The Role of the Integrin αvβ6 in Regulating the Epithelial to Mesenchymal Transition in Oral Cancer

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Abstract. *In this study, we evaluated whether the forced expression of β6 integrin would modulate the epithelial to mesenchymal transition (EMT). When the full length β6 integrin was expressed in poorly invasive squamous cell carcinoma SCC9 cells, the resulting SCC9β6 cells acquired a fibroblast-like morphology, increased expression of the mesenchymal marker vimentin and reduced expression of the epithelial markers keratin and E-cadherin. SCC9β6D1 cells, which express a truncated form of β6 subunit lacking the C-terminal 11 amino acids (AA), retained their epithelial morphology and did not alter vimentin or E-cadherin expression. This suggests that the full-length β6 subunit can induce EMT in oral SCC cells. We previously showed that expression of β6 increases both MMP-3 activation and tenascin-C expression and we now show that both molecules are MEK dependent. These results also demonstrate that the terminal 11 AA of β6 contain information important for establishing an epithelial to mesenchymal transition.*

Metastasis remains the most poorly understood aspect of oral cancer, which continues to have an extremely poor prognosis (5-year survival rate, 50%) (1). During metastatic progression, polarized epithelial tumor cells are converted into motile mesenchymal cells, invade the basement membrane beneath, enter blood vessels and disseminate into a secondary site. The initial stage of these processes is associated with morphogenetic changes referred to as the epithelial to mesenchymal transition (EMT) and it has long been suggested to be an important process in many types of epithelial cancer. The molecular basis of the EMT process involves changes in the expression, distribution and function of a number of proteins that play a role in extracellular matrix (ECM) remodeling or in cell–cell adhesion, such as matrix metalloproteinases (MMPs), E-cadherin, and integrins (2).

Loss of epithelial cell polarity and acquisition of the motile phenotype requires loss of cell–cell adhesion, reorganization of the cytoskeleton, and alterations in the gene expression profiles (3). E-cadherin, a key "read-out molecule" in the EMT, mediates cell–cell adhesion between adjacent epithelial cells and helps maintains epithelial integrity. E-cadherin expression and its subcellular localization is altered during tumor progression, as cells change shape and become more like mesenchymal cells. Cell-surface receptors, such as the integrins, bind to ECM components and play a major role in modifying the cell attachment required for cell motility and invasion, and they can also serve as transducers of cell survival signals.

An invasive carcinoma cell must acquire the ability to interact with distinct interstitial matrices subsequent to its transgression of the basement membrane. Accordingly, the integrin family of adhesion molecules represents the major receptors that mediate attachment to the ECM, with ligand occupancy triggering critical intracellular signaling pathways (4). Surprisingly little is known about how integrin expression and function are regulated during the EMT. The upregulation of ECM molecules such as tenascin-C (TN-C) is repeatedly seen in epithelial tumors of the head and neck, thereby assigning a mesenchymal property to the cells (5). The integrin αvβ6 is an epithelial specific integrin and has been shown to be a receptor for Arg–Gly–Asp (RGD) sites in fibronectin, TN-C and vitronectin. Activation of TGFβ1 occurs through the binding of αvβ6 to the latency-associated peptide (LAP) (6). This sustains the EMT and is also required for the migration of post-EMT cells on fibronectin and TN-C. Both αvβ6 and TN-C have been shown by our laboratory to be up–regulated in oral cancer and are indicative of aggressive disease with high recurrence rate. Work leading up to this point demonstrates that expression of β6 promotes cell motility (7). We also found that expression of full length β6, but not the truncated variant, confers a fibroblast-like morphology to the cells.

Although the genetic and phenotypic makeup of a tumor contribute to tissue-specific metastasis, it is equally necessary

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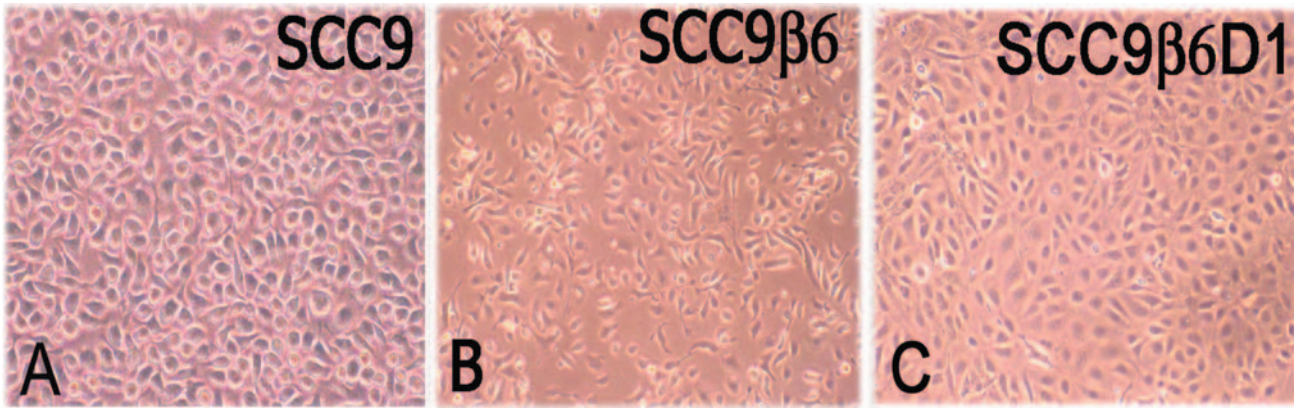

Figure 1. *Expression of β6 alters oral SCC morphology. A total of 2×10^5 SCC cells were plated onto tissue culture plastic and allowed to form a monolayer overnight (24 h). To evaluate cell morphology, micrographs were taken using a Nikon TE300 inverted microscope. A, SCC9SN; B, SCC9β6 and C, SCC9β6D1 cells. Note the cobblestone appearance of the SCC9SN (A) and the SCC9β6D1 (C) cells as compared to the SCC9β6 cells which have a more elongated, fibroblast-like morphology (B).*

to recognize the involvement of the tumor microenvironment in tumor dissemination. Early changes observed in tissue before evidence of carcinogenesis suggest that local tissue environmental changes can be a critical component of tissue specific metastasis (8). In our study, we investigated the role αvβ6 integrin plays in mediating the EMT.

Materials and Methods

Cell culture. The SCC9 cell line (derived from a tongue lesion) was obtained from Dr James Reinwald (Brigham and Woman's Hospital, Harvard School of Medicine) and has been described elsewhere (9). SCC9β6 and SCC9β6D1 cells were generated in our laboratory through retroviral transduction with the full-length β6 and β6D1 cDNA (7, 10). The full-length β6 and the construct carrying the β6 C-terminal deletion D1 were generous gifts of Dr Dean Sheppard (UCSF). C-Terminal deletion: D1: β6 lacking the C-terminal 11 amino acids (^{778}EKQKVDLSTDC^{788}). The D1 construct was used to establish the SCC9β6D1 cell line. Neither the SCC9β6 nor the SCC9β6D1 cell lines showed changes to integrins other than β6. The empty vector expressing SCC9SN cells were also established in our laboratory as described elsewhere (10).

Growth conditions. Cells were routinely cultivated in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. Previous work from our laboratory demonstrated that transfection with β6 promotes MMP-3 activation (7, 10). In order to determine if MAP kinase was upstream of MMP-3, the SCC9β6 cells were plated on FN for 4 h in the presence (+) or absence (–) of the MEK inhibitor U0126 (0.1 μM). The conditioned medium was collected and concentrated 50-fold, then analyzed by Western blot using anti-MMP-3 antibodies. We have previously shown that TN-C (a mesenchymal protein highly expressed during development) is neoexpressed in oral cancer and it may regulate EMT by establishing a microniche for tumor cell proliferation.

Briefly, SCC9SN and SCC9β6 cells were grown under serum free conditions for 24 h. The media was concentrated, separated by gel electrophoresis and analyzed by Western blot using anti-TN-C antibodies. The gel was quantified by densitometry and assigned relative value units (rvu). To determine if MAPK was involved in TN-C secretion, SCC9β6 cells were grown serum-free for 24 h at 37˚C in 5% CO_2. The MEK inhibitor U0126 (0.1 μM) was added to half of the cultures for 24 h whereas the remaining cultures received no treatment. The cultures were then terminated and the conditioned medium was analyzed by Western blotting.

Reagents. Mouse monoclonal antibodies to TN-C (BC-4) were kindly provided by Dr Luciano Zardi (Istituto Nazionale, Genoa, Italy). Mouse monoclonal antibodies to vimentin (clone 3B4) and to actin (mAb 3128) were purchased from Chemicon International (Temecula, CA, USA). Rabbit polyclonal antibodies to cytokeratin and function-blocking anti-E-cadherin antibodies (clone She-78-7) were from Zymed Laboratories (South San Francisco, CA, USA).

Immunofluorescence microscopy. A total of 2×10^5/ml cells were plated onto fibronectin (FN)-coated glass coverslips (10 μg/ml) for 24 h, serum–free and fixed with 3% paraformaldehyde, permeabilized with 0.1% Triton® X-100. The cells were incubated first with anti-mAb for 1 h and rinsed with phosphate-buffered saline (PBS) and incubated with biotin-conjugated goat anti-mouse IgG (1:50) for 30 min at room temperature followed by an additional rinse with PBS. The cultures were then incubated with fluorescein isothiocyanate (FITC)-conjugated streptavidin (1:100) (Amersham, Piscataway, NJ, USA) for 30 min at room temperature, washed with PBS and mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA). The cultures were then examined for expression of green-fluorescence using immunofluorescence microscopy.

Western blotting. Cells were serum-starved for 24 h and then plated onto FN (10 μg/ml) for 24 h. The cells were then lysed in Nonidet P-40 lysis buffer (1.5% Nonidet P-40, 150 mM, NaCl, 0.2% SDS, 1 mM EDTA, 20 mM Tris-HCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM Na_3VO_4, 50 mM NaF).

Protein concentration was determined by BCA Protein Assay Kit (Pierce, Rockford, IL, USA). The proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Micron Separation Inc, Westborough,

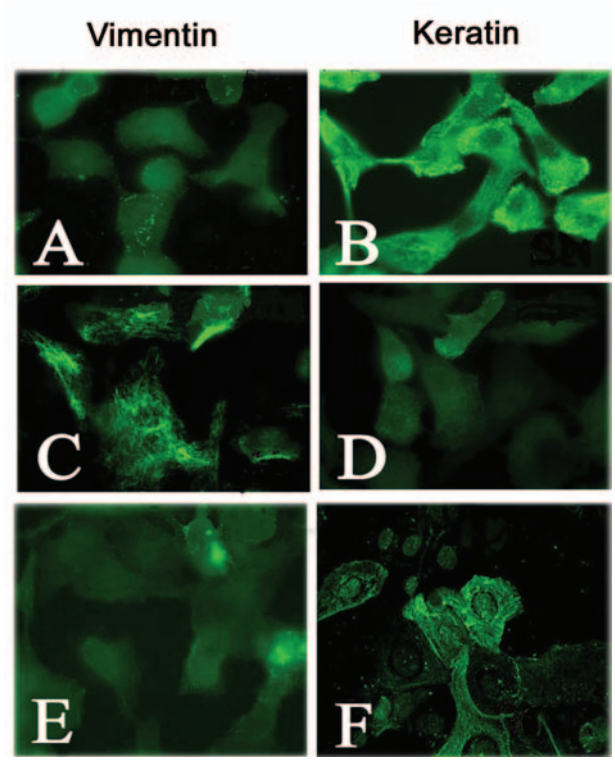

Figure 2. *Integrin β6 modulates vimentin/keratin expression. Three oral cancer cell lines SCC9SN (A, B), SCC9β6 (C, D) and SCC9β6D1 (E, F) cells were plated onto FN (10 μg/ml) for 24 h, under serum-free conditions. The cells were processed for immunofluorescence microscopy using antibodies to vimentin (A, C, E) and cytokeratin (B, D, F). SCC9SN cells reacted strongly with the anti-cytokeratin (B) but not with the anti-vimentin antibodies (A). The SCC9β6 cells reacted very strongly with antibodies to vimentin (C) but not at all with antibodies to keratin (D). SCC9β6D1 stained with the anti-cytokeratin (F) but not the anti-vimentin antibodies (E).*

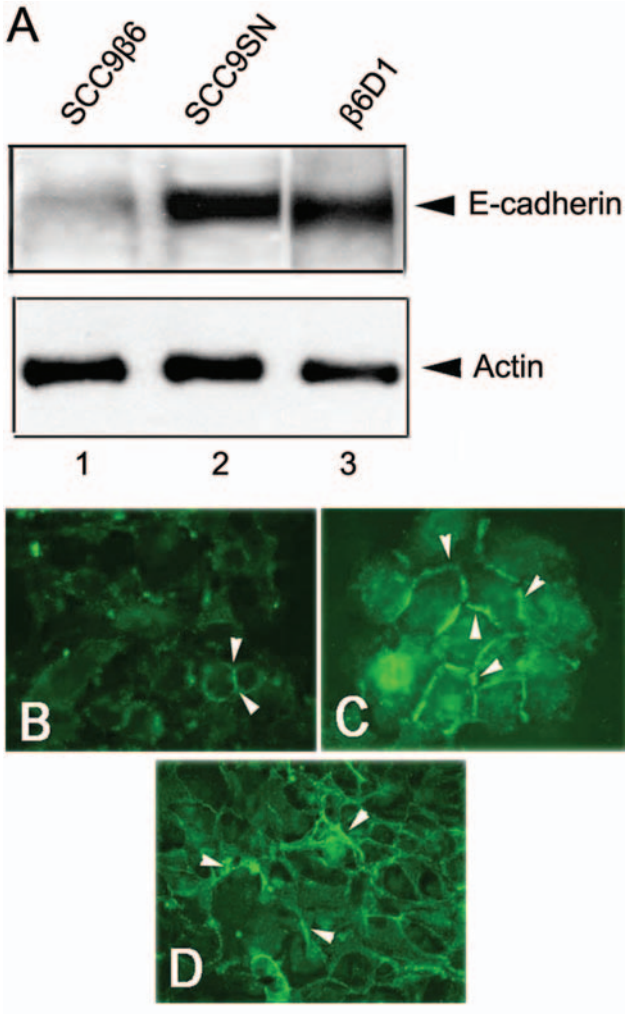

Figure 3. *E-cadherin is modulated by expression of β6. A, Western blotting was performed to evaluate the expression of E-cadherin. A total of 2×10^5 oral SCC cells were plated on FN for 24 hours, lysed and analyzed by Western blotting for expression of E-cadherin. Note the loss of E-cadherin in the SCC9β6 cells (lane 1); in contrast, note the elevated level of E-cadherin in SCC9SN and SCC9β6D1 (lanes 2 and 3, respectively). Immunofluorescence was performed to localize E-cadherin in cultured cells. A total of 2×10^5 cells were plated on FN for 24 hours and then fixed, stained and processed for immunofluorescence microscopy with anti-E-cadherin antibodies. As determined by Western blotting, the SCC9β6 cells were essentially negative for E-cadherin (B). In sharp contrast, SCC9SN and SCC9β6D1 cells had high level expression of E-cadherin (C, D, respectively).*

MA, USA) using a semi-dry blotting apparatus (Bio-Rad, Hercules CA, USA) as described elsewhere (11). The membranes were then developed using the ECL Chemiluminescence Kit (Amersham) and bands were detected by exposure to X-ray film. The blots were quantified and assigned rvu using an image analysis program (NIH Image, http://www.rsb.info/nih-image).

Results

Expression of β6 alters oral SCC morphology. Previous work from our laboratory demonstrated that overexpression of the β6 integrin in poorly invasive oral SCC9 cells increased cell migration and invasion (7, 10). Using αvβ6 function-blocking antibodies, we determined this enhanced motility was β6 dependent. Typically motile cells have morphology distinct from stationary cells. When the SCC9 cells were transfected with β6, cell motility of the transfected cells (SCC9β6) was dramatically increased (7, 10). We therefore next wished to determine if cell shape was affected by expression of β6. We compared the cell morphology between the SCC9SN (Figure 1A) and the SCC9β6 cells (Figure 1B). Upon examination, the SCC9SN cells had the typical "cobblestone" appearance indicative of quiescent, stationary epithelial cells (Figure 1A). In contrast, the SCC9β6 cells (Figure 1B) appeared more fibroblast-like and spindle-shaped.

A construct in which the terminal 11 AA of β6 were deleted (β6D1) was used to transfect the SCC9 cells to derive the SCC9β6D1 cell line (Figure 1C). The terminal 11 AA in β6 integrin is a unique stretch not found in other integrin β subunits. The cell morphology of the SCC9β6D1 cells was compared to the invasive SCC9β6 cells expressing the full-length β6. In contrast to the fibroblast-like morphology presented by the SCC9β6 cells, the SCC9β6D1 cells were more cobblestone in appearance, similar to that exhibited by the parental SCC9SN cells (Figure 1 C). It appears that the mesenchymal phenotype, attributed to the transfection with β6, is modulated through the 11 terminal residues.

Integrin β6 modulates vimentin/keratin expression. Cytokeratin and vimentin are intermediate filaments found typically in epithelial and mesenchymal cells, respectively. Expression of vimentin is correlated with the EMT whereas E-cadherin is a marker of the epithelial phenotype. Therefore, we evaluated the cells for expression of both vimentin and cytokeratin. Briefly, 2×10^5 SCC9SN cells were plated on FN for 24 h, fixed, permeabilized and stained for vimentin (Figure 2A) or cytokeratin (Figure 2B). Almost no staining was detected using antibodies to vimentin (Figure 2 A); whereas, a strong reaction was detected when the SCC9SN cells were processed using antibodies to cytokeratin (Figure 2 B). This indicated that the SCC9SN cells retained a great deal of their epithelial characteristics. In contrast, when the SCC9β6 cells (2×10^5) (Figure 2 C, D) were examined, a converse pattern of vimentin/keratin expression was found. The expression of cytokeratin was barely detectable (Figure 2 D), whereas vimentin was highly expressed in the SCC9β6 cells (Figure 2 C). These results suggest that the mesenchymal phenotype maybe regulated by β6 expression.

Based on the apparent shift in cell morphology, we wished to evaluate intermediate filament (vimentin and keratin) composition in response to β6 truncation. Expression of vimentin was virtually undetectable in SCC9β6D1 cells (Figure 2 E) whereas keratin was highly expressed (Figure 2 F). This confirms our morphological data and indicates that the terminal 11 AA of β6 contains information critical to the EMT.

E-Cadherin is modulated by expression of β6. Cell lysate from the SCC9β6 and the SCC9SN cells was compared for expression of E-cadherin (Figure 3A; lanes 1 and 2 upper panel, respectively). A dramatic decrease in E-cadherin expression was seen in the lysate from the SCC9β6 cells (Figure 3A, lane 1, upper panel) as compared to the SCC9SN cells. When the SCC9β6D1 cell lysate was examined, the expression of E-cadherin was comparable to that found in the SCC9SN cells (Figure 3A, lane 3, upper panel). These results demonstrate that the terminal 11 AA found in the β6 subunit was critical in modulating E-cadherin expression. Actin was used as a loading control (Figure 3A, lower panel).

Immunofluorescence microscopy was used as a visual adjunct to the Western blot to localize structures (Figure 3). Although SCC9β6 cells did not readily form cell–cell junctions, E-cadherin was evident from some sites of cell contact (see arrows, Figure 3B). However, the SCC9SN cells expressed high levels of E-cadherin at all the cell–cell junctions (Figure 3C). This exactly matched the results shown by Western blot. When the SCC9β6D1 cells were examined, E-cadherin was detected at all cell–cell junctions (Figure 3D). These results when combined with those achieved by Western blot strongly indicate that the terminal 11 AA of the β6 integrin acts as an inhibitor of the epithelial phenotype.

Suppression of MEK inhibits β6-mediated MMP-3 activation and blocks TN-C secretion. Expression of the active 48 kDa form of MMP-3 was decreased by incubation with the MEK inhibitor, U0126 (Figure 4A, lane 2). This suggests that activation of MMP-3, which is initiated by αvβ6 ligand binding, is mediated *via* MAPK.

TN-C expression was increased 6-fold in SCC9β6 cells as compared to that of SCC9SN cells (Figure 4B, lanes 1 and 2, respectively). Actin served as a control (Figure 4B, bottom panel).

There was a six-fold decrease in TN-C secretion in the cultures incubated in the presence of the MEK inhibitor U0126 (Figure 4C, lane 2). This demonstrates that the MEK/MAPK pathway modulates the secretion of TN-C by oral SCC cells and is an important component of the ECM development.

Discussion

A body of literature has been published with respect to tumor cell passage through EMT. Epithelial-mesenchymal transition (EMT) may also represent a key step in the progression of carcinoma towards invasive and metastatic disease (4). The process of EMT permits epithelial cells to escape the structural constraints imposed by tissue architecture and adopt a phenotype more amenable to cell movement. The progression to invasive and metastatic oral SCC involves profound alterations in epithelial structure and function. In the clinical scenario, the loss of epithelial features in colon cancer at the invasive front, typified by lack of E-cadherin and increased vimentin expression, is strong evidence for EMT in carcinoma (12). Dedifferentiation and redifferentiation is a dynamic process regulated by the tumor environment (12). These authors suggest that the tumor cells can fluctuate between a differentiated and dedifferentiated state *in vivo* and is dependent upon the state or composition

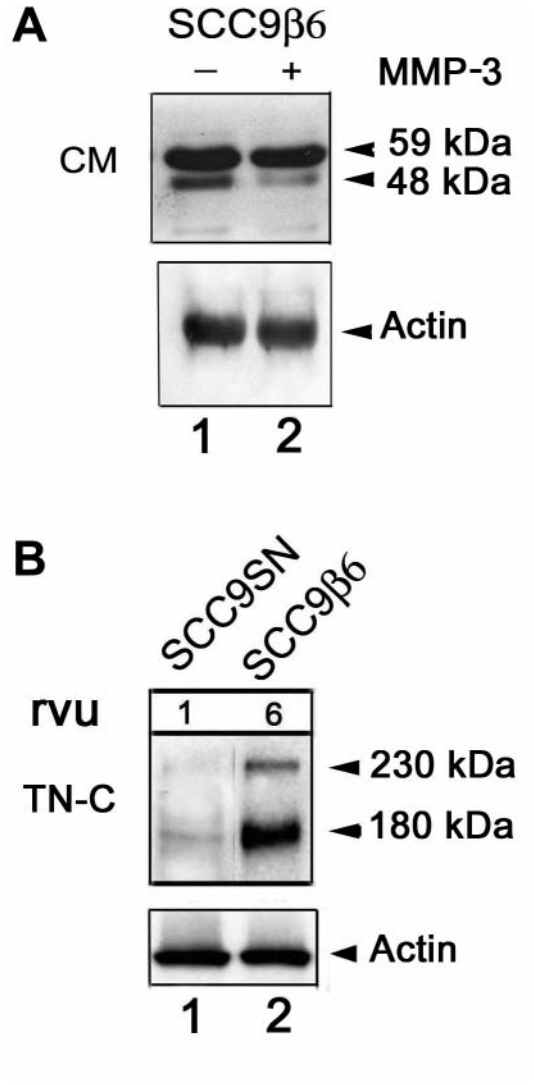

Figure 4. Inhibiting MEK suppresses β6-mediated MMP3 activation and secretion of TN-C. A, Suppression of MEK suppresses MMP3 activation. SCC9β6 cells were grown on FN for 24 h in the presence (+) (0.1 μM) or absence (–) of the MEK inhibitor U0126. The culture media were collected, concentrated and analyzed by Western blot for the expression of MMP3. Note the dramatic decrease in the active 48 kDa form of the molecule in the presence of the MEK inhibitor U0126. B, Expression of β6 promotes TN-C secretion. SCC9SN (lane 1) and SCC9β6 (lane 2) were plated on FN for 24 h under serum-free conditions. The conditioned media were collected and concentrated, followed by Western blotting for TN-C. The membrane was scanned using dosimetry and assigned relative value units (rvu). Note the 6-fold increase in TN-C expression in the conditioned media of the SCC9β6 cells (lane 2) when compared to the SCC9SN conditioned media (lane 1). Actin was used as a loading control (bottom panel). C, Inhibition of MEK suppresses TN-C secretion. SCC9β6 cells were grown as described on FN for 24 in the presence (+) and absence (–) of the MEK inhibitor U0126. The conditioned media were concentrated and analyzed for TN-C secretion. Note the 6-fold decrease in TN-C secretion when the cells were incubated with U0126 (lane 2). Actin was used as a loading control (bottom panel).

of the extracellular matrix. However, the actual data supporting the occurrence of EMT is specific solid tumors and its relevance to the process of progression of these types of cancer has been scant (13).

We show in this study that the expression of β6 provides the necessary cues to promote the EMT. Epithelial cells receive important stimuli from the environment through soluble growth factors and insoluble extracellular matrix proteins (14). In concert with growth factors, the ECM can profoundly influence cell phenotype and behavior (14). Our laboratory and others have shown that high expression of αvβ6 can be used to predict aggressiveness of a tumor (7). We suggest that the induced expression of αvβ6 helps establish a tumor microenvironment by the induction of molecules such as MMP-3 and TN-C. The ability to regulate the expression of a specific fibronectin receptor as a consequence of the EMT confers a selective advantage on the tumor cells.

Cell motility requires a degree of independence from the surrounding cells (13). Much attention has been focused on trying to determine what is the molecular signature of cells undergoing EMT. A prime example relates to the intercellular adhesion molecule E-cadherin, whose targeted disruption during tumor progression is one of the most common alterations in cancer (13). One way that we suggest this proceeds is *via* the expression of the αvβ6 integrin. Bates *et al*. (4) established that EMT induced a specific transcriptional up-regulation of the αvβ6 integrin. We found that the expression of the full-length β6 results in conversion from an epithelial phenotype into a more mesenchymal type cell, with fewer cell–cell junctions and a more fibroblastic, spindle-shaped appearance. In this study, we used a β6 construct lacking the *C*-terminal 11 AA to generate the SCC9β6D1 cell line with a truncated β6 integrin. The removal of the terminal 11AA in the SCC9β6D1 cells results in an epithelial morphology which is cobblestone-like and

rich in E-cadherin. There is also a dramatic switch in the composition of the intermediate filaments. The SCC9β6 cells express significant levels of vimentin and extremely low levels of keratin. This suggested that the β6 integrin may be a prominent contributor to the EMT seen in many types of tumors including various oral cancer lesions. Therefore when we observed the decreased vimentin and increased keratin in the SCC9β6D1 cells, it suggested that perhaps expression of the terminal 11 AA was important for maintenance of the less-differentiated, mesenchymal phenotype and that its deletion shifted the cells back towards the epithelial phenotype. To further prove that αvβ6 was involved in the EMT, we evaluated the cell lines for expression of E-cadherin. E-cadherin was reduced in the SCC9β6 cells. This is what we expected in that invasive tumor cells would not have a need for strong cell-cell associations. However, we were surprised when we evaluated the SCC9β6D1 cells and found that E-cadherin was strongly up-regulated. This suggested to us that removal of the terminal 11 AA derails the default pathway which promotes the EMT.

Our laboratory previously showed that both MMP-3 and TN-C are up-regulated in response to expression of the β6 integrin (10, 15). In this study, we determined that suppressing the function of MEK inhibits both MMP3 and TN-C expression. This is an important finding in that the regulation of two key molecules known to play a role in invasion and metastasis which are also implicated as part of the EMT cascade are regulated through MAPK.

These results further demonstrate that the expression of characteristic mesenchymal markers is apparently regulated by β6 expression and MAPK. The fact that expression of the αvβ6 is associated with EMT is confirmatory of the work of Bates *et al*. (4). However, the novel aspect of this study is that the information contained within the terminal 11 AA of β6 completely alters the morphology and expression of the mesenchymal phenotype. This is particularly exciting and suggests that the terminal 11 AA of β6 are important for regulating invasion and metastasis by modulating EMT.

Acknowledgements

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