Expression of the αvβ6 Integrin Promotes Migration and Invasion in Squamous Carcinoma Cells

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The integrin αvβ6 is a fibronectin receptor whose expression is not detectable on normal oral epithelium but is increased significantly in healing and in oral epithelial dysplasia and oral squamous cell carcinoma, suggesting it may promote changes associated with tumor development. To study whether αvβ6 may drive invasive behavior we have used transfection and retroviral infection to create a panel of epithelial cell lines expressing various levels of αvβ6. We report that increased expression of αvβ6 in malignant keratinocytes promotes invasion and leads to an increased capacity for migration towards fibronectin. αvβ6 expression may have a significant role in contributing to the malignant behavior of epithelial cells. Key words: integrins/invasion/keratinocytes/migration. J Invest Dermatol 117:67±73, 2001

In increased epithelial mobility and migration play a fundamental role in normal and pathologic processes such as development, wound healing, and malignant transformation. Most dramatically this is seen in carcinogenesis, where epithelial cells cross the basement membrane and proliferate and migrate within the connective tissues. The process of invasion involves altered cell–cell and cell–matrix interactions, loss of growth control, and acquisition of the ability to degrade and invade stromal tissues. Frequently these changes are accompanied by altered expression of cell adhesion molecules, of which the integrins are the most important extracellular matrix (ECM) receptors (Juliano and Varner, 1993; Giancotti and Mainiero, 1994; Ben-Ze’ev, 1997).

Worldwide, oral squamous cell carcinoma (SCC) is the sixth most common cancer, representing about 5.5% of all malignancies (Parkin et al, 1993). In the U.K. there are over 3000 new cases each year with a mortality rate that has remained at over 50% for decades and is not improving (Johnson and Warnakulasuriya, 1993; Hindle et al, 1996). One integrin heterodimer, αvβ6, is not expressed in normal epithelium but is consistently found in oral epithelial dysplasia and oral SCC, suggesting it may play a role in the development and progression of such tumors.

Integrins are transmembrane cell surface receptors, composed of noncovalently linked heterodimers of α and β chains (Hynes, 1992; Sonnenberg, 1993). Integrins mediate adhesion to the ECM and function as coordinators of signaling pathways that regulate a diverse range of cell functions including motility, proliferation, differentiation, and apoptosis (Hynes, 1992; Sonnenberg, 1993; Frisch and Francis, 1994; Clark and Brugge, 1995; Yamada, 1997). These intracellular signals are generated as a result of ligand binding and in this way cells may respond to ECM-specific interactions (Damsky and Werb, 1992). Different integrins generate different signals and some of these may have profound effects on the biologic behavior of tumors.

De novo expression of αvβ6 is seen in a number of epithelial malignancies, particularly oral SCC, which may be correlated with a downregulation of αvβ3 (Agrez et al, 1996; Jones et al, 1997). More recently, Hamidi et al (2000) found that αvβ6 was expressed in a high percentage of oral epithelial dysplasias where it correlated with disease progression, suggesting that it may promote transition to a malignant phenotype. Intriguingly, increased αvβ6 expression also is seen on wound keratinocytes suggesting that αvβ6 normally plays a role in tissue repair and/or remodeling (Haapasalmin et al, 1996).

To address the potential role of αvβ6 in oral SCC, we have examined the functional significance of high αvβ6 expression using a range of SCC cell lines, which have been created by transfection and retroviral infection. We report that increased expression of αvβ6 promotes invasion and leads to an increased capacity for migration towards fibronectin, perhaps suggesting that αvβ6 may have a role in tumor progression and may enhance the aggressive behavior of carcinoma.

MATERIALS AND METHODS

Antibodies and reagents Eight monoclonal antibodies (all of murine origin unless stated) were used in this study. L230 (anti-human αv) was prepared in our laboratory from hybridoma cells obtained from the American Type Culture Collection (Rockville, MD) (Weinacker et al, 1994). E7P6 and R6G9 against the β6 subunit (Weinacker et al, 1994) and 10D5 against αvβ6 (Huang et al, 1998) were prepared in the laboratories of Dean Sheppard. P1F6 against αvβ5 was obtained from Life Technologies, Paisley, U.K. P1F6 against α5β1 was purchased from Chemicon International (Harrow, U.K.). Fluorescein isothiocyanate (FITC) conjugated rabbit antimouse antibodies were purchased from Dako (High Wycombe, U.K.). Genetecin (neomycin analog G418), puromycin, type I collagen, cellular fibronectin, and bovine serum...
Table I. Flow cytometric analysis of keratinocyte cell lines derived from oral SCC

<table>
<thead>
<tr>
<th>α1</th>
<th>α2</th>
<th>α3</th>
<th>α4</th>
<th>α5</th>
<th>α6</th>
<th>αv</th>
<th>β1</th>
<th>β4</th>
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<tr>
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<td>ND</td>
<td>142.4</td>
<td>181.8</td>
<td>ND</td>
<td>8.7</td>
<td>581.0</td>
<td>ND</td>
<td>270.1</td>
<td>199.0</td>
<td>ND</td>
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<tr>
<td>V3</td>
<td>170.5</td>
<td>211.3</td>
<td>ND</td>
<td>8.18</td>
<td>662.3</td>
<td>12.6</td>
<td>346.3</td>
<td>201.0</td>
<td>ND</td>
<td>5.3</td>
<td>1.8</td>
</tr>
<tr>
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<td>163.4</td>
<td>165.5</td>
<td>ND</td>
<td>9.1</td>
<td>689.7</td>
<td>157.1</td>
<td>383.2</td>
<td>229.7</td>
<td>ND</td>
<td>6.8</td>
<td>174.6</td>
</tr>
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<td>116.4</td>
<td>210.9</td>
<td>ND</td>
<td>10.9</td>
<td>542.0</td>
<td>7.2</td>
<td>338.1</td>
<td>218.0</td>
<td>ND</td>
<td>2.4</td>
<td>2.5</td>
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*Cell lines H357, V3, VB6, and C1 were detected by trypan/EDTA and immunolabeled with antibodies to α1 (TS2/7), α2 (P1E6), α3 (P1B5), α4 (7.2), α5 (P1D6), α6 (GOH1), β1 (TS2/16), β4 (3E1), αv (L230), αβ3 (LM609), αβ5 (P1F6), or αβ6 (E7P6). Bound antibody was detected by FITC-conjugated rabbit antiamouse antisera. Negative control had secondary antibody only and has been subtracted from the results. The table shows a representative experiment (ND = not detected). Flow cytometry confirmed high αβ6 in the VB6 cells and showed that V3 and C1 cells express predominantly αβ5. The null transfectant control C1 cells are the same as the V3 parental confirming that the transfection and infection processes had not altered levels of other integrins expressed by the cells. The integrins α1, α4, and the αβ3 heterodimer were not detectable in the cell lines. The αv subunit or αv heterodimers were not detectable in the original H357 cells.*
Figure 1. VB6 cells do not express the α5β1 heterodimer. Immunoprecipitation of αα and β1 integrins expressed by keratinocyte cell lines derived from oral SCC. Antibodies against β1 or αα were added to Nonidet P-40 detergent lysates of surface-ioldinated [125I] H357, V3, VB6, and C1 cells. Immunocomplexes were captured with rabbit antinouse IgG and protein A sepharose. Samples were separated on 6% SDS-PAGE gels under nonreducing conditions. Cells were exposed to XAR-5 film. As β1 is expressed at very high levels it was necessary to combine a short and a long exposure of the gel in order to clearly see the electrophoretic mobility of the β1 subunit. Immunoprecipitation with two different anti-αα antibodies (L230, P2W7) failed to coprecipitate β1.

(Sharfe System, Germany). Readings were taken every day until day 10. Experiments were repeated a minimum of four times in triplicate.

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) or collagen I (50 μg per ml) in PBS for 1 h at 37°C and blocked with migration buffer (0.5% BSA in αα-MEM) for 30 min at 37°C. For blocking experiments, cells were incubated with antibody for 30 min at 4°C prior to seeding. 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 1 × 10^5 in 100 μl of migration buffer, and incubated at 37°C for 3 h. Following incubation, Transwell inserts were fixed in 10% formalin, stained with 0.5% crystal violet in 10% ethanol for 10 min, and washed. Cells in the upper compartment were removed using a cottonwool swab and the filter was mounted in DPX on a microscope slide. Cells, which had migrated to the lower surface of the filter, were counted by microscopy, using multiple random high-powered fields (at least six fields per filter as determined by cumulative frequency analysis). Between 200 and 800 cells were counted routinely per filter. The experiments were repeated four times in triplicate.

Invasion assays Cell invasion assays were performed using matrigel-coated polycarbonate filters (8 μm pore size, Transwell; Becton Dickinson). Matrigel (70 μl; 1:2 dilution in αα-MEM) was added to the upper membrane and allowed to gel for 1 h at 37°C. For blocking experiments, cells were incubated with anti-integrin antibody for 30 min at 4°C prior to seeding. Additional antibody was added to the assay at 24 h intervals. To act as a chemoattractant, 500 μl of KGM was placed in the lower chamber. Cells were plated in the upper compartment of quadruplicate wells at a density of 5 × 10^4 in 200 μl of αα-MEM and incubated at 37°C for 72 h. The cells in the lower chamber (including those attached to the undersurface of the membrane) were then trypsinnized and counted on a Casy 1 counter (Sharfe System). Experiments were repeated six times in quadruplicate. Adhesion assays on matrigel were also carried out at this time to ensure that any reduction of invasion produced by anti-integrin blocking antibodies was not simply due to inhibition of initial cell attachment.

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 Tukey’s pairwise comparison (set at 5% significance). A p-value of <0.05 was considered to be significant.

RESULTS

The VB6 cell line expresses high levels of ααβ6 V3 cells were infected with a retrovirus containing β6 cDNA to create VB6. Flow cytometry confirmed that the C1 null transfectants showed

Figure 2. VB6 cells adhere to fibronectin using both ααβ6 and α5β1. Adhesion of C1 (A) and VB6 (B) cells to fibronectin. Chromium 51 (Cr)-labeled cells (1.5 × 10^5) were added to fibronectin-coated 96-well plates containing an irrelevant control antibody (GOH3, 1:100 anti-α6) or test antibodies against αα (L230, 20 μg per ml), ααβ6 (10D5, 20 μg per ml), or α5β1 (P1D6, 1:100). Adhesion to fibronectin of C1 and VB6 cells in the presence of GOH3 was 34% and 40%, respectively. Background binding to BSA-coated wells was 14% and 13%, respectively, and has been subtracted from the results. Results are expressed relative to GOH3. Adhesion of C1 cells was blocked by antibodies to α5β1 (P1D6) whereas a similar inhibition of adhesion of VB6 cells required antibodies to both ααβ6 and α5β1. The figure shows a representative experiment performed in quadruplicate. Error bars represent standard deviation.
unaltered αv (or other integrin) expression compared with the V3 parental cells (Table I). Although a low level of αvβ6 was expressed in C1 and V3 cells, the predominant αv-containing heterodimer was αvβ5, which we have shown previously to be a functional vitronectin receptor (Jones et al, 1996). In contrast, αvβ6 expression on VB6 cells showed a 100-fold increase compared with the V3 and C1 cells (Table I). The level of αvβ5 expression in VB6 cells remained unaltered, as did the levels of the fibronectin receptor α5β1 and other integrins (Table I). Fluorescence-activated cell sorter analysis also confirmed that these cells do not express αvβ3 (Table I).

In order to determine whether V3, VB6, and C1 cells expressed αvβ1, another potential fibronectin receptor, cells were analyzed by immunoprecipitation. Figure 1 shows that immunoprecipitation with two different anti-αv antibodies failed to coprecipitate β1. As a comparison, β1 integrins were immunoprecipitated with a β1-specific antibody (TS2/16) and electrophoresed on the same gel. Thus the combined results from flow cytometry and immunoprecipitation show that the only αv integrins expressed by V3, C1, and VB6 cells are αvβ5 and αvβ6 and that VB6 cells express high levels of αvβ6.

Adhesion of VB6 cells to fibronectin is mediated by α5β1 and αvβ6. To confirm that αvβ6 in VB6 cells was functional, the cell lines were plated onto fibronectin in the presence or absence of blocking antibodies against the αv subunit (L230), αvβ6 (10D5), α5β1 (P1D6), or an irrelevant antibody against α6 (GOH3). Combinations of these antibodies were also used. Adhesion of C1 cells (Fig 2A) and V3 cells (data not shown) to fibronectin could be blocked using P1D6 alone. Maximal inhibition of VB6 adhesion to fibronectin, however, required a combination of 10D5 and P1D6 (or L230 and P1D6) indicating that αvβ6 is functional and that the VB6 cells bind to fibronectin using both α5β1 and αvβ6 (Fig 2B).

Subcellular distribution of αvβ6. Immunofluorescence for β6 in VB6 cells showed that β6 colocalized with actin in focal adhesions. This was confirmed by colocalization of β6 with the focal adhesion protein talin (data not shown).

Increased αvβ6 expression has no effect on cell growth on uncoated or matrigel-coated tissue culture plastic. The growth rates of the V3, VB6, and C1 lines were compared on uncoated versus matrigel-coated tissue culture plastic. Cell proliferation was also measured within matrigel gels. G418 and puromycin were removed from the medium for the duration of the experiment. αvβ6 had no effect on cell growth on uncoated or matrigel-coated tissue culture plastic, or within matrigel gels (data not shown).

Increased expression of αvβ6 increases migration towards fibronectin. To determine whether αvβ6 is involved in keratocyte migration, haptotactic migration assays were performed using fibronectin-coated Transwell filters. Over four separate experiments, migration towards fibronectin was significantly increased in VB6 cells compared with either V3 (p < 0.001) or C1 cells (p < 0.001). Figure 4A shows a representative experiment. No difference in migration was observed between the V3 parental cells and C1 null transfectants. The cell lines showed no significant differences when migrating towards collagen I, confirming that the differences in migration potential were specific for fibronectin (Fig 4B).

The migration towards fibronectin of V3 and C1 cells was inhibited by 79% and 68%, respectively, using antibodies against α5β1 (P1D6) (Fig 4C, D) but not by antibodies against the αv integrins (L230, P1F6, 10D5). In marked contrast, antibodies against αv (L230), αvβ6 (10D5), or α5β1 (P1D6) inhibited migration of the VB6 cells (19%, 52%, and 31%, respectively) (Fig 4E). To block migration completely, however, a combination of 10D5 and P1D6 (or L230 and P1D6) was necessary. Migration towards fibronectin in the presence of P1D6 (anti-α5β1) in VB6 cells shows that αvβ6 alone is capable of mediating migration towards fibronectin, although to a lesser extent than when both receptors are available.

Increased expression of αvβ6 increases invasion through matrigel. To determine whether αvβ6 is involved in cell invasion, cells resuspended in α-MEM were added to matrigel-coated Transwell filters and allowed to invade for 72 h toward KGM placed in the lower chamber. In six separate experiments, invasion consistently was increased significantly in VB6 cells compared with V3 (p = 0.001) or C1 cells (p < 0.001). This increased invasion was not due to differences in growth rates of cells in matrigel, which were similar for all the SCC cell lines (data not shown). No significant difference in invasion was observed between the V3 parental cell line and C1 null transfectants. Figure 5A shows a representative experiment.

The invasion of V3 and C1 cells was inhibited by 61% and 65%, respectively, using antibodies against α5β1 (P1D6) (Fig 5B, C) but not by antibodies against the αv integrins (L230, P1F6, 10D5). In contrast, antibodies against αv (L230) and αvβ6 (10D5) dramatically inhibited invasion of VB6 cells (77% and 73%, respectively) whereas antibodies against α5 (P1D6) had no significant effect (Fig 5D). Invasion of matrigel by all cell lines could be blocked with antibody against β1 (P4C10). Adhesion to matrigel in these lines also, however, is blocked completely by anti-β1 (data not shown) and it is probable that P4C10 inhibits cell attachment to matrigel rather than invasion (10D5 has no effect on VB6 adhesion to matrigel – data not shown).
To date there are few data available on the biologic role of αvβ6 in oral SCC. As de novo expression of αvβ6 in oral epithelial dysplasia and SCC has been implicated in disease progression we tested whether induced increases in this heterodimer affected biologic behavior of transformed keratinocytes. Using transfection and retroviral infection we generated a series of SCC cell lines, derived from a single αv-negative precursor, which express varying levels of αvβ6. We show that high αvβ6 expression is associated with a more invasive and more migratory phenotype. As these processes are fundamental to epithelial malignancy this may explain, in part, how upregulation of αvβ6 in oral SCC (Breuss et al., 1995; Jones et al., 1997) could promote malignant behavior.

Altered integrin expression occurs in many epithelial tumors (Peltonen et al., 1989; Pignatelli et al., 1990; Hall et al., 1991; Koretz et al., 1991; Downer et al., 1993; Jones et al., 1993; Thomas et al., 1997) and although these changes are not always consistent, certain trends are evident. Loss of normally expressed integrins may be a common finding, particularly in poorly differentiated lesions. Conversely, other integrins, such as αvβ3, are upregulated significantly during the invasive stages of certain tumors like melanoma and glioblastoma (Marshall and Hart, 1996).

Upregulation of αvβ6 in SCC suggests that this integrin has an active role in tumor progression through inappropriate activity of tissue repair mechanisms. At present, however, there are few data on the biologic functions of αvβ6 in squamous cell or other carcinomas. Neo-expression of αvβ6 in the SW480 colon carcinoma cell line mediated ligand-independent signaling for MMP-9 secretion (Niu et al., 1998), and Agrez et al (1994) reported an αvβ6-dependent increase in proliferation in collagen gels by the same cells, as well as enhanced xenograft growth in nude mice. In contrast to these findings high αvβ6 expression in VB6 cells did not affect cell proliferation within matrigel. This suggests that enhanced proliferation may be collagen I dependent but also raises the possibility that the functional effect of αvβ6 expression may be cell-type specific.

Figure 4. Increased expression of αvβ6 increases migration towards fibronectin. Cells were allowed to migrate towards fibronectin in haptotactic migration assays. After 3 h the samples were fixed and the cells were counted by microscopy. (A) Comparison of migration of V3, VB6, and C1 cells towards fibronectin (FN). (B) Comparison of migration of V3, VB6, and C1 cells towards collagen I (COL I). To assess integrin specificity of migration, integrin-blocking antibodies against αv (L230), αvβ5 (P1F6), αvβ6 (10D5), and αβ1 (P1D6) were added to V3 cells (C), C1 cells (D), and VB6 cells (E), prior to plating into wells (results for both cell lines are expressed relative to an irrelevant control antibody against class I MHC, W6/32 = 100). Figures show representative experiments performed in triplicate. Error bars represent standard deviation.
Several in vivo studies have suggested that upregulation of αvβ6 by keratinocytes in wounds and tumors is associated with downregulation of αvβ5 (Clark et al, 1996; Jones et al, 1997) possibly due, in part, to sequestration of all the available αv subunits as αvβ6 heterodimers. In contrast to these findings, the levels of αvβ5 expressed by VB6 cells were comparable to V3 and C1 cells. As expression of αv in these cells is controlled by a viral (CMV) promoter, however, the αv subunit is constitutively overexpressed and therefore not a limiting factor for αvβ5 expression.

Effect of αvβ6 on cell migration Huang et al (1998) demonstrated that αvβ6 mediated mouse keratinocyte migration on fibronectin and also on vitronectin substrates. In agreement with these findings, migration of VB6 cells towards fibronectin was significantly higher than either V3 (p < 0.001) or C1 cells (p < 0.001) (Fig 4A). VB6 cells adhered and migrated on fibronectin using both αvβ6 and αvβ1, and migration towards fibronectin could only be blocked completely using antibodies against both integrins (Fig 4E). It is not yet evident whether these integrins cooperate in the VB6 cells or whether one is used preferentially. Migration towards fibronectin in V3 and C1 cells was abolished by antibodies against α5β1 (Fig 4C, D). Adhesion to fibronectin, however, was also blocked by anti-α5β1 and it is possible that the results in the migration assay were partly due to inhibition of adhesion to the filters rather than a genuine inhibition of migration.

Effect of αvβ6 on cell invasion The upregulation of αvβ6 expression in oral SCC has been consistently demonstrated in vivo where expression is often strongest at the invasive front of the tumor (Hamid et al, 2000). High expression of αvβ6 in VB6 cells led to a more invasive phenotype (Fig 5A). Thus VB6 cells were significantly more invasive than V3 (p = 0.001) and C1 cells (p < 0.001). These data describing the invasion-promoting activity of an αv integrin in malignant epithelial cells might be compared with the studies of Daden et al (1995) who demonstrated that malignant melanoma cell lines expressing αvβ3 were more invasive than lines expressing αvβ5.

Invasion by the VB6 cells could be reduced dramatically using anti-αv or anti-α5β6 antibodies whereas anti-α5 or anti-αvβ6 had no effect on the C1 null transfectants or the V3 parental cells (Fig 5B, C, D), confirming that the increased invasion by VB6 cells was mediated through αvβ6. Intriguingly, invasion in the C1 and V3 cells appeared to be partly modulated through α5β1, whereas inhibition of this integrin produced no significant effect on VB6 invasion.

The mechanism for the increased invasiveness of VB6 cells is, at present, unclear. The increased invasiveness of VB6 cells is not simply a reflection of a general increase in cell motility but rather a specific motility advantage as a consequence of αvβ6 expression as all the cell lines migrate towards collagen I at similar levels. It is possible that αvβ6 may modulate protease expression in a similar fashion to colon carcinoma cells (Niu et al, 1998).

In summary, the upregulation of αvβ6 in oral SCC has been demonstrated in several clinical studies but there are few data to suggest a possible biologic role (Breuss et al, 1995; Jones et al, 1997; Hamid et al, 2000). In this paper we show, for the first time, that in malignant keratinocytes, high αvβ6 expression promotes invasion and migration. This suggests that αvβ6 may have a specific role in driving oral SCC progression. These results indicate that integrin changes in tumor tissue may not simply reflect cancer development but may have a causative role in this process.

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