

Loss of Adhesion-Regulated Proteinase Production Is Correlated with Invasive Activity in Oral Squamous Cell Carcinoma

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BACKGROUND. Oral squamous cell carcinoma (OSCC) is the most common malignancy of the oral cavity. However, the cellular and biochemical factors that underlie locoregional and distant spread of the disease are poorly understood. Invasion of OSCC requires multiple cellular events including dissolution of cell-cell junctions, basement membrane attachment, extracellular matrix proteolysis, and migration.

METHODS. We evaluated these properties in vitro using premalignant gingival keratinocytes (ppl26) and two OSCC lines (SCC15 and SCC68). Expression of adhesion molecules integrins and cadherins, cytoplasmic intermediate filaments (IF) vimentin and keratin as well as matrix degrading proteins were evaluated. Moreover, regulation of protease production by adhesion molecules was tested.

RESULTS. All cell lines contained comparable levels of the epithelial cell-cell adhesion molecule, E-cadherin. Differential expression of cytoplasmic IF was evident between premalignant ppl26 cells and OSCC cell lines. Expression levels of the $\alpha3\beta1$ integrin, utilized for attachment to laminin-5 and other matrix proteins, was high in SCC68 cells, moderate in SCC15 cells, and low in ppl26 cells. $\alpha3\beta1$ integrin clustering up-regulates expression of urinary-type plasminogen activator (uPA) in ppl26 cells via a mechanism involving ERK activation. Both ppl26 and SCC15 cells were responsive to $\alpha3\beta1$ clustering, resulting in enhanced uPA expression. However, basal uPA levels were high in SCC68 cells and integrin clustering did not further stimulate uPA production. ERK was constitutively activated in SCC68 cells and treatment of cells with an inhibitor of ERK activation (PD98059) reduced uPA expression. Consistent with the enhanced proteolytic potential, SCC68 cells readily penetrated Matrigel and invasion was blocked by an anticatalytic uPA antibody.

CONCLUSIONS. These data suggest that loss of adhesion-regulated proteinase production may lead to elevated pericellular proteinase activity and coincident alterations in cytoskeletal IF protein expression, thereby contributing to the invasive potential of OSCC. *Cancer* 2002;95:2524-33. © 2002 American Cancer Society.

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Oral squamous cell carcinoma (OSCC) is the most common malignancy of the oral cavity, causing more deaths than any other oral disease.¹ Treatment of advanced OSCC is associated with high mortality rates and is complicated by disruption of speech and swallowing that accompany surgical resection.² The cellular and biochemical factors that underlie locoregional and distant spread of the disease are poorly understood. Invasion and metastasis of OSCC require

multiple cellular events including cytoskeletal alterations, disruption of cell-cell adhesive contacts and basement membrane attachment, matrix protein proteolysis, and migration.³ A more detailed analysis of the molecular events that contribute to OSCC invasion is a necessary prerequisite to the development of novel treatment strategies. We evaluated these properties *in vitro* using premalignant gingival keratinocytes and OSCC cells.

Intermediate filaments (IF) are one of the three major cytoskeletal systems found in mammalian cells.⁴ Virtually all epithelial cells express keratin IF, which play important roles in many physiologic functions, including maintenance of cell shape and assembly of epithelial cell-substrate (hemidesmosome) and cell-cell (desmosome) adhesion sites.⁵ In contrast, vimentin is assembled into homopolymer IF only in mesenchymal cells.^{6,7}

Integrins are a family of heterodimeric transmembrane glycoprotein receptors comprised of α and β subunits that mediate cell-matrix interactions. Compared with normal oral mucosa in which integrins are expressed predominantly in basal keratinocytes, OSCC display highly varied patterns of integrin expression.⁸ Although disorganization of integrin expression is an early event in OSCC progression, the functional contribution of integrins to regulation of dissemination is unknown.

Following adhesion, invading OSCC cells secrete proteolytic enzymes including urinary-type plasminogen activator (uPA), which catalyzes activation of the zymogen plasminogen into the broad-spectrum proteinase, plasmin. In human OSCC tumor specimens, staining for both uPA and its receptor (uPAR) correlates with the mode of invasion and lymph node metastasis.⁹⁻¹¹ Multivalent $\alpha\beta 1$ integrin aggregation up-regulates uPA expression via a MEK/ERK1/2-dependent pathway in premalignant oral keratinocytes,¹² suggesting that cell-matrix adhesion may promote subsequent invasive behavior.

In the current study, we evaluated the expression of cytoskeletal IF proteins, integrins, and uPA using premalignant gingival keratinocytes and OSCC-derived cell lines. Our data demonstrate differential expression of these proteins in the tested cell types. Both premalignant and moderately invasive cells expressed low levels of uPA, but were responsive to integrin clustering-induced ERK activation and subsequent proteinase induction. In contrast, highly invasive cells displayed constitutively active ERK, elevated uPA expression that is no longer regulated by integrins, and proteinase-dependent invasion. These data suggest that loss of adhesion-regulated proteinase production may lead to constitutively elevated pericellular pro-

teinase activity, thereby contributing to the invasive potential of OSCC.

MATERIALS AND METHODS

Materials

Bovine serum albumin (BSA), the plasmin substrate Val-Leu-Lys-p-nitroanilide (VLKpNA), and peroxidase conjugates of secondary antibodies were purchased from Sigma (St. Louis, MO). Plasminogen was purified by affinity chromatography from outdated human plasma as previously described.¹² Antihuman integrin $\alpha 3$ (MAb 2056) and $\beta 1$ (P4C10) antibodies were obtained from Chemicon (Temecula, CA) and Life Technologies (Gaithersburg, MD), respectively. Affinity-purified polyclonal antibody specific for phosphorylated p42/p44 MAP kinase (anti-ACTIVE MAPK p42/p44) was purchased from Promega (Madison, WI). Anti-ERK1/2 (anti-p42/p44), which recognizes both phosphorylated and nonphosphorylated p42/p44, was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The anticatalytic uPA antibody (no. 394) was obtained from American Diagnostica (Greenwich, CT). The MEK1 inhibitor, PD98059, was purchased from New England Biolabs (Beverly, MA). Polyclonal rabbit antikeratin antibody was generated against bovine tongue keratin in the Goldman laboratory. Monoclonal murine antivimentin antibody was purchased from Sigma. Alexa Fluor 594-labeled goat antimouse and Alexa Fluor 488-labeled goat antirabbit secondary antibodies were purchased from Molecular Probes (Eugene, OR). Hydrobond-P:polyvinylidene difluoride (PVDF) membrane and SuperSignal enhanced chemiluminescence reagents were obtained from Amersham (Arlington Heights, IL) and Pierce (Rockford, IL), respectively. Matrigel and Biocoat invasion wells were acquired from Becton Dickinson (Bedford, MA). The Diff-Quik stain set was purchased from Dade Behring (Dudingen, Switzerland). The laminin-5 $\beta 3$ chain-specific antibody, B1K, was purchased from Transduction Laboratories (San Diego, CA).

Cell Culture

Premalignant oral keratinocytes (pp126 cells) were a gift from Dr. D. Oda (University of Washington, Seattle, WA).¹³ pp126 cells were obtained by immortalization of normal human gingival keratinocytes with human papillomavirus 16 DNA. These cells do not stratify in liquid-air interphase organotypic culture models. However, they display normal keratin synthesis and some degree of differentiation and are representative of a premalignant transformation to OSCC. Cells were maintained in keratinocyte-SFM (Gibco BRL, Gaithersburg, MD) supplemented with 100 U/mL penicillin, 100 μ g/mL streptomycin, 5 ng/mL epidermal growth

factor (EGF), 50 $\mu\text{g}/\text{mL}$ bovine pituitary extract (BPE), and 0.09 mM CaCl_2 .¹³ SCC15 and SCC68 cells, established from squamous cell carcinoma of the oral cavity, were generously provided by Dr. J Rheinwald (Brigham & Women's Hospital, Harvard Institutes of Medicine, Boston).¹⁴ These cells do not stratify in organotypic culture, display growth factor-independent growth, and are tumorigenic in nude mice. Cells were maintained in keratinocyte-SFM supplemented with 100 U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, 25 $\mu\text{g}/\text{mL}$ BPE (supplied with the medium), 0.2 ng/mL EGF, and 0.4 mM CaCl_2 .

Before treatment with antibody-conjugated latex beads (described below), cell monolayers were released from culture flasks by the addition of trypsin/EDTA, seeded at a constant density of 0.7×10^5 cells per well into 24-well tissue culture plates, and allowed to attach overnight in the medium described above. Cells were then washed twice with phosphate-buffered saline (PBS), incubated for 12 hours in medium lacking BPE and EGF, and supplemented with fresh BPE/EGF-free medium before treatment with antibody-conjugated latex beads (8–10 $\mu\text{g}/\text{mL}$). Following incubation, conditioned media were collected for uPA activity determination as described below.

Immunofluorescence

For detection of IF, cells were grown on glass coverslips for 48 hours, fixed in cold, dehydrated methanol for 3 minutes, and stained with rabbit anticytokeratin or murine antivimentin at 37 °C for 30 minutes as previously described.¹⁵ Cells were then incubated with Alexa Fluor 594-labeled goat antimouse secondary antibody at a dilution of 1:200 for 30 minutes at 37 °C.¹⁶ Coverslips were washed as above, mounted, and examined with a Zeiss LSM510 scanning confocal microscope (Zeiss, Thornwood, NY).

Analysis of Integrin Expression

Total expression levels of the $\alpha 3\beta 1$ integrin were evaluated by Western blotting of whole cell lysates and surface expression was determined using flow cytometry. For flow cytometry, 1.8×10^5 cells were suspended in an equal volume (100 μL) of medium, incubated with antibodies against integrin $\alpha 3$ (1:50 goat polyclonal IgG, SC-6592, Santa Cruz Biotechnology), $\alpha 2$ (1:100 mouse monoclonal IgG, MoAb1384, Chemicon), $\beta 1$ (mouse monoclonal IgG, MoAb2252, Chemicon), and $\beta 4$ (1:20 mouse monoclonal IgG, MoAb1964, Chemicon) for 30 minutes at room temperature. Cells were then washed with PBS and incubated with the corresponding fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (donkey anti-goat IgG FITC SC-2024 [Santa Cruz Biotechnology] at a 1:20

dilution for integrin $\alpha 3$ and anti-mouse IgG FITC [Boehringer Mannheim, Indianapolis, IN] at a 1:100 dilution for integrins $\alpha 2$, $\beta 1$, and $\beta 4$) for 30 minutes in the dark at room temperature. Cells were washed again with PBS and resuspended with 100 μL of medium for fluorescence analysis on an Epics XL-MCL flow cytometer (Beckman Coulter, Hialeah, FL). Control experiments contained only the appropriate secondary antibody.

Analysis of Laminin-5 Expression Level

The extracellular matrix (ECM) was generated as described previously.¹⁶ Briefly, pp126, SCC15, and SCC68 cells were grown for 48–72 hours until cells were confluent. The cells were then removed by treatment for approximately 7 minutes with 20 mM NH_4OH followed by three rapid washes in sterile distilled water and 3 washes with sterile PBS. The matrix was collected in 10 mM Tris-HCl, pH 6.8, containing 8 M urea, 1% sodium dodecyl sulfate (SDS), and 15% β -mercaptoethanol. Samples were then electrophoresed on 7.5% SDS polyacrylamide gels, electroblotted to PVDF membrane, and probed with an anti-laminin-5 $\beta 3$ chain-specific MoAb (B1K). Blots were then incubated with alkaline phosphatase-conjugated secondary antibody and developed with 5-bromo-4-chloro-3-indolyl phosphate and Nitro-blue tetrazolium (0.25 mg/mL) in 0.1 M Tris, pH 9.5, containing 0.1 M NaCl and 5 mM MgCl_2 .

Preparation of Latex Bead-Immobilized Antibodies

Antiintegrin subunit-specific MoAbs or control isotype-matched IgGs were passively adsorbed onto 2.97- μm latex beads (Sigma) as described^{12,17,18} with the following modifications. A 1% (final concentration) suspension of latex beads was incubated in 50 mM 4-morpholineethanesulfonic acid buffer (pH 6.1) with 75 $\mu\text{g}/\text{mL}$ of the appropriate antibody overnight at 4 °C with gentle agitation. Antibody-conjugated beads were blocked with 10 mg/mL BSA for 90 minutes at room temperature, centrifuged for 3 minutes at 3000 rpm, and washed twice by resuspension in two volumes of culture medium. Bead-immobilized antibodies were resuspended in BPE/EGF-free medium at a final concentration of 1% by volume. Determination of total protein concentration in unblocked bead suspensions using a bicinchoninic acid detection kit (Sigma) indicated that 60–70% of immunoglobulins were adsorbed, resulting in a final concentration of 8–10 $\mu\text{g}/\text{mL}$ antibody beads in culture wells.

Analysis of uPA Activity

Net plasminogen activator (PA) activity in conditioned media was quantified using a coupled assay to moni-

tor plasminogen activation and the resulting plasmin hydrolysis of a colorimetric substrate (VLKpNA) as previously described.¹⁹ Control reactions contained 10 $\mu\text{g}/\text{mL}$ of the anticatalytic uPA antibody 398 (American Diagnostica). Statistical analyses were performed using Graph Pad Prizm (GraphPad software, San Diego, CA).

Detection of Total and Active (Phosphorylated) ERK

To evaluate ERK (MAP kinase) activation, cells (0.7×10^5) were cultured overnight in growth factor-free medium, lysed as previously described,¹² and extracted on ice for 30 minutes. The lysates were centrifuged, the protein concentration was determined using the Bio-Rad (Richmond, CA) protein assay kit, and equal amounts of cellular protein (20 μg) were electrophoresed on SDS polyacrylamide gels and electroblotted to Immobilon. Blots were probed with an anti-ERK1/2 antibody (1:1000) to detect total ERK1/2 expression or with anti-ACTIVE MAPK p42/p44 (1:5000) to detect the phosphorylated, active forms of ERK. In control experiments, ERK phosphorylation was blocked using the MEK inhibitor, PD98059, at the indicated concentrations.

Analysis of Matrigel Invasion

Cellular invasive capacity was assessed using a Boyden chamber (8- μm pore size) coated with Matrigel (10 μg). Cells (1.5×10^5) were added to the chamber in 500 μL of growth factor-free medium in the presence or absence of anticatalytic uPA antibody (25 $\mu\text{g}/\text{mL}$), nonspecific control mouse IgG (25 $\mu\text{g}/\text{mL}$), or PD98059. Following incubation at 37 °C for 24 hours, noninvading cells were removed from the top of the filters with a cotton swab, filters were fixed and stained with Diff-Quik stain, and migrating cells adherent to the underside of the filter were enumerated using an ocular micrometer and counting a minimum of 10 high-powered fields. All experiments were done in triplicate.

RESULTS

Expression of IFs and Integrins

Expression of vimentin IF in cancerous oral epithelial cells, which normally only express keratin, is correlated with the epithelial/mesenchymal transition and an increase in metastatic behavior. Some studies suggest that vimentin expression can be detected even before the tumor cells exhibit a mesenchymal morphology.^{20,21} To determine whether vimentin expression may be involved in the increased motility of invasive oral epithelial carcinoma cells, the IF expression patterns of premalignant pp126 cells and carcinoma cell lines were evaluated and compared by

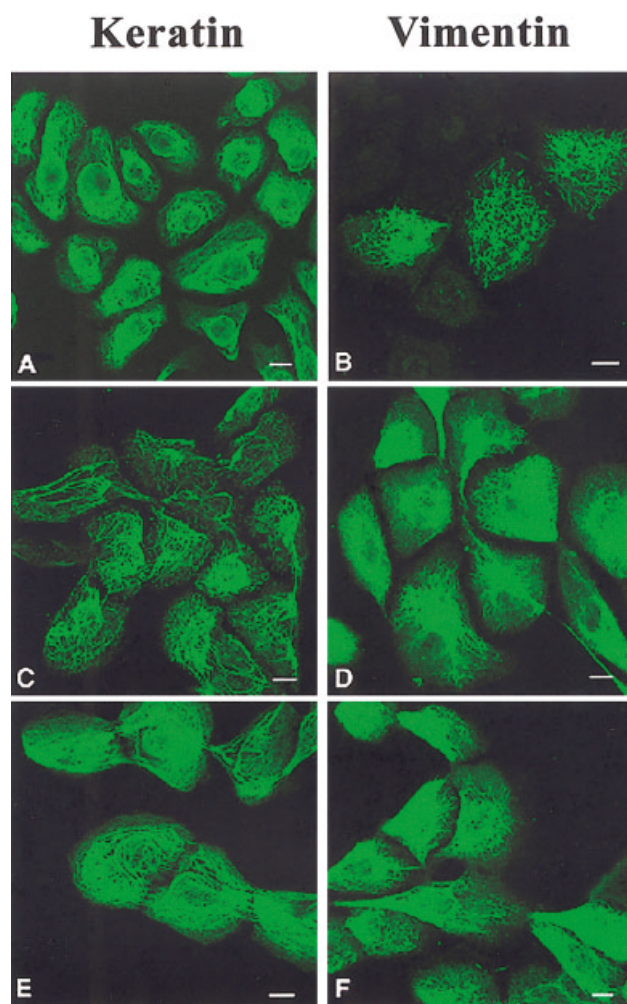


FIGURE 1. Immunofluorescence analysis of keratin and vimentin expression. pp126 cells (A,B), SCC15 cells (C,D), and SCC68 cells (E,F) were grown on glass coverslips for 48 hours, fixed as described in the Materials and Methods, and processed for indirect immunofluorescence with rabbit anticytokeratin (A,C,E) or murine antivimentin antibodies (B,D,F). After washing, cells were incubated with Alexa Fluor 594 secondary antibody. Coverslips were washed and mounted. Images were captured with a Zeiss LSM 510 confocal microscope. Scale bar = 10 μm .

immunofluorescence. All three epithelial cell lines stained positively for keratin (Fig. 1A,C,E) and for the epithelial cell marker, E-cadherin (data not shown). However, vimentin expression differed significantly among the cell lines tested. A relatively small number of pp126 cells (10–20%) expressed vimentin IF (Fig. 1B). In general, these were short and did not form the extensive networks found in mesenchymal cells that express vimentin, such as fibroblasts. They were very similar to the “squiggles” believed to be precursors to longer IF observed among fibroblasts.²² In contrast, the majority of SCC15 (Fig. 1D) and SCC68 (Fig. 1F)

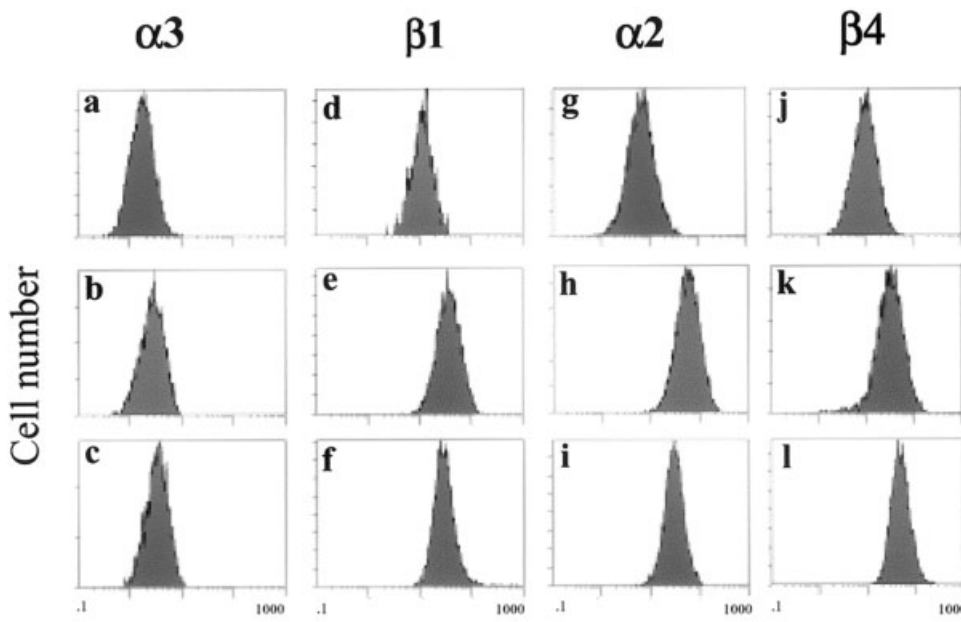


FIGURE 2. Expression of laminin-5 binding integrin subunits. (A,B) Flow cytometric analysis of $\alpha 3$, $\beta 1$, $\alpha 2$, and $\beta 4$ integrin expression. 1.8×10^5 cells (pp126 in panels a, d, g, and j; SCC15 in panels b, e, h, and k; and SCC68 in panels c, f, i, and l) were incubated at room temperature for 30 minutes with monoclonal antibodies (MoAb) against $\alpha 3$ (SC6592, 1:50, panels a–c), $\beta 1$ (MoAb2252, 1:50, panels d–f), $\alpha 2$ (MoAb1384, 1:100, panels g–i), and $\beta 4$ (MoAb1964, 1:20, panels j–l) integrins. Fluorescein isothiocyanate-conjugated secondary antibodies were added for 30 minutes and integrin-expressing cells quantified by fluorescence-activated cell sorting using a Beckman Coulter Epics XL-MCL flow cytometer. Relative fluorescence units for the three cell lines are presented in panel B.

A	LOG FITC				
	$\alpha 3$	$\beta 1$	$\alpha 2$	$\beta 4$	
pp126	1.3	11.4	6.7	9.3	
SCC15	2.1	35	59	29.2	
B	SCC68	2.7	30	29	49.4

cells exhibited extensive vimentin IF networks that extended from the nucleus to the cell periphery.

Immunohistochemical studies of human tumors have shown persistent expression of the $\alpha 3$ and $\beta 1$ integrin subunits.^{8,23} Multivalent aggregation of the $\alpha 3\beta 1$ integrin by bead-immobilized laminin-5 or laminin-5 binding integrin subunit-specific antibodies participates in proteinase regulation in premalignant pp126 cells.¹² However, additional integrins including $\alpha 2\beta 1$ and $\alpha 6\beta 4$ also bind laminin-5. Therefore, total expression levels of the $\alpha 2$, $\alpha 3$, $\beta 1$, and $\beta 4$ integrin subunits were evaluated by Western blotting of whole cell lysates of pp126, SCC15, and SCC68 cells. The premalignant pp126 cells express lower total levels of all integrin subunits compared with SCC15 and SCC68 cells (data not shown). To evaluate cell surface integrin localization, flow cytometric analysis was utilized. These results confirm the Western blotting data and demonstrate that pp126 cells express lower cell surface levels of $\alpha 3$, $\beta 1$, $\alpha 2$, and $\beta 4$ integrins (Fig. 2A, panels

a,d,g,j) compared with SCC15 and SCC68 cells (panels b,e,h,k and c,f,i,l, respectively). Relative fluorescence units for pp126, SCC15, and SCC68 integrin expression are summarized in Figure 2B.

Analysis of uPA Activity and ERK Phosphorylation

The serine proteinase, uPA, is not prevalent in the normal oral mucosa.⁹ However, staining for both uPA and uPAR correlates with the mode of invasion and lymph node metastasis in human tumor specimens.^{9–11} Using a coupled colorimetric assay to quantify uPA-catalyzed plasminogen activation,¹⁹ the conditioned medium of pp126 cells demonstrated low levels of PA activity, SCC15 cells displayed moderate activity, and SCC68 PA levels were approximately 10-fold higher than in pp126 cells (Fig. 3A). In control experiments, PA activity was blocked using anticatalytic uPA antibodies, confirming its identity as uPA rather than as a tissue-type activator. As it has been previously demonstrated that ERK phosphorylation induces uPA promoter activation,²⁴ both ERK activa-

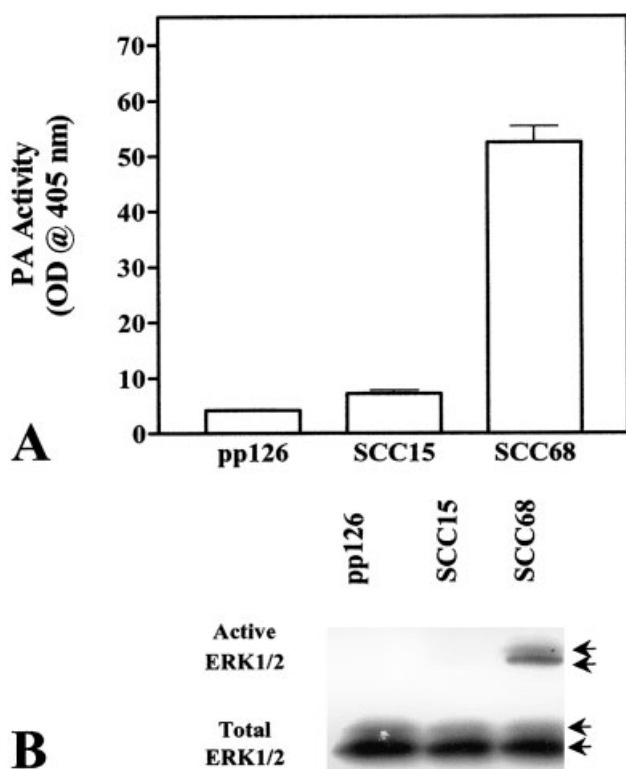


FIGURE 3. Urinary-type plasminogen activator (uPA) activity and ERK phosphorylation. (A) Relative uPA activity. An equal number (2.5×10^5) of pp126, SCC15, and SCC68 cells were plated, cultured overnight, washed twice with phosphate-buffered saline, and cultured for an additional 24 hours in growth factor-free medium. Conditioned medium was then collected, clarified by centrifugation, and plasminogen activator activity was determined using a coupled colorimetric plasminogen activation assay based on plasmin hydrolysis of VLKpNA as described in the Materials and Methods. (B) Detection of total and phosphorylated ERK1/2. Cells were lysed with RIPA buffer and lysates ($15 \mu\text{g}$) were evaluated by Western blotting for ERK activation (top) or ERK expression (bottom). Total cellular protein was collected and quantitated as described in the Figure 2 legend. Blots were probed with anti-ACTIVE MAPK p42/p44 (1:5000) to detect the phosphorylated, active form of ERK (top) or with anti-ERK1/2 antibody (1:1000) to detect total ERK1/2 expression (bottom), followed by peroxidase-conjugated secondary antibody and enhanced chemiluminescence detection. The arrows designate the migration positions of p42 and p44.

tion and total ERK expression were evaluated by Western blotting. Whereas all three cell lines exhibited similar levels of ERK expression (Fig. 3B, bottom panel), only SCC68 cells displayed constitutively active (phosphorylated) ERK (Fig. 3B, top panel).

Integrin Regulation of uPA Activity

$\alpha 3\beta 1$ integrin aggregation induces expression of uPA via a MEK-ERK-dependent pathway in pp126 cells.¹² To determine whether OSCC cell lines are responsive

to adhesion-regulated proteinase expression, cells were incubated with latex bead-immobilized $\alpha 3$ integrin subunit-specific MoAbs to induce multivalent integrin aggregation¹² and conditioned medium was evaluated for uPA. A significant increase in uPA activity was observed only in the conditioned medium of pp126 and SCC15 cells, whereas SCC68 cells were unresponsive to integrin clustering (Fig. 4A). Similar results were obtained using other clones of $\alpha 3$ or $\beta 1$ subunit-specific antibodies (e.g., P1B5, ASC6 for $\alpha 3$ and 21C8, P5D2 for $\beta 1$). Clustering of other integrins such as $\alpha 2$, $\alpha 5$, $\alpha 6$, or $\beta 4$ was ineffective at inducing uPA expression.¹² In control experiments using isotype-matched control IgG-coated beads, no induction was observed (Fig. 4A). As ERK phosphorylation is correlated with uPA promoter activation²⁴ and SCC68 cells exhibit constitutively active ERK and high basal levels of uPA, the effect of the MEK inhibitor, PD98059, on SCC68 uPA expression was evaluated. Concomitant with the PD98059-induced inhibition of ERK phosphorylation (Fig. 4C), a significant reduction in the uPA activity was observed (Fig. 4B). These results suggest that constitutive ERK activation abrogates the ability of SCC68 cells to regulate proteinase expression in response to environmental cues such as integrin aggregation.

To determine whether the increased level of uPA activity in SCC68 cells is due to a relative increase in the expression of endogenous laminin-5, a natural ligand of $\alpha 3\beta 1$, laminin-5 deposition was compared. Western blot analysis using a mouse MoAb against the $\beta 3$ subunit of human laminin-5 demonstrated that all three cell lines deposited comparable levels laminin-5 (Fig. 5). Therefore, the observed differences in uPA expression do not reflect constitutive $\alpha 3\beta 1$ integrin activation due to enhanced expression of the endogenous ligand. Furthermore, incubation of SCC68 cells with an $\alpha 3$ function blocking antibody did not alter the uPA activity level in these cells (data not shown).

Analysis of Invasion

Cellular invasion through ECM protein barriers requires proteolytic activity. Previous studies have implicated the uPA/plasmin system in tumor invasion and metastasis.²⁵ As SCC68 cells displayed the highest levels of uPA activity, uPA-dependent invasion was evaluated. Cells were added to a porous polycarbonate filter coated with Matrigel in the presence or absence of anticatalytic uPA function blocking or control antibodies and invasion was quantified after a 36-hour incubation period. Inhibition of uPA activity significantly reduced invasion (Fig. 6A), supporting a functional role for uPA catalytic activity in SCC68 cell invasion. Compared with the premalignant pp126 cells,

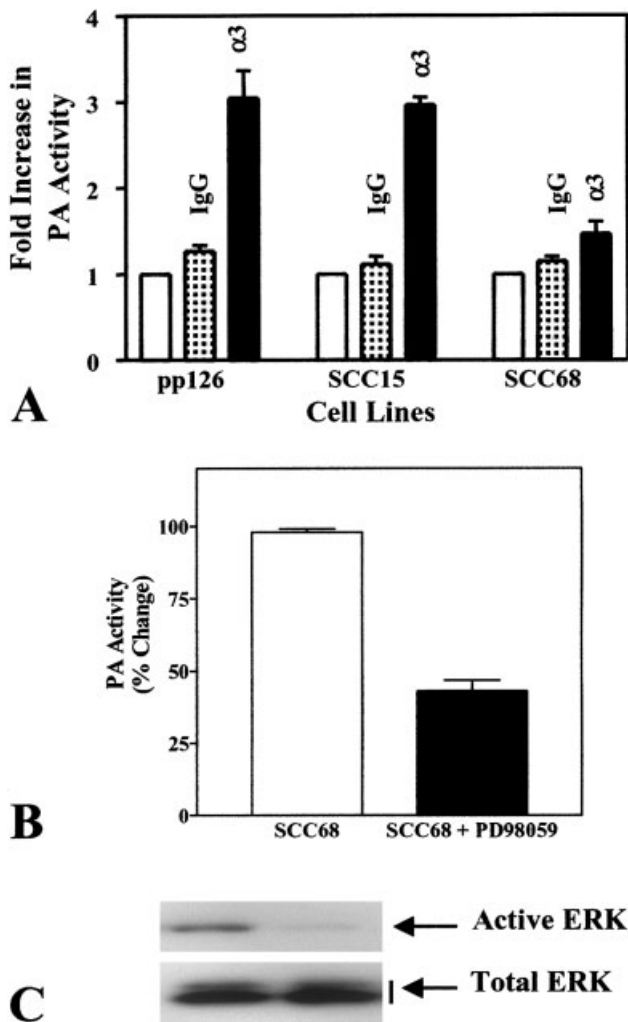


FIGURE 4. Effect of $\alpha 3$ integrin clustering on urinary-type plasminogen activator (uPA) expression and ERK activation. (A) Cells (pp126, SCC15, SCC68, as indicated, 0.7×10^5) were left untreated (open bars) or were treated with IgG control beads (striped bars) or with $\alpha 3$ integrin antibody beads (solid bars) as described in Materials and Methods. After 24 hours, conditioned media were analyzed for uPA activity using a coupled colorimetric plasminogen activation (PA) assay. Results are expressed as the fold increase in PA activity (compared with untreated controls, designated as 1). (B) Inhibition of constitutive ERK phosphorylation in SCC68 cells down-regulates uPA activity. SCC68 cells were cultured in six-well tissue culture dishes for 12 hours under their normal culture conditions, followed by incubation in growth factor-free medium for an additional 24 hours in the presence of the MEK inhibitor, PD98059 ($10 \mu\text{M}$), or an equal concentration of dimethylsulfoxide vehicle. Conditioned media ($20 \mu\text{L}$) were evaluated for uPA activity as described above. Results are expressed as percent change, relative to untreated cells, designated as 100%. (C) Analysis of ERK activation. Lysates were collected from the cells described in (B) and protein ($15 \mu\text{g}$) was analyzed by Western blotting to confirm that PD98059 treatment inhibited ERK phosphorylation. Blots were probed with anti-ACTIVE MAPK p42/p44 (1:5000) to detect the phosphorylated, active form of ERK (top) or with anti-ERK1/2 antibody (1:1000) to detect total ERK1/2 expression (bottom), followed by peroxidase-conjugated secondary antibody and enhanced chemiluminescence detection. The arrows designate the migration positions of p42 and p44.

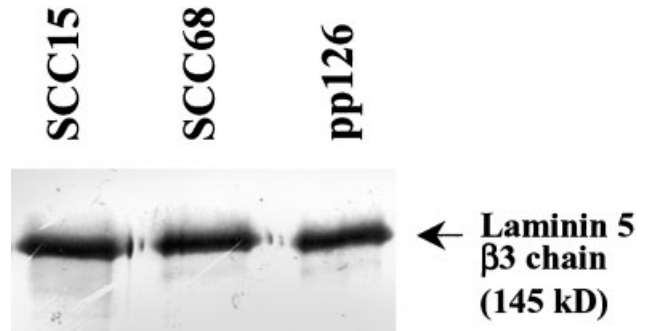


FIGURE 5. Analysis of laminin-5 deposition. pp126, SCC15, and SCC68 cells were grown for 48–72 hours until cells were confluent. The cells were then removed by treatment for approximately 7 minutes with 20 mM NH_4OH followed by three rapid washes in sterile distilled water and three washes with sterile phosphate-buffered saline. The matrix was collected as described in the Materials and Methods. Samples were then electrophoresed on 7.5% sodium dodecyl sulfate polyacrylamide gels, electroblotted to PVDF membrane, and probed with an antilaminin-5 $\beta 3$ chain-specific monoclonal antibody (B1K). Blots were then incubated with alkaline phosphatase-conjugated secondary antibody and developed colorimetrically.

SCC68 cells displayed an approximately threefold higher invasive activity (Fig. 6B). Treatment of SCC68 cells with the MEK inhibitor, PD98059, blocks uPA expression and reduces invasion in a dose-dependent manner, supporting the importance of ERK phosphorylation and uPA expression in OSCC invasion.

DISCUSSION

Although OSCC is the most common malignancy of the oral cavity, the cellular and biochemical factors that underlie locoregional and distant spread of the disease are poorly understood. We evaluated multiple cellular properties correlated with invasion and metastasis, including expression of cell-matrix adhesion proteins, cytoskeletal composition and organization, proteinase regulation, and invasive behavior. Our data demonstrate that although pp126, SCC15, and SCC68 cells retain comparable levels of E-cadherin expression (data not shown), they differ in their expression of IF, integrins, and proteases and in their regulation by environmental cues.

Oral squamous cell carcinomas are derived from squamous epithelial cells, as determined by their keratin IF composition.²⁶ Acquisition of an invasive and migratory oral carcinoma cell phenotype correlates with an abnormal expression pattern of cytoskeletal IF. Specifically, vimentin IF are expressed in these cells, which contain the Type I (K10, K14, K16) and Type II (K1, K5, K6) keratins.^{20,21,26} A comparison of cytoskeletal IF expression in SCC15, SCC68, and pp126 cells distinguishes the premalignant keratinocytes

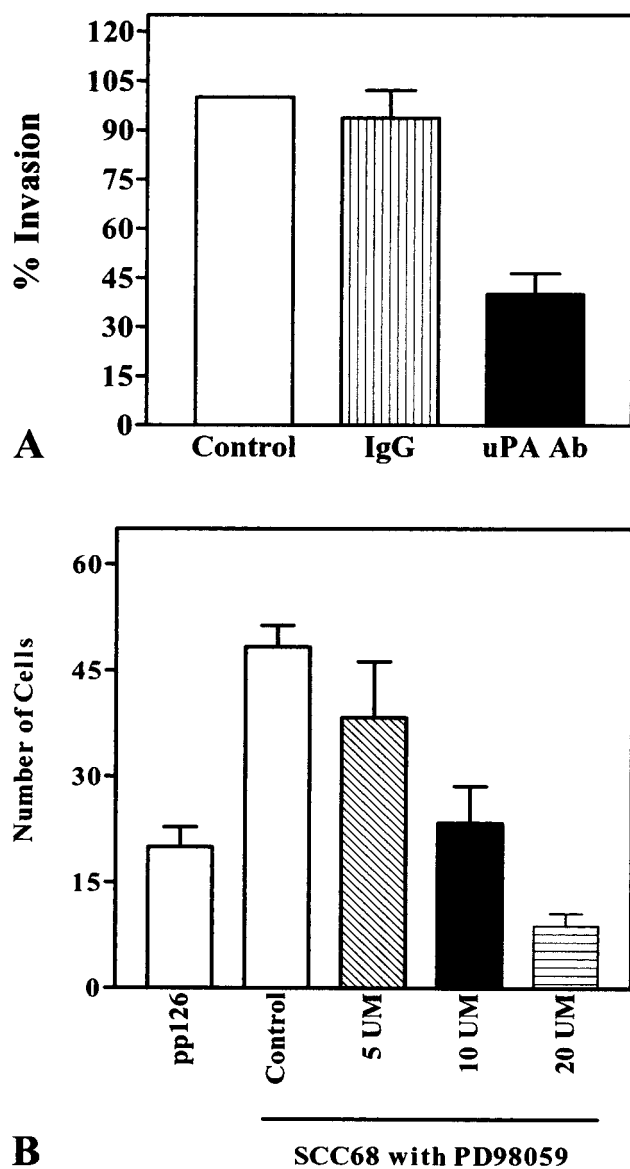


FIGURE 6. Analysis of invasion. (A) SCC68 cells (2×10^5) were seeded onto a porous polycarbonate filter overlaid with Matrigel ($11 \mu\text{g}$), allowed to invade for 36 hours, and the invading cells adherent to the lower surface of the filter were enumerated using an ocular micrometer. Inclusion of anticatalytic urinary-type plasminogen activator antibodies ($30 \mu\text{g}$), but not control IgG ($30 \mu\text{g}$), significantly inhibits invasion. Results are expressed as percent invasion relative to untreated control (designated as 100%). (B) Cells (pp126 or SCC68, as indicated, 2×10^5) were seeded onto a porous polycarbonate filter overlaid with Matrigel in the presence or absence of the MEK inhibitor, PD98059, at the concentrations indicated, allowed to invade for 24 hours, and the invading cells were quantified as described above. Results are expressed as relative invasion (number of cells per high-powered field).

from the more invasive carcinoma cells. Very few pp126 cells stain positively for vimentin by indirect immunofluorescence criteria. Those that do express vimentin show primarily short, discontinuous IF that are similar to the squiggles believed to be the precursors of longer IF.²² Conversely, the majority of SCC15 and SCC68 cells contain extensive vimentin IF networks. This aberrant expression of vimentin is linked to changes in cell morphology and behavior known as the epithelial/mesenchymal transition and is concurrent with a loss of cell anchorage and increased motility of many types of metastatic epithelial cells.²⁷⁻²⁹ This is consistent with the results of the current study. We show that vimentin-positive cells readily penetrate Matrigel, supporting the hypothesis that the alterations in the IF cytoskeleton in OSCC are correlated with invasive carcinomas.

Immunohistochemical studies of human tumors have demonstrated that numerous changes in the expression of integrins are associated with advanced-stage OSCC. Immunohistochemical analyses of human OSCC lesions are complex and difficult to interpret. In advanced lesions, staining for $\alpha 6$ and $\beta 4$ is commonly lost, whereas persistent or up-regulated, albeit disorganized, staining of $\alpha 2$, $\alpha 3$, and $\beta 1$ is observed consistently.^{8,23} Compared with premalignant pp126 cells, SCC15 and SCC68 cells exhibited a two to fivefold increase in cell surface expression of the $\alpha 2$, $\alpha 3$, and $\beta 1$ integrin subunits, supporting the published immunohistochemical data. $\beta 4$ integrin staining persists in cultured cells. However, the polarity of the expression may be altered compared with the normal oral mucosa. Although the functional contribution of integrins to regulation of OSCC dissemination is unknown, pp126 cells preferentially adhere to the basement membrane protein laminin-5 via the $\alpha 3\beta 1$ integrin.¹² Laminin-5 or integrin antibody-induced clustering of the $\alpha 3\beta 1$ integrin resulted in ERK activation and a subsequent increase in uPA expression. In addition, aggregation of the $\alpha 3\beta 1$ integrin in the presence of PD98059 blocked both ERK phosphorylation and uPA expression. This result indicates that $\alpha 3\beta 1$ integrin aggregation induces a signal propagated through the MEK-ERK pathway and up-regulates uPA expression, whereas clustering of other integrins such as $\alpha 2$, $\alpha 5$, $\alpha 6$, and $\beta 4$ was ineffective.¹² Consistent with this observation are data that demonstrate that inhibition of ERK activity using a dominant-negative mutant blocks transcription from the uPA promoter.²⁴

In the current study, uPA expression by pp126 cells was low, whereas expression by SCC15 and SCC68 cells was moderate and high, respectively. Both pp126 and SCC15 cells were responsive to $\alpha 3\beta 1$ integrin clustering, resulting in ERK phosphorylation (data

not shown) and stimulation of uPA expression. However, SCC68 cells, which were nonresponsive to integrin clustering-induced proteinase expression, displayed constitutively active ERK. In addition, inhibition of ERK activation with PD98059 significantly blocked uPA expression. Thus, it is noteworthy that premalignant and moderately invasive OSCC cells such as pp126 and SCC15, respectively, may respond to signals transduced by integrin-mediated matrix binding to promote proteolysis and invasion. In contrast, highly invasive OSCC cells such as SCC68 may have lost this regulatory mechanism, resulting in high basal levels of proteolysis and contributing to an enhanced invasive potential. We are currently investigating whether the constitutive activation of the MEK/ERK pathway and the subsequent activation of the uPA promoter in SCC68 cells are related to sustained integrin activation or involve alternative signaling pathways.

To assess the functional contribution of uPA expression to invasive activity, the ability of SCC68 cells to penetrate a Matrigel barrier was evaluated in the presence of anticatalytic uPA antibodies. Inhibition of uPA significantly decreased invasive activity, supporting the requirement for proteolytic modification of Matrigel components for invasion. In contrast to invasion, migration of SCC68 cells is unaffected by anticatalytic uPA antibodies (S. Ghosh and M.S. Stack, unpublished observation), indicating that motility per se is proteinase independent. In addition to direct inhibition of uPA function, blocking uPA expression using PD98059 also decreased invasion, providing additional evidence that ERK activation contributes to the invasive activity of SCC68 cells via a uPA-dependent mechanism. This conclusion is supported by a study using OSCC cells orthotopically implanted into the floor of the mouth in a murine model. Injection of the resultant tumors with PD98059 significantly blocked invasion of the underlying musculature.³⁰ These data suggest that loss of adhesion-regulated proteinase production may lead to elevated pericellular proteinase activity, thereby contributing to the invasive potential of OSCC.

The results of the current study support a working hypothesis that expression of uPA is under control of a variety of factors, including integrin aggregation-induced signaling in premalignant oral mucosa. Progression to a more malignant phenotype may be accompanied by constitutive activation of signaling pathways such as the MEK/ERK cascade, leading to loss of adhesion-regulated proteinase expression. In support of this hypothesis, we observed the presence of activated ERK and high uPA levels in primary OSCC tumor specimens compared with the adjacent tissue

(S. Ghosh and M.S. Stack, unpublished observation). Although we do not understand the mechanism by which activation of the MEK/ERK pathway is sustained in SCC68 cells, ligand (i.e., uPA) occupation of the uPAR promotes a physical interaction between the uPA/uPAR complex and $\beta 1$ integrin-containing heterodimers.^{12,31,32} A functional consequence of this interaction is the enhancement of both the magnitude and duration of ERK activation.³² These data suggest a potential autocrine regulatory loop wherein uPA expression results in sustained uPA/uPAR ligation and uPA/uPAR/integrin interaction, potentiating integrin signaling, ERK activation, and subsequent uPA transcription. The resulting increase in uPA activity promotes pericellular proteolysis, thereby contributing to the invasive potential of OSCC.

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