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The Expression of Integrin αvβ6 Promotes the Epithelial Cell Morphology and Suppresses Invasive Behavior in Transformed Oral Keratinocytes

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Abstract. *The expression of the integrin αvβ6 has been correlated with oral SCC invasion. We evaluated its expression in three 4NQO transformed murine oral keratinocyte cell lines (B7E3, B7E11and B4B8). The B7E3 cells were negative for β6, whereas the B7E11 and theB4B8 cells were both positive. The β6 negative B7E3 cells were fibroblast-like in appearance, whereas the B7E11 cells were more epithelial-like. The B4B8 cells were a mixture of the two cell types. Using immunofluorescent microscopy, we found that vimentin was highly expressed in the B7E3 cells, whereas the B7E11 cells keratin positive. The B4B8 cells expressed both filaments. The B7E3 cells formed large tumors when injected into nude mice, whereas the B4B8 cells formed small tumors and the B7E11 cells formed none. These results suggest that the expression of the αvβ6 integrin suppresses tumor formation and may promote the epithelial phenotype in 4NQO-transformed murine oral keratinocytes.*

Oral squamous cell carcinoma (OSCC) is the most common neoplasm arising in the head and neck region. Worldwide, the annual incidence of new cases exceeds 300,000 (1). The 5-year survival rate for OSCC has not improved in more than two decades (1). It is known that the cellular invasion to the surrounding tissue correlates with metastasis, and the mode of invasion is one of the markers of the cellular malignancy and prognosis of the oral cancer. Many factors are associated with local invasion, such as cell-cell adhesion molecules, matrix proteinases and cytokines, which change the mobility and scattering of the cells (1, 2).

Loss of the classical, cobblestone epithelial phenotype and acquisition of fibroblastoid, mesenchymal characteristics, is referred to collectively as epithelial-mesenchymal transition (EMT). This transition to a less differentiated state as reflected by EMT may be related to tumor progression, local invasion and metastasis (3).

Integrins are cell-surface adhesion receptors for the extracellular matrix (ECM) (6). Integrins have been shown to be important for cell motility during keratinocyte wound healing (7). For example, αvβ6 which is a fibronectin (FN)-binding integrin, is barely detectable in epithelial cells, but it is strongly induced during wound healing, inflammation, and, in some cases, tumorgenesis (8, 9). In several types of human cancers it is known that tumors expressing high levels of αvβ6 are more invasive than those expressing low levels of αvβ6 (2). More recently, several studies have shown that the expression of the integrin αvβ6 is an important contributor to the invasive phenotype in oral SCC (2, 10). However, the relationships between the integrin molecule and the morphological changes during EMT have not been determined.

In this study, we examined how αvβ6 expression correlated with cell morphology, cellular invasiveness and EMT in transformed mouse oral keratinocyte cell lines.

Materials and Methods

Cell lines. Transformed oral murine keratinocytes (B7E3, B7E11 and B4B8) were provided Dr. Carter Van Waes (National Institutes of Health). Keratinocytes were transformed by 4-nitroquinolone-1-oxide (4-NQO) *in vitro* as described (Thomas *et al.*, 1999). The keratinocytes were obtained from the tongues of BALB/c male mice and established as primary cultures (Thomas *et al.*, 1999). Cultures were exposed to

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Table I. *Expression of integrin molecule on transformed mouse keratinocyte cells.*

	αV	α3	α5	α6	β1	β3	β6	Morphology
B7E3	+/-	-	+	+	+	-	-	Spindle
B7E11	+/-	-	+	+	+	-	++	Cobblestone
B4B8	+/-	-	+	+	+	-	+	Cobblestone Spindle

2.6 μM 4-NQO for 3 hr (Crane *et al.*, 1998). 4-NQO has been shown to induce multiple SCC in the oral mucosa of CBA/J oral keratinocytes *in vitro* (Crane *et al.*, 1998). The cells were immortalized and transformed as determined by growth in soft agar and rhodamine retention. The cells were maintained in Dulbecco's Modified Eagle Medium/F-12 (DMEM/F-12) containing 10% fetal bovine serum (FBS), glutamine and antibiotics.

Reagents. Polyclonal antibodies to human α3 (cat # AB1920), α5 (cat # AB1928), and β5 (cat # AB1926), and monoclonal antibodies to α6 (cat # MAB1982) were purchased from Chemicon International (Temecula, CA, USA). Monoclonal antibodies to αv (CD51) (cat # 553241), β1 (CD29) (cat # 553715, 555004), and β3 (CD61) (cat # 553344) were obtained from PharMingen (San Diego, CA, USA). Mouse monoclonal antibodies to αvβ6 (10D5) were a generous gift of Dr. Dean Sheppard (University of California, San Francisco, CA, USA). Anti-vimentin monoclonal antibody (VIM13.2) was purchased from Sigma-Aldrich. Rabbit anti-mouse keratin 5 (AF138) was purchased from Covance Research Products (Columbia, MO, USA).

Fluorescein-conjugated secondary antibodies (goat anti-mouse IgG, goat anti-rat IgG and goat anti-rabbit IgG) were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Flow cytometry. Cultured cells were harvested by trypsinization and washed twice with phosphate buffered saline (PBS). Non-specific binding was blocked with normal goat serum at 4˚C for 10 minutes. Cells were then incubated with primary antibodies for 30 minutes at 4˚C, washed with PBS and incubated with secondary antibodies conjugated to fluorescein. Stained cells were resuspended in 100 μl of PBS and analyzed by FACScan (2, 13).

Immunofluorescence microscopy. To evaluate vimentin or cytokeratin expression, cells were seeded onto glass coverslips and incubated at 37˚C for 12 hours. The cells were then fixed with 2% paraformaldehyde and permeabilized with 0.1% Triton X-100 (Sigma, St. Louis, MO, USA) for 10 minutes. Non-specific binding was blocked with 3% BSA, and the coverslips were incubated with antibodies to vimentin or keratin for 60 minutes at room temperature, followed by rinsing with PBS. The cultures were then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG or anti-rabbit IgG (1:100) (Amersham) for 30 minutes at room temperature, washed with PBS, and mounted with Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, USA). The cultures were then evaluated by immunofluorescence microscopy (14, 15).

Cell migration assay. Cell migration assays were carried out as described previously (2). Briefly, the lower surface of Transwell cluster

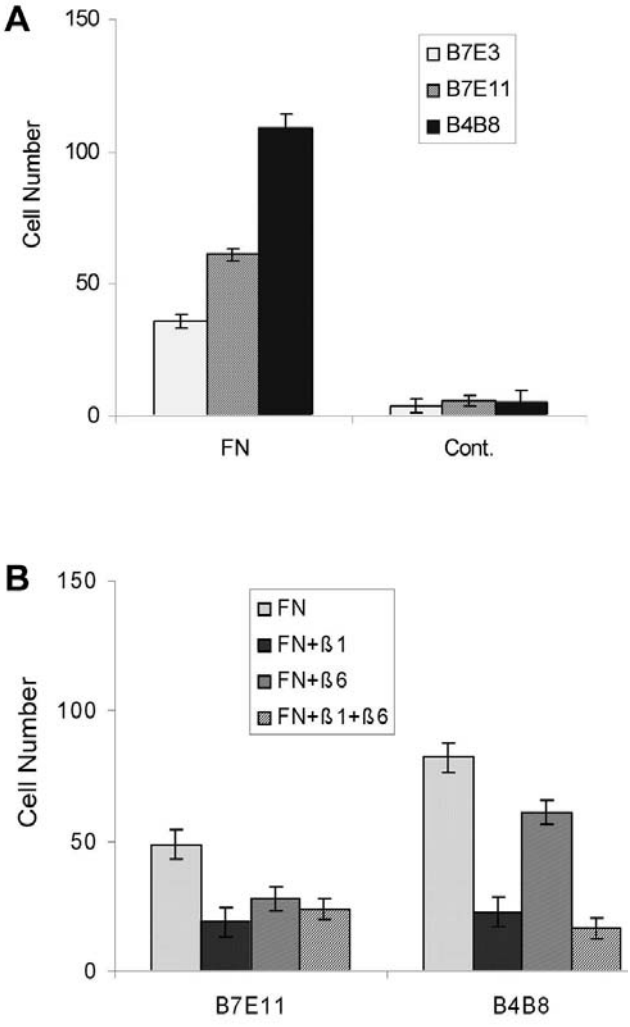

Figure 1. *Differential migration of transformed oral keratinocytes on FN. 4x10^4 B7E3, B7E11, B4B8 cells were analyzed for migration on FN (10 μg/ml). Cells were allowed to migration for 2.5 h(a). Effects of anti-beta1 and beta6 antibodies on migration of these cells on FN. B7E11, B4B8 cells were analyzed for migration on FN (10 μg/ml) in the absence and presence of neutralizing antibodies to beta1 (HA/2, 10 μg/ml), and beta6 (10D5, 10 ug/ml)(b) . The number of cells migrating was determined by counting five random fields of each Transwell filter were counted at 200x magnification, and the cell numbers were calculated as total migrated cells per filter and expressed as mean±SD.*

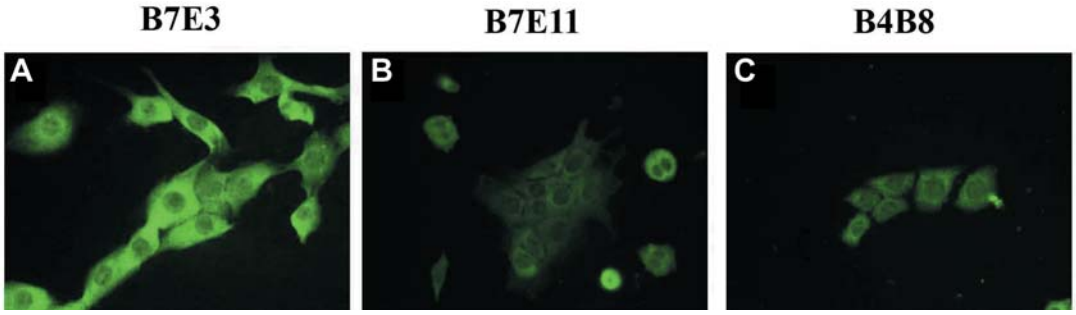

Figure 2. *Detection of vimentin in transformed murine oral keratinocytes. Transformed mouse keratinocyte cells were grown overnight on glass cover slides and processed for immunofluorescence microscopy . B7E3(A), B7E11(B) and B4B8(C) cells were plated on glass coverslips for 12hr and evaluated using anti-vimentin mAb (VIM1.2).*

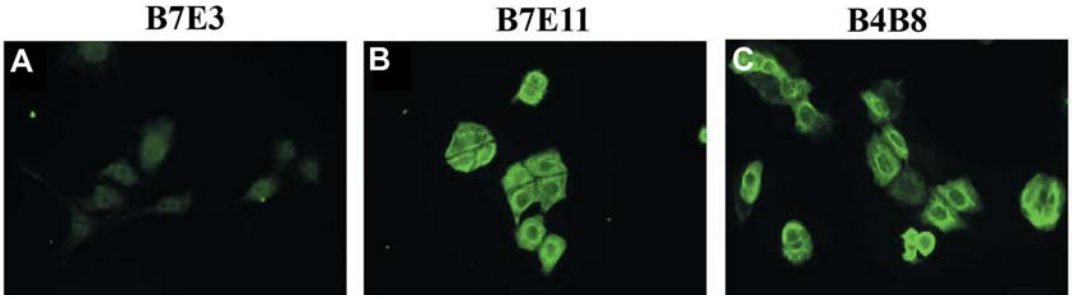

Figure 3. *Expression of keratin is differentially expressed in transformed murine oral keratinoctyes. B7E3 (A), B7E11 (B) and B4B8 (C) cells were plated on glass coverslips for 12hr and evaluated using anti-keratin mAb (AF138)(b).*

plates (8.0 μm pore size; Costar, Cambridge, MA, USA) were coated with FN (10 μg/ml) at 37˚C for 1 hour. Cells were detached with 0.025% trypsin and 0.53 mM EDTA (Invitrogen) and neutralized with trypsin inhibitor (Sigma). Cells were then resuspended in serum-free DMEM. 5 x 10^4 cells in 100 μl of serum-free DMEM containing 0.1% BSA were added to the upper chamber and allowed to migrate for 2.5 hours at 37˚C. Cells on the upper surface were removed. The filters were fixed and stained with Crystal Violet. Each experiment was repeated in triplicate. Five random fields of each filter were counted at 200x magnification, and the cell numbers were calculated as total migrated cells per filter. In some experiments function-blocking monoclonal antibodies to αvβ6 (10D5), or to β1 (Ha2/5, Pharmingen cat # 555004), were incubated with cells.

In vivo assays. Footpad injection of athymic mice was next used to analyze *in vivo* cell behavior. Eight week-old nu/nu BALB/c mice (Charles River, Wilmington, MA, USA) were used for the experiments. These cells were detached from tissue culture plates and suspended in PBS. Mice were injected with 1x10^6 cells/0.05 ml of cell suspension into one hind footpad. After 7 weeks, the mice were sacrificed by cervical dislocation and the size of the primary tumor was determined. The lesions were excised and prepared for microscopic studies (2, 17).

Results

Expression of integrins on transduced oral murine keratinocytes. Using flow cytometry an integrin receptor profile was established for the B7E11, B7E3 and the B4B8 cells. Expression of αv, α5, α6 and β1 were essentially the same for each cell line (Table I). None of the cells tested expressed significant levels of the integrin α3β1 (Table I). In contrast, the expression of αvβ6 varied amongst the cell lines: B7E11>>B4B8>>B7E3 (Table I). These results indicate that the three transformed oral murine keratinocytes have a differential expression profile for the β6 integrin.

β6 is associated with epithelial morphology in oral murine keratinocytes. The cell morphology of the three cell lines was examined by phase contrast microscopy. The β6 negative B7E3 cells were spindle-shaped whereas the B7E11 cells were cobblestone in appearance (Table I). The B4B8 cells which express intermediate levels of β6 consist of a mixed population of cells with both a fibroblast and cobblestone appearance (Table I). These results suggest that the absence of β6 in this system promotes mesenchymal morphology. In contrast, the expression of β6 promotes the classical cobblestone appearance of epithelial cells.

β1 and β6 mediate migration of transformed oral murine keratinocytes on fibronectin. We wished to determine if cell migration on FN differed amongst the transformed oral keratinocytes. The B7E11 and the B4B8 cells were

Table II. *Tumor formation in mouse foot-pad.*

	Tumor site (width, mm)	Control (width, mm)
B7E3	4.3	1.8
B7E11	2.0	1.9
B4B8	3.7	1.8

Athmic nu/nu mice in groups of five were injected with 1 x 10^6 cells/mouse of B7E3, B7E11 and B4B8 cells and tumor growth was measured as described in the Materials and methods.

significantly more migratory on FN than the β6 negative B7E3 cells (Figure 1A). When the cells were incubated with anti-β1 or anti-β6 antibodies cell migration was partially suppressed (Figure 1B). When the two antibodies were added together the effect was significantly enhanced and migration was almost totally blocked (Figure 1B). These results indicate that migration of transformed murine oral keratinocytes on FN is dependent upon both β1 and β6 integrins.

Vimentin expression is conversely correlated with αvβ6 in transformed oral murine keratinocytes. Epithelial to mesenchymal transition is a characteristic of invasive epithelial tumors. In Table I we document the differential cell morphology displayed by the cells and wished to correlate the expression of intermediate filaments with the described morphology. We also wished to determine if there was a correlation between αvβ6 and vimentin or keratin expression. Less differentiated fibroblastic cells are typically rich in vimentin, whereas an epithelial cell is typically rich in keratin expression. The cell lines were evaluated by immunofluorescence microscopy using anti-vimentin and anti-keratin monoclonal antibodies (Figures 2 and 3 respectively). Vimentin was expressed differentially amongst the cell lines: B7E3>B4B8>B7E11 (Figure 2). This suggested that the absence of β6 promoted a mesenchymal phenotype. In contrast, expression of keratin filaments was exactly the opposite: B7E11>B4B8>B7E3 (Figure 3). The expression of keratin correlated with β6 expression. These results indicate that vimentin and keratin may be differentially regulated by the β6 integrin.

αvβ6 suppresses tumor growth by transformed murine oral keratinocytes. The cells were next evaluated for tumor growth *in vivo*. We injected the three cell lines into the footpad of athymic (nu/nu) mice at 1 x 10^6 cells per injection site. Tumor development was monitored several times a week. At seven weeks the animals were sacrificed and examined for the presence of tumors. Animals injected with the B7E3 and B4B8 cells formed tumors, but not in those injected with B7E11 cells (Table II). A comparison of tumor growth, measured as the diameter of the footpad, is shown in Table II. Tumors derived from the B7E3 cells and the B4B8 cells averaged 4.3 and 3.7 mm, respectively. The B7E11 cells did not produce tumors. These results indicate that the expression of αvβ6 in transformed murine oral keratinocytes suppresses tumor growth *in vivo*.

Discussion

Oral SCC has poor prognosis due to its invasive and metastatic behavior (1, 11, 18, 19). Late detection coupled with early invasion is responsible for much of the high morbidity and mortality associated with disease. The invasive phenotype is mediated by shifts in the profile of adhesion molecules, altered ECM production and differential production of MMPs. Invasive SCC has been associated with upregulation of MMP3 and MMP9 (2). Invasive SCC, has a fibroblastic morphology with high level expression of the mesenchymal marker vimentin, suggesting that the cells have undergone epithelial to mesenchymal transformation (EMT) (20, 21).

Tumor cells must adhere to the ECM before they can modify and migrate upon it. Previous work from our laboratory indicates that the expression of αvβ6 does not affect the initial attachment of tumor cells to the ECM. Rather, we found that cell migration and invasion are modulated as a result of β6 expression. In human oral SCC the expression of this integrin complex is correlated with the mesenchymal phenotype.

Work from our laboratory also indicates the forced expression of the β6 integrin promotes tumor cell growth *in vitro* and *in vivo*. Oral SCC tumor cell migration and invasion of reconstituted basement membrane is facilitated through the expression of β6 in human oral SCC.

In this study, we evaluated three transformed murine oral keratinocyte cell lines for the expression of αvβ6. We suspected that the expression of β6 would be correlated with the mesenchymal phenotype and ability to form tumors in the cell lines used. We used flow cytometry to characterize their expression of αvβ6. The B7E3 cells were negative for β6 whereas the B7E11 cells expressed high levels of β6. Expression of β6 in the B4B8 cells was intermediate. It was unexpected that the expression of β6 promoted the epithelial/cobblestone morphology in transformed murine oral keratinocytes. Equally as unexpected, as the level of β6 decreased in the cell lines they took on a more spindle-like morphology. The B7E11 cells were typically epithelial, whereas the β6 negative B7E3 cells were the most mesenchymal exhibiting a pure spindle-shaped morphology. The B4B8 are composed of a mixture of cell types.

Interestingly we found that cell migration on FN was not related to morphology in transformed murine oral keratinocytes. The B7E11 and the B4B8 were significantly more migratory than the B7E3 cell line.

The expression of vimentin was exactly the opposite to that of αvβ6. The B7E3 cells strongly expressed vimentin but little or no keratin. In contrast, the B7E11 cells expressed keratin

with little or no expression of vimentin. B4B8 cells showed intermediate expression of both markers. This suggests that in this system the expression of the β6 integrin is not responsible for the mesenchymal phenotype. This is in direct contrast to what our laboratory and others have previously shown in human oral SCC. We were extremely interested to determine if formation of tumors was dependent upon the expression of β6 as found in the human SCC cells we have previously evaluated (2). We therefore compared αvβ6 expression in our cell lines with cell morphology and their invasiveness *in vivo*. Both the B7E3 and the B4B8 cells formed tumors *in vivo*, with the B7E3 cells forming the larger tumors.

We believe that the results presented in this current work represent a novel finding in that the positive expression of the integrin αvβ6 is associated with the suppression of the progress of EMT and tumor growth. This paper is the first report of an association between the expression of αvβ6 and the suppression of tumor growth in using transformed oral keratinocyte. We also believe this is the first report that the expression of αvβ6 promotes the epithelial phenotype. We suggest that expression of αvβ6 promotes the noninvasive epithelial phenotype in oral SCC.

Our results suggest that the possibility that the expression of αvβ6 in transformed murine oral keratinocytes may suppress tumor growth and may suppress epithelial-mesenchymal transition (EMT). Our results justify further investigation to elucidate in detail the relationship between EMT in oral cancer cell biology.

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