

# $\alpha$ v $\beta$ 6 Integrin Upregulates Matrix Metalloproteinase 9 and Promotes Migration of Normal Oral Keratinocytes

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The integrin  $\alpha$ v $\beta$ 6 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  $\alpha$ v $\beta$ 6 and this integrin has a functional role in both cell adhesion and migration towards fibronectin. We provide experimental evidence that the increased expression of  $\alpha$ v $\beta$ 6 by normal human oral keratinocytes results in coordinate changes, which promote a more migratory phenotype. Thus increased expression of  $\alpha$ v $\beta$ 6 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  $\alpha$ v $\beta$ 6 in these processes and indicate a coordinated link between  $\alpha$ v $\beta$ 6 expression and upregulation of matrix metalloproteinase 9. It appears that  $\alpha$ v $\beta$ 6 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*

**M**igration 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  $\alpha$ v $\beta$ 6 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  $\alpha$ v $\beta$ 5 expression to  $\alpha$ v $\beta$ 6 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  $\alpha$ v $\beta$ 5 and *de novo* expression of  $\alpha$ v $\beta$ 6 (Breuss *et al*, 1994; Jones *et al*, 1997). Thus squamous cell carcinoma may represent a process of uncontrolled tissue remodeling.

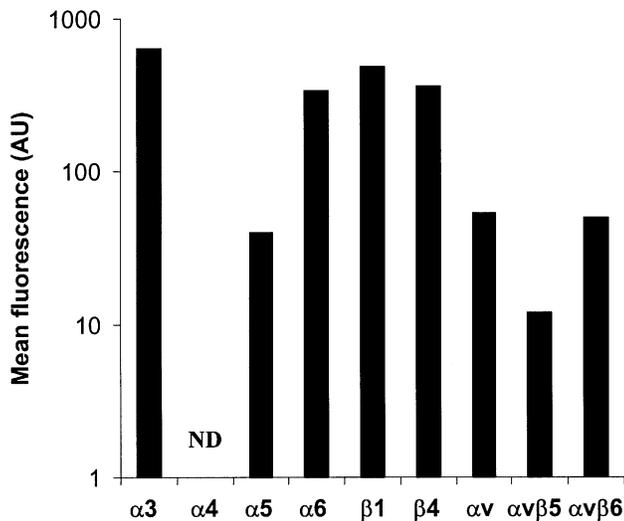
The upregulation of  $\alpha$ v $\beta$ 6 by wound keratinocytes suggests that this integrin heterodimer could have an active role in cell movement. Huang *et al* (1998) reported greatly reduced migration on fibronectin and vitronectin by keratinocytes from  $\beta$ 6 knockout mice. Restoration of  $\alpha$ v $\beta$ 5 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  $\beta$ 6 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.

<sup>1</sup>This project was supervised jointly by P.M. Speight and J.F. Marshall.



**Figure 1. Flow cytometric analysis of integrin expression by NHK.** The geometric mean fluorescence (arbitrary 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  $\alpha$ v $\beta$ 6 expression by NHK and demonstrated that NHK also express the  $\alpha$ 5 $\beta$ 1 fibronectin receptor and show high expression of  $\alpha$ 6,  $\beta$ 4,  $\alpha$ 3, and  $\beta$ 1 integrin subunits.

In this study we have examined the functional significance of  $\alpha$ v $\beta$ 6 expression by primary oral keratinocytes established in tissue culture. Our results show that such cultured keratinocytes express high levels of functional  $\alpha$ v $\beta$ 6 and that MMP-9 upregulation by keratinocytes plated on fibronectin is  $\alpha$ v $\beta$ 6 dependent. Increased expression of  $\alpha$ v $\beta$ 6 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  $\alpha$ v $\beta$ 6 in these processes and indicate a coordinated link between  $\alpha$ v $\beta$ 6 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  $\alpha$ v) was prepared in our laboratory from hybridoma cells obtained from the American Type Culture Collection (Rockville, MD) (Weinacker *et al.*, 1994). The anti- $\alpha$ v $\beta$ 6 (E7P6 and R6G9) (Weinacker *et al.*, 1994), anti- $\alpha$ v $\beta$ 6 (10D5; Huang *et al.*, 1998), anti- $\alpha$ 5 $\beta$ 1 (P1D6), and anti- $\beta$ 1 (P4C10) antibodies were purchased from Chemicon International (Harrow, U.K.). Anti- $\alpha$ v $\beta$ 5 (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<sup>2</sup> flasks in keratinocyte growth medium (KGM) as described. KGM comprised  $\alpha$ -modified Eagle's medium ( $\alpha$ -MEM) containing 10% fetal bovine serum (GlobePharm, Surrey, U.K.) supplemented with 100 IU per liter penicillin, 100  $\mu$ g per liter streptomycin, and 2.5  $\mu$ g per liter amphotericin B (Gibco BRL),  $1.8 \times 10^{-4}$  M adenine, 5  $\mu$ g per ml insulin,  $1 \times 10^{-10}$  M cholera toxin,

0.5  $\mu$ g 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.

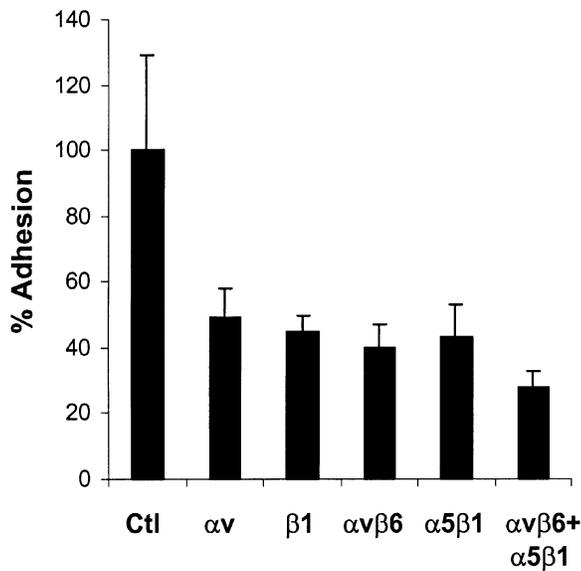
**Flow cytometry** Subconfluent cells were washed with phosphate-buffered saline (PBS) and harvested by trypsin/ethylenediamine tetraacetic acid (0.25% wt/vol, 5 mM). Cells were incubated with primary antibody for 40 min at 4°C and washed twice with PBS. FITC-conjugated secondary antibody was applied 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. Labeled cells were scanned on a FACSCalibur cytometer (Becton Dickinson) and analyzed using Cellquest software, acquiring  $1 \times 10^4$  events.

**Adhesion assays** Ninety-six well plates (Falcon 3912; Becton Dickinson) were coated with plasma fibronectin (Sigma). A 50  $\mu$ l fibronectin solution at a concentration of 10  $\mu$ g 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 [<sup>51</sup>Cr] labeled (Brunner *et al.*, 1976), washed, and resuspended in  $\alpha$ -MEM ( $1.5 \times 10^4$  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<sub>2</sub> and 0.5 mM MgCl<sub>2</sub>). 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  $\alpha$ v** Two  $\times 10^4$  cells were plated onto 13 mm glass coverslips coated with fibronectin (10  $\mu$ g per ml) and blocked with 0.5% BSA in PBS. Coverslips were incubated for 24 h at 37°C in 5% CO<sub>2</sub>. For MMP-9 analysis cells were incubated for 24 h in serum-free medium containing 1  $\mu$ M 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- $\alpha$ v; 18  $\mu$ g 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  $\mu$ l of fibronectin at 10  $\mu$ g 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  $\alpha$ -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<sub>2</sub>, 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

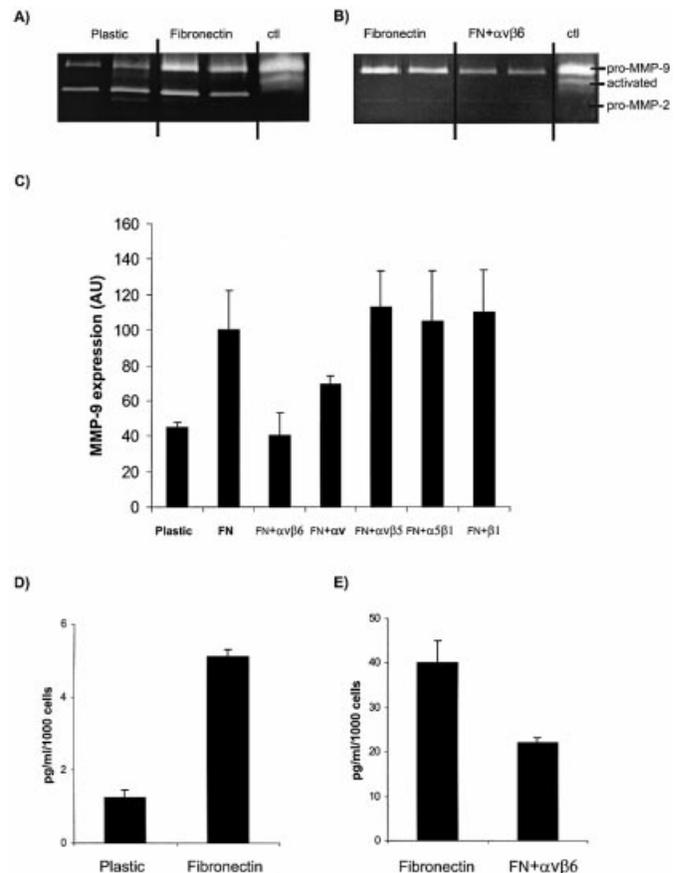


**Figure 2** NHK adhere to fibronectin using both  $\alpha v\beta 6$  and  $\alpha 5\beta 1$  integrins. Adhesion to fibronectin of NHK in the presence of control antibody was 24% of the plated cells. Background binding to BSA-coated wells was 5% and has been subtracted from the results. Results are expressed relative to control antibody (= 100%). Adhesion of NHK was blocked by antibodies to both  $\alpha v\beta 6$  (60% inhibition) and  $\alpha 5\beta 1$  (57% inhibition), as well as against the  $\alpha v$  and  $\beta 1$  subunits (51% and 55% inhibition, respectively). Maximal inhibition of adhesion was obtained using a combination of anti- $\alpha v\beta 6$  and anti- $\alpha 5\beta 1$  (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.

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  $\mu$ l 1 M  $H_2SO_4$  and the resultant color change was read at 450 nm on a spectrophotometer (SLT Lab Instruments, 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  $\mu$ m pore size, Transwell<sup>®</sup>, Becton Dickinson). The membrane undersurface was coated with fibronectin (10  $\mu$ g per ml) in PBS for 1 h at 37°C and blocked with migration buffer (0.5% BSA in  $\alpha$ -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  $\mu$ l of migration buffer, following which cells were plated in the upper chamber of triplicate wells, at a density of  $4 \times 10^4$  in 100  $\mu$ l of migration buffer, and incubated at 37°C for 20 h. After 20 h, the cells in the lower chamber (including those



**Figure 3.** Upregulation of MMP-9 by NHK on fibronectin is  $\alpha v\beta 6$  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  $\mu$ g 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- $\alpha v\beta 6$  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- $\alpha v\beta 6$  antibody. Error bars represent standard deviation.

attached to the undersurface of the membrane) were trypsinized and counted on a Casy 1 counter (Sharfe System, Germany).

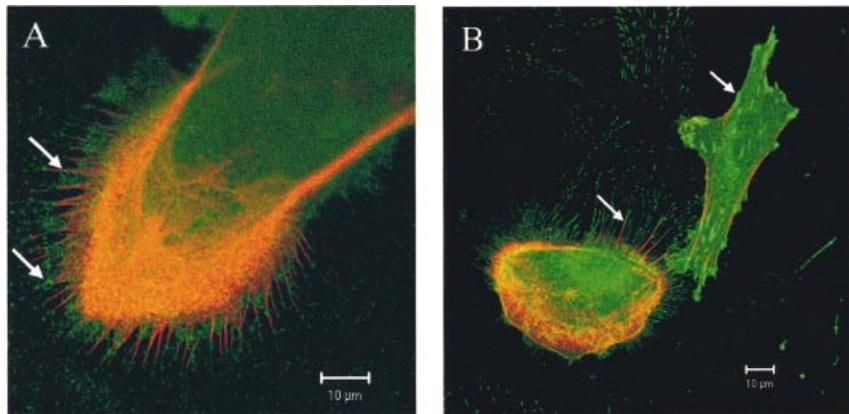
**Statistical analysis** Data are expressed as the mean  $\pm$  SD of a given number of observations. Where appropriate, one-way analysis of variance was used to compare multiple groups. Comparisons between groups were by Fisher's PLSD (set at 5% significance). A p-value of less than 0.05 was considered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

## RESULTS

**Cultured keratinocytes show relatively high  $\alpha v\beta 6$  expression** Levels of surface expression of integrins were determined by flow cytometry. NHK express low levels of  $\alpha v\beta 5$  (mean fluorescence 12) but much greater levels of  $\alpha v\beta 6$  (mean fluorescence 50) (Fig 1). Flow cytometry demonstrated that NHK also express the  $\alpha 5\beta 1$  fibronectin receptor and show high expression of  $\alpha 6$ ,  $\beta 4$ ,  $\alpha 3$ , and  $\beta 1$  integrin subunits.

**Adhesion of NHK to fibronectin is mediated by  $\alpha v\beta 6$  and  $\alpha 5\beta 1$**  NHK were plated onto fibronectin in the presence or

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



absence of blocking antibodies against the  $\alpha v$  subunit,  $\alpha v\beta 6$ ,  $\alpha 5\beta 1$ ,  $\beta 1$ , or an irrelevant antibody (W6/32) against class I major histocompatibility complex (Fig 2). Combinations of these antibodies also were used. Antibodies directed against  $\alpha v\beta 6$  (60% inhibition) and  $\alpha 5\beta 1$  (57% inhibition) significantly inhibited adhesion of NHK to fibronectin (as did antibodies against the  $\alpha v$  and  $\beta 1$  subunits; 51% and 55%, respectively). Maximal inhibition of NHK adhesion to fibronectin, however, required a combination of antibodies against  $\alpha v\beta 6$  and  $\alpha 5\beta 1$  (72% inhibition). This demonstrates a functional role for  $\alpha v\beta 6$  in NHK adhesion to fibronectin and that NHK may adhere through both  $\alpha 5\beta 1$  and  $\alpha v\beta 6$ . 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  $\alpha v\beta 6$ -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 3A).

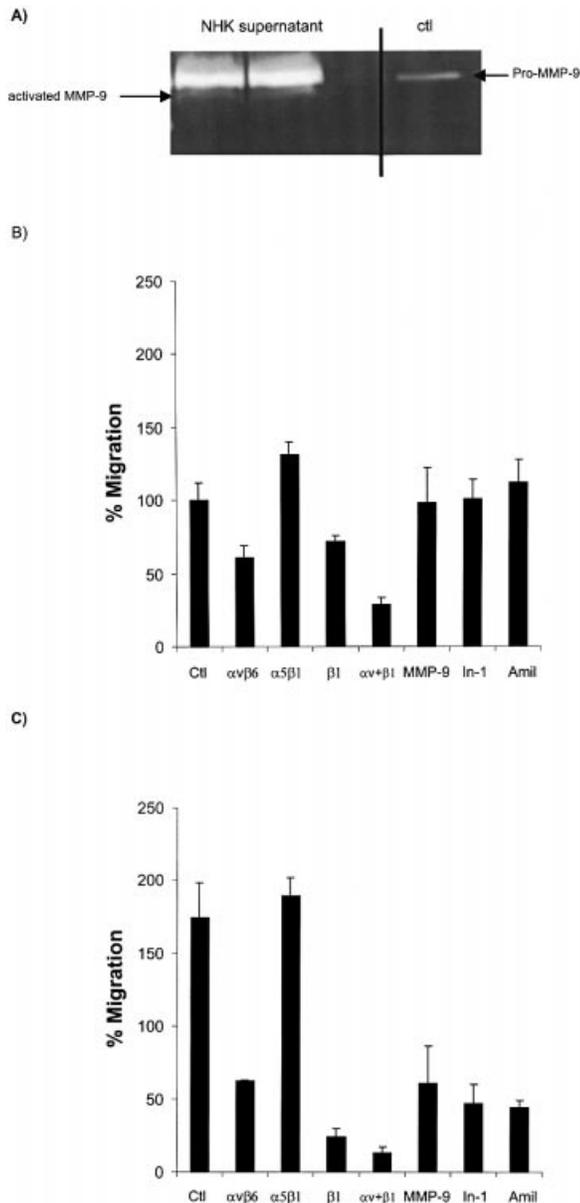
To test the integrin dependence of the MMP-9 upregulation on fibronectin NHK were incubated with specific integrin-blocking antibodies against  $\alpha v$ ,  $\alpha v\beta 5$ ,  $\alpha v\beta 6$ ,  $\alpha 5\beta 1$ , or  $\beta 1$  prior to plating. A significant reduction in MMP-9 levels was observed when  $\alpha v$  or  $\alpha v\beta 6$  was inhibited ( $p = 0.03$ ,  $p < 0.001$ , respectively; Fig 3B). In contrast, expression of MMP-9 was not affected by the other integrin-blocking antibodies ( $\alpha v\beta 5$ ,  $\alpha 5\beta 1$ , or  $\beta 1$ ; Fig 3C), confirming the specificity of  $\alpha v\beta 6$  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  $\alpha v\beta 6$  integrin.

Expression of MMP-9 was also quantitated by ELISA (Fig 3D, E). This confirmed the  $\alpha v\beta 6$ -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  $\alpha v$**  We examined the subcellular distribution of MMP-9 in NHK. Indirect immunocytochemistry on NHK revealed that MMP-9 located to the terminal aspects of filopodia in monensin-treated cells (Fig 4A, arrow) or in larger submembranous vesicles (data not shown) suggesting that alteration in the cell morphology affects MMP-9 distribution. The  $\alpha v$  subunit was detected in focal adhesions (Fig 4B, upper arrow) and, interestingly, was also expressed strongly on filopodia (Fig 4B, lower arrow).

**$\alpha v\beta 6$  functions in NHK migration towards fibronectin** To determine whether  $\alpha v\beta 6$  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- $\alpha v\beta 6$  antibody (39% inhibition;  $p = 0.014$ ) (Fig 5B). Interestingly, inhibition of  $\alpha 5\beta 1$  increased migration (30% increase;  $p = 0.003$ ) (Fig 5B), suggesting that this integrin may function in a purely adhesive capacity and may be inhibitory to cell migration. Migration was also reduced by anti- $\beta 1$  antibodies (28% inhibition;  $p = 0.023$ ) suggesting that integrins of the  $\beta 1$  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- $\alpha v$  and anti- $\beta 1$  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 5B).

**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 5C). 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 5C). 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 5C; 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- $\alpha v\beta 6$  antibodies inhibited migration by 62%, whereas anti- $\alpha 5\beta 1$  antibodies again produced an increase in migration (Fig 5B). Interestingly, blocking  $\beta 1$  integrins produced more effective inhibition of migration in the presence of activated MMP-9, suggesting a more prominent role for  $\beta 1$  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- $\alpha v\beta 6$ ,  $\alpha v$ , and  $\beta 1$  antibodies. Maximum inhibition was produced by a combination of anti- $\alpha v$  and anti- $\beta 1$ . Anti- $\alpha 5\beta 1$  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- $\alpha v$  and anti- $\beta 1$  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  $\alpha v\beta 6$  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,  $\alpha v\beta 6$  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  $\alpha v\beta 6$  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  $\alpha v\beta 6$  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  $\alpha v\beta 6$  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  $\beta 6$ -transfected squamous carcinoma cells. Huang *et al* (1998) found that  $\alpha v\beta 6$  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  $\beta 6^{-/-}$  mice (Huang *et al*, 1996), though it might be that early deletion of  $\beta 6$  leads to other integrin receptors fulfilling the role of the  $\alpha v\beta 6$  heterodimer.

In this report we demonstrate  $\alpha v\beta 6$ -dependent upregulation of MMP-9 by NHK on fibronectin. This upregulation could be blocked with specific anti- $\alpha v\beta 6$  or anti- $\alpha v$  antibodies but not by antibodies against  $\alpha v\beta 5$ ,  $\alpha 5\beta 1$ , or  $\beta 1$ . 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  $\alpha 5\beta 1$  they upregulated MMP-9, whereas adhesion via  $\alpha 4\beta 1$  decreased MMP-9 expression. Melanoma cells modulate MMP-2 through  $\alpha v\beta 3$  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  $\alpha 3$  and  $\beta 1$  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).  $\alpha v\beta 5$ ,  $\alpha v\beta 6$ ,  $\alpha 2\beta 1$ ,  $\alpha 3\beta 1$ , and  $\alpha 5\beta 1$  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,  $\alpha$ v $\beta$ 6 functions in both adhesion to and migration towards fibronectin. Interestingly, inhibition of  $\alpha$ 5 $\beta$ 1 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- $\beta$ 1 antibody, however, indicating that other integrins of the  $\beta$ 1 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  $\alpha$ v $\beta$ 6 and MMP-9 raises the possibility of a similar interaction (Tarui *et al*, 2001).

In summary, the upregulation of  $\alpha$ v $\beta$ 6 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  $\alpha$ v $\beta$ 6 by NHK results in coordinate changes, which promote a more migratory phenotype. Thus we show that increased expression of  $\alpha$ v $\beta$ 6 results in a fibronectin-dependent increase in pro-MMP-9, that MMP-9 activity increases  $\alpha$ v $\beta$ 6-dependent migration, and that this may be further dependent on

plasmin activation. The results suggest a key role for  $\alpha$ v $\beta$ 6 in these processes, and indicate a coordinated link between  $\alpha$ v $\beta$ 6 expression and upregulation of MMP-9.

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