolished by an anti-MMP-9 specific antibody (61% inhibition; $p < 0.001$) and a specific MMP-9 chemical inhibitor (Calbiochem; called in-1 in **Fig 5*C***; 70% inhibition; $p < 0.001$). This indicates that the observed increase in migration due to addition of plasminogen was MMP-9 dependent, and demonstrates a functional role for MMP-9 in NHK migration. Addition of the uPA inhibitor amiloride produced a similar level of inhibition (72% inhibition; $p < 0.001$) suggesting that MMP-9 is activated through uPA-dependent generation of plasmin. Integrin inhibition produced a similar pattern of migration to that seen in assays without plasminogen, although in general the level of inhibition was greater. Thus anti-αvβ6 antibodies inhibited migration by 62%, whereas anti-α5β1 antibodies again produced an increase in migration (**Fig 5*B***). Interestingly, blocking β1 integrins produced more effective inhibition of migration in the presence of activated MMP-9, suggesting a more prominent role for β1 integrins in cell migration towards fibronectin under these conditions. Once again

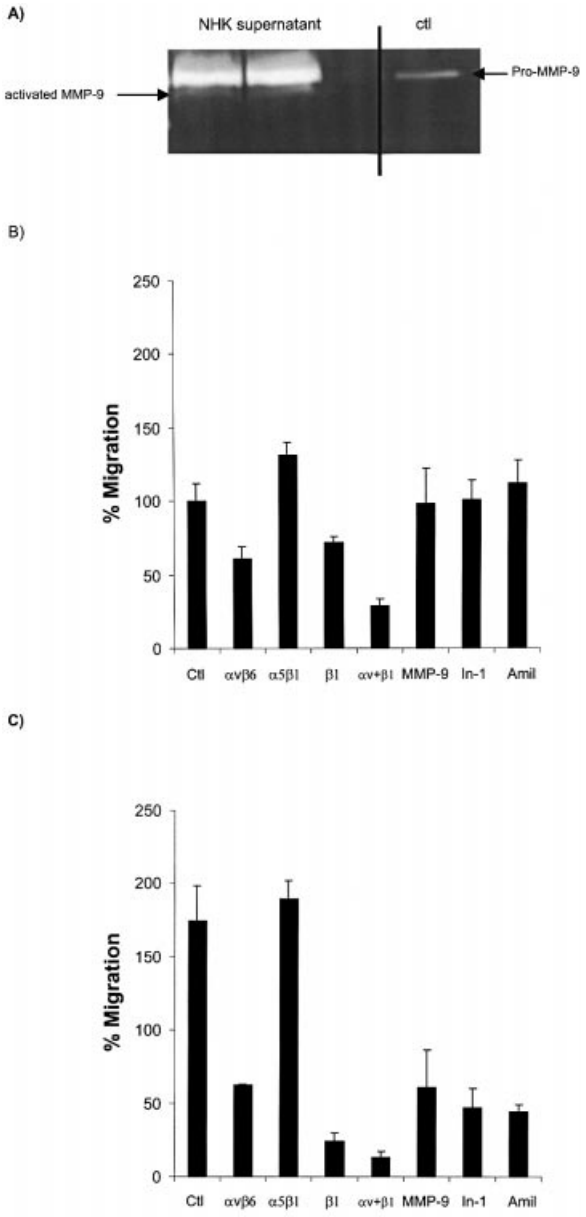

Figure 5. Activation of MMP-9 increases NHK migration. Cell migration assays were performed in the presence of specific integrin and MMP-9 blocking antibodies, and specific MMP-9 and uPA chemical inhibitors (in-1 and amiloride, respectively). Under serum-free conditions NHK secrete pro-MMP-9 and so plasminogen was added to the assay leading to activation of MMP-9. *Error bars* represent standard deviation. (*A*) Zymogram demonstrating activation of MMP-9 by NHK on addition of plasminogen to the migration assay. Control is supernatant from a human OSCC line VB6, which produces large amounts of pro-MMP-9. (*B*) Migration of NHK is inhibited by anti-αvβ6, αv, and β1 antibodies. Maximum inhibition was produced by a combination of anti-αv and anti-β1. Anti-α5β1 produced an increase in cell migration. MMP-9 and uPA inhibitors did not affect migration. Results are expressed relative to an irrelevant control antibody (= 100). Error bars represent standard deviation. (*C*) Addition of plasminogen increases NHK migration. Results are expressed relative to the plasminogen-free migration of cells treated with control antibody. Specific anti-MMP-9 antibody and an MMP-9 chemical inhibitor (in-1) abolished the increase in migration. Furthermore, the uPA inhibitor amiloride (Amil) also significantly inhibited migration suggesting that MMP-9 is activated through an enzymatic cascade involving the catalytic conversion of plasminogen to plasmin.

the maximal inhibition was produced when a combination of anti-αv and anti-β1 antibodies was used (90% inhibition; $p < 0.001$).

DISCUSSION

During the process of cutaneous and oral wound repair, keratinocytes migrate over a provisional matrix of fibrin, fibronectin, vitronectin, and tenascin (Clark *et al*, 1982; Cavani *et al*, 1993). Keratinocyte locomotion is an essential process during early wound healing, and cellular division only occurs after a lag phase (Clark, 1985). Migrating and proliferating keratinocytes degrade and reduce their contact with the basement membrane allowing exposure to components of the extracellular matrix and blood clot (Clark, 1985). Accordingly, keratinocytes modify the expression, cell-surface distribution, and cytoskeletal association of integrin receptors (Haapasalmi *et al*, 1996) and also gain the ability to degrade connective tissue (Ravanti and Kahari, 2000).

Dissolution of the basement membrane by keratinocytes is thought to be the prerequisite for epithelial cell migration into the wound area. Gelatinases, which degrade type IV collagen (the major component of basement membrane) and which also have a broad substrate specificity, are believed to be important in the initial degradation of basement membrane and in the pericellular lysis that occurs in the wound bed (Salo *et al*, 1991). *In situ* hybridization studies of MMP-2 or MMP-9 expression in skin and oral mucosal wounds have localized MMP-9 mRNA expression to keratinocytes and MMP-2 mRNA to fibroblasts in the wound bed (Oikarinen *et al*, 1993; Salo *et al*, 1994).

Expression of the integrin αvβ6 appears to be restricted to a subset of epithelial cells (Breuss *et al*, 1993, 1995) and in adult tissues, with the exception of secretory phase endometrium, is expressed at very low or undetectable levels (Breuss *et al*, 1995). In contrast to this, αvβ6 expression is increased dramatically during development, following injury or inflammation, and in a variety of epithelial neoplasms (Breuss *et al*, 1995; Agrez *et al*, 1996; Jones *et al*, 1997). Several studies have demonstrated the upregulation of αvβ6 integrin by keratinocytes in both cutaneous and oral wounds (Breuss *et al*, 1994; Clark *et al*, 1996; Haapasalmi *et al*, 1996) although the exact function of this heterodimer remains unclear. Whereas αvβ6 expression is not detectable on normal keratinocytes *in vivo*, strong expression is found in cultured cells where conditions may mimic a wound environment (Huang *et al*, 1998).

De novo expression of αvβ6 has been shown to modulate several cellular processes in colon carcinoma cells including cell adhesion and spreading on fibronectin, proliferation within collagen gels, and MMP-9 upregulation (Agrez *et al*, 1994; Weinacker *et al*, 1994; Niu *et al*, 1998). Similarly, Thomas *et al* (2001) demonstrated upregulation of MMP-9 in β6-transfected squamous carcinoma cells. Huang *et al* (1998) found that αvβ6 has a critical role in keratinocyte migration on fibronectin (and vitronectin) and that this effect is enhanced by protein kinase C activation. Apparently somewhat paradoxically cutaneous wounds heal uneventfully in β6−/− mice (Huang *et al*, 1996), though it might be that early deletion of β6 leads to other integrin receptors fulfilling the role of the αvβ6 heterodimer.

In this report we demonstrate αvβ6-dependent upregulation of MMP-9 by NHK on fibronectin. This upregulation could be blocked with specific anti-αvβ6 or anti-αv antibodies but not by antibodies against αvβ5, α5β1, or β1. Several other studies have shown integrin-dependent modulation of MMPs upon interaction with ligands. For example, Huhtala *et al* (1995) reported that when rabbit synovial fibroblasts bound to fibronectin via α5β1 they upregulated MMP-9, whereas adhesion via α4β1 decreased MMP-9 expression. Melanoma cells modulate MMP-2 through αvβ3 and bind the active form of the enzyme on the cell surface via this integrin (Seftor *et al*, 1993; Brooks *et al*, 1996). Larjava *et al* (1993) found that MMP-9 expression in keratinocytes could be stimulated using antibodies against α3 and β1 subunits.

The role of integrins in cell migration is well documented. Keratinocytes freshly isolated from skin are relatively immobile and their ability to migrate develops with time in culture (Guo *et al*, 1990). αvβ5, αvβ6, α2β1, α3β1, and α5β1 have all been shown to play a functional role in keratinocyte migration on their respective

ligands, although many of these studies have been somewhat contradictory (Toda *et al*, 1987; Adams and Watt, 1991; Kim *et al*, 1992a, b, 1994; Haapasalmi *et al*, 1996; Huang *et al*, 1998). We demonstrate that, in NHK, αvβ6 functions in both adhesion to and migration towards fibronectin. Interestingly, inhibition of α5β1 reduced NHK adhesion to fibronectin but produced an increase in cell migration possibly suggesting that the function of this integrin may be to anchor cells to fibronectin. A reduction in migration was observed using anti-β1 antibody, however, indicating that other integrins of the β1 family may actively be involved in modulating this cellular behavior.

The role of activated MMPs in cell migration where cells are moving over a substrate (as opposed to cell invasion where cells move through a substrate) is not clear, although several studies have demonstrated a potential role for these enzymes in cell migration (Pilcher *et al*, 1997; Giannelli *et al*, 1997). McCawley *et al* (1998) found that EGF- and HGF-mediated keratinocyte migration was coincident with induction of MMP-9. They further demonstrated that inhibitors of MMP activity or addition of an MMP-9 neutralizing antibody prevented growth-factor-induced colony dispersion, suggesting a functional role for MMP-9 induction during this response (McCawley *et al*, 1998). Makela *et al* (1999) recently demonstrated that MMP-2 is involved in random keratinocyte migration on uncoated coverslips. Previous studies have suggested that the role of MMP-2 in cell migration is not to destroy matrix proteins but to modify them, exposing cryptic sites that trigger cell migration (Giannelli *et al*, 1997). It is also possible that adherent cells use activated enzyme to degrade the matrix (endogenous or exogenous) to which they are attached, making cells less adherent and allowing them to move more freely. Palecek *et al* (1997) demonstrated the role of cell adhesion in migration and showed that optimal migration was reached at a point between cells being ''too adherent'' and ''not adherent enough''.

Activation of MMP-9 produced a significant increase in NHK migration towards fibronectin. MMP-9 is produced in proenzyme form by NHK and under serum-free conditions, as in migration assays, is not activated. Previous studies have suggested that MMP-9 may be activated through an enzymatic cascade involving the generation of plasmin by the uPa (Juarez *et al*, 1993; Ramos-DeSimone *et al*, 1999). Confirming these previous studies, addition of plasminogen to the migration assays resulted in activated MMP-9 and produced a significant increase in migration towards fibronectin (**Fig 5**). The increased migration was inhibited by an anti-MMP-9 antibody (which prevents activation of the enzyme), a specific MMP-9 chemical inhibitor, and a uPA inhibitor, amiloride, which suggests that activation of MMP-9 is catalyzed, at least partially, through the conversion of plasminogen to plasmin. These data suggest that, whereas migration of NHK is not MMP-9 dependent, activation of MMP-9 does increase migration. As wounds contain significant amounts of plasmin and plasminogen (Lund *et al*, 1999), this could provide a mechanism to activate MMP-9 *in vivo*. Furthermore, the subcellular localization of MMP-9 to the terminal aspects of filopodia in monensin-treated cells suggests a cellular mechanism for controlling the location of MMP-9 activity, and that filopodia may play a role in keratinocyte movement. Intriguingly, a recent study has demonstrated that urokinase receptor (uPAR) may act as a ligand for several integrins and modulates cell behavior accordingly. The similar subcellular distribution of αvβ6 and MMP-9 raises the possibility of a similar interaction (Tarui *et al*, 2001).

In summary, the upregulation of αvβ6 by wound keratinocytes has been demonstrated by immunohistochemistry in several clinical studies but there are few data to suggest a possible biologic role (Breuss *et al*, 1995; Clark *et al*, 1996; Haapasalmi *et al*, 1996). For the first time, we provide experimental evidence that the increased expression of αvβ6 by NHK results in coordinate changes, which promote a more migratory phenotype. Thus we show that increased expression of αvβ6 results in a fibronectin-dependent increase in pro-MMP-9, that MMP-9 activity increases αvβ6-dependent migration, and that this may be further dependent on plasmin activation. The results suggest a key role for αvβ6 in these processes, and indicate a coordinated link between αvβ6 expression and upregulation of MMP-9.

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REFERENCES

Adams JC, Watt FM: Expression of β1, β3, β4 and β5 integrins by human epidermal keratinocytes and non-differentiating keratinocytes. *J Cell Biol* 115:829–841, 1991

Agrez MV, Bates RC, Mitchell D, Wilson N, Ferguson N, Anseline P, Sheppard D: Multiplicity of fibronectin-binding alpha V integrin receptors in colorectal cancer. *Br J Cancer* 73:887–892, 1996

Agrez M, Chen A, Cone RI, Pytela R, Sheppard D: The αvβ6 integrin promotes proliferation of colon carcinoma cells through a unique region of the β_6 cytoplasmic domain. *J Cell Biol* 127:547–556, 1994

Breuss JM, Gallo J, Delisser HM, *et al*: Expression of the β6 subunit in development, neoplasia and tissue repair suggests a role in epithelial remodeling. *J Cell Sci* 108:2241–2251, 1995

Breuss JM, Gillett N, Lu L, Sheppard D, Pytela R: Restricted distribution of integrin beta 6 mRNA in primate epithelial tissues. *J Histochem Cytochem* 41:1521–1527, 1993

Brooks PC, Stromblad S, Sanders LC, *et al*: Localization of matrix-metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin αvβ3. *Cell* 85:683–693, 1996

Brunner KT, Enger HD, Cerottini J-C: The ^{51}Cr-release assay as used for the quantitative measurement of cell-mediated cytolysis *in vitro*. In: Bloom BR, David JHR, eds. *In Vitro Methods in Cell-Mediated and Tumour Immunity* New York: Academic Press, 1976:pp 423–436

Cavani A, Zambruno G, Marconi A, Manca V, Marchetti M, Giannetti A: Distinctive integrin expression in the newly forming epidermis during wound healing in humans. *J Invest Dermatol* 101:600–604, 1993

Clark RAF: Cutaneous wound repair: basic biological considerations. *J Am Acad Dermatol* 13:701–725, 1985

Clark RAF, Lanigan JM, Dellepella P, Manseau E, Dvorak HF, Colvin RB: Fibronectin and fibrin provide a provisional matrix for epidermal cell migration during wound reepithelialisation. *J Invest Dermatol* 79:264–269, 1982

Clark RAF, Ashcroft GS, Spencer MJ, Larjava H, Ferguson MWS: Re-epithelialization of normal human excisional wounds is associated with a switch from αvβ5 to αvβ6 integrins. *Br J Dermatol* 135:46–51, 1996

Damsky CH, Werb Z: Signal transduction by integrin receptors for extracellular matrix: cooperative processing of extracellular information. *Curr Opin Cell Biol* 4:772–781, 1992

Freshney RI: Disaggregation of the tissue and primary culture. In: Freshney RI, ed. *Culture of Animal Cells: a Manual of Basic Technique* 2nd edn. New York: Wiley-Liss, 1987:pp 107–126

Giannelli G, Falk-Marzillier J, Schriraldi O, Stetler-Stevenson WG, Quaranta V: Induction of cell migration by matrix metalloproteinase-2 cleavage of laminin-5. *Science* 277:225–228, 1997

Guo M, Toda K-I, Grinnell F: Activation of human keratinocyte migration on type I collagen and fibronectin. *J Cell Sci* 96:197–205, 1990

Haapasalmi K, Zhang K, Tonnesen M, *et al*: Keratinocytes in human wounds express αvβ6 integrin. *J Invest Dermatol* 106:1–7, 1996

Huang XZ, Wu J, Cass D, *et al*: Inactivation of the integrin β6 subunit reveals a role of epithelial integrins in regulating inflammation in the lungs and skin. *J Cell Biol* 133:921–928, 1996

Huang X, Wu J, Spong S, Sheppard D: The integrin αvβ6 is critical for keratinocyte migration on both its known ligand, fibronectin, and on vitronectin. *J Cell Sci* 111:2189–2195, 1998

Huhtala P, Humphries MJ, McCarthy JB, Tremble PM, Werb Z, Damsky CH: Cooperative signaling by α5β1 and α4β1 integrins regulates metalloproteinase gene expression in fibroblasts adhering to fibronectin. *J Cell Biol* 129:867–879, 1995

Hynes RO: Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* 69:11–25, 1992

Jones J, Sugiyama M, Speight PM, Watt FM: Restoration of αvβ5 integrin in neoplastic keratinocytes results in increased capacity for terminal differentiation and suppression of anchorage independent growth. *Oncogene* 12:119–126, 1996

Jones J, Watt FM, Speight PM: Changes in the expression of α_v integrins in oral squamous cell carcinoma. *J Oral Path Med* 26:63–68, 1997

Juarez J, Clayman G, Nakajima M, Saya KK, Nicolson GL, Boyd D: Role and regulation of expression of 92-kDa type-IV collagenase (MMP-9) in 2 invasive squamous cell carcinoma cell lines of the oral cavity. *Int J Cancer* 55:10–18, 1993

Kim JP, Zhang K, Chen JD, Wynn KC, Kramer RH, Woodley DT: Mechanism of human keratinocyte migration on fibronectin: unique roles of RGD site and integrins. *J Cell Physiol* 151:443–450, 1992a

Kim JP, Zhang K, Kramer RH, Schall TJ, Woodley DT: Integrin receptors and

RGD sequences in human keratinocyte migration: unique anti-migratory function of alpha 3 beta 1 epiligrin receptor. *J Invest Dermatol* 98:764–770, 1992b

Kim JP, Zhang K, Chen JD, Kramer RH, Woodley DT: Vitronectin-driven human keratinocyte locomotion is mediated by the alph v beta 5 integrin receptor. *J Biol Chem* 269:26926–26932, 1994

Kobayashi T, Hattori S, Nagai Y, Sakuraoka K, Nishikawa T: Secretion of different types of gelatinases from cultured human keratinocytes. *J Dermatol* 24:213–216, 1997

Kobayashi T, Hattori S, Nagai Y, Tajima S, Nishikawa T: Differential regulation of MMP-2 and MMP-9 gelatinases in cultured human keratinocytes. *Dermatology* 197:1–5, 1998

Larjava H, Lyons JG, Salo T, *et al*: Anti-integrin antibodies induce type-IV collagenase expression in keratinocytes. *J Cell Physiol* 157:190–200, 1993

Lund LR, Romer J, Bugge TH, *et al*: Functional overlap between two classes of matrix-degrading proteases in wound healing. *EMBO J* 18:4645–4656, 1999

Makela M, Larjava H, Pirila E, Maisi P, Salo T, Sorsa T, Uitto V-J: Matrix metalloproteinase-2 (gelatinase A) is related to migration of keratinocytes. *Exp Cell Res* 251:67–78, 1999

McCawley LJ, O'Brien P, Hudson LG: Epidermal growth factor (EGF)- and scatter factor/hepatocyte growth factor (SF/HGF)-mediated keratinocyte migration is coincident with induction of matrix metalloproteinase (MMP)-9. *J Cell Physiol* 176:255–265, 1998

Niu J, Gu X, Turton J, Meldrum C, Howard EW, Agrez M: Integrin mediated signalling of gelatinase B secretion in colon carcinoma cells. *Biochem Biophys Res Comm* 249:287–291, 1998

Oikarinen A, Kylmaniemi M, Autio-Harmainen H, Autio P, Salo T: Demonstration of 72-kDa and 92-kDa forms of type IV collagenase in human skin: variable expression in various blistering diseases, induction during re-epithelialization, and decrease by topical glucocorticoids. *J Invest Dermatol* 101:205–210, 1993

Palecek SP, Loftus JC, Ginsberg MH, Lauffenberger DA, Horwitz AF: Integrin-ligand binding properties govern cell migration speed through cell-substratum adhesiveness. *Nature* 385:537–540, 1997

Pilcher BK, Dumin JA, Sudbeck BD, Krane SM, Welgus HG, Parks WC: The activity of collagenase-1 is required for keratinocyte migration on a type I collagen matrix. *J Cell Biol* 137:1445–1457, 1997

Ramos-DeSimone N, Hahn-Dantona E, Sipley J, Nagase H, French D, Quigley JP: Activation of matrix metalloproteinase-9 (MMP-9) via a converging plasmin/stromelysin-1 cascade enhances tumor cell invasion. *J Biol Chem* 274:13066–13076, 1999

Ravanti L, Kahari V: Matrix metalloproteinases in wound repair. *Int J Mol Med* 6:391–407, 2000

Salo T, Lyons JG, Rahemtulle F, Birkedal-Hansen H, Larjava H: Transforming growth factor-β1 upregulates type IV collagenase expression in cultured human keratinocytes. *J Biol Chem* 266:11436–11441, 1991

Salo T, Makela M, Kylmaniemi M, Autio-Harmainen H, Larjava H: Expression of matrix metalloproteinase-2 and -9 during early human wound healing. *Lab Invest* 70:176–182, 1994

Seftor REB, Seftor EA, Stetler-Stevenson WG, Hendrix MJC: The 72kDa type IV collagenase is modulated via differential expression of alpha v beta 3 and alpha 5 beta 1 integrins during human melanoma cell invasion. *Cancer Res* 53:3411–3415, 1993

Tarui T, Mazar AP, Cines DB, Takada Y: Urokinase receptor (uPAR/CD87) is a ligand for integrins and mediates cell–cell interaction. *J Biol Chem* 276:3983–3990, 2001

Thomas GJ, Lewis MP, Hart IR, Marshall JF, Speight PM: αvβ6 integrin promotes invasion by squamous carcinoma cells through upregulation of. MMP-9. *Int J Cancer* in press, 2001

Toda K-I, Tuan T-L, Brown PJ, Grinnell F: Fibronectin receptors of human keratinocytes and their expression during cell culture. *J Cell Biol* 105:3097–3104, 1987

Weinacker A, Chen A, Agrez M, *et al*: Role of the integrin alpha v beta 6 in cell attachment to fibronectin. Heterologous expression of intact and secreted forms of the receptor. *J Biol Chem* 269:6940–6948, 1994