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Association Between αvβ6 Integrin Expression, Elevated p42/44 kDa MAPK, and Plasminogen-Dependent Matrix Degradation in Ovarian Cancer

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Abstract Altered expression of αv integrins plays a critical role in tumor growth, invasion, and metastasis. In this study, we show that normal human epithelial ovarian cell line, HOSE, and ovarian cancer cell lines, OVCA 429, OVCA 433, and OVHS-1, expressed αv integrin and associated β1, β3, and β5 subunits, but only ovarian cancer cell lines OVCA 429 and OVCA 433 expressed αvβ6 integrin. The expression of αvβ6 in OVCA 429 and OVCA 433 was far higher than αvβ3 and αvβ5 integrin and correlated with high p42/p44 mitogen activated protein kinase (MAPK) activity and high secretion of high molecular weight urokinase plasminogen activator (HMW-uPA), pro-metalloproteinase 2 and 9 (pro-MMP-9 and pro-MMP-2). In contrast to HOSE and OVHS 1, OVCA 433 and OVCA 429 exhibited approximately 2-fold more plasminogen-dependent [^{3}H]-collagen type IV degradation. Plasminogen-dependent [^{3}H]-collagen IV degradation was inhibited by inhibitor of uPA (amiloride) and MMP (phenanthroline) and by antibodies against uPA or MMP-9 or αvβ6 integrin, indicating the involvement of αvβ6 integrin, uPA and MMP-9 in the process. The αvβ6 correlated increase in HMW-uPA and pro-MMP secretion could be inhibited by tyrosine kinase inhibitor genistein or the MEK 1 inhibitor U0126, consistent with a role of active p42/44 MAPK in the elevation of uPA, MMP-9, and MMP-2 secretion. Under similar conditions, genistein and U0126 inhibited plasminogen-dependent [^{3}H]-collagen type IV degradation. These data suggest that sustained elevation of p42/44 MAPK activity may be required for the co-expression of αvβ6 integrin, which in turn may regulate the malignant potential of ovarian cancer cells via proteolytic mechanisms. J. Cell. Biochem. 84: 675–686, 2002.

Key words: αvβ6 integrin; epithelial cells; ovarian cancer; metastasis

The integrins constitute a large family of related αβ cell surface heterodimers widely found on many cell types that mediate cell-matrix and cell–cell interactions [Hynes, 1992]. At least 21 different integrins are currently known; most of which belong to one of two major subfamilies defined by the β1 and αv subunits [Hynes, 1992]. It is the combination of specific α and β pairings that determines ligand specificity. Both α and β subunits contribute to ligand binding but subunit switching may change this specificity. The αv integrins pair with β1, β3, β5, β6, and β8 subunits and have been implicated in cell attachment and spreading [Moser et al., 1996], regulation of extracellular proteases [Brooks et al., 1996], angiogenesis [Brooks et al., 1995], regulation of tumor progression [Petitclerc et al., 1999], tumor invasion, and metastasis [Gasparini et al., 1998].

In ovarian cancer cell lines, β1 and αvβ3 mediate in vitro adhesion to collagen, laminin, and fibronectin [Cannistra et al., 1995]. Localization of the α6β4 laminin receptor within the cell membrane in poorly differentiated ovarian carcinomas is changed compared with normal epithelia [Bottini et al., 1993]. This change is associated with topological alterations of laminin within the cell membrane of ovarian cancer cells, suggesting a relationship between altered

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production or degradation of extracellular matrix components of ovarian cancer cells and the α6β4 receptor. The expression of β1 and αvβ3 integrins have been shown in ovarian cancer cell lines and cancer tissues [Cannistra et al., 1995], while subunits β2, β4, and β7 were absent. αvβ3, α5, and α6 showed higher immunoreactivity compared with α2, α3, and α4 subunits [Cannistra et al., 1995]. Furthermore, peritoneal seeding of ovarian cancer was successfully blocked by using RGD binding peptide, suggesting a role for β integrin subunits in ovarian cancer metastasis [Yamamoto et al., 1991].

αvβ6 is an epithelial cell restricted integrin. It has been shown to be expressed in malignant colonic and oral epithelium but not in normal epithelium [Breuss et al., 1995; Jones et al., 1997]. In healthy adult primate tissues, β6 mRNA and protein are not detected [Breuss et al., 1993]. In contrast, β6 is expressed during fetal development during wound healing and in a variety of epithelial tumors [Agrez et al., 1994; Haapasalmi et al., 1996]. Although 90% of ovarian cancer arise from malignant transformation of the surface epithelium and this tissue constantly undergoes changes akin to wound healing after ovulation, an evaluation of the role of αvβ6 is required. Interestingly, αvβ6 is concentrated at the invading margins of epithelial malignancies, the site where numerous proteolytic activities are concentrated [Brooks et al., 1996]. Recently, it has been shown that αvβ6 expression in oral keratinocytes and colon cancer cells that do not normally express αvβ6 leads to enhanced secretion of MMP-9 [Agrez et al., 1999; Thomas et al., 2001b]. αvβ6-induced secretion of MMP-9 in oral keratinocytes is associated with increased migration [Thomas et al., 2001a] and in colon cancer cells increased plasminogen-dependent proteolysis of denatured collagen [Agrez et al., 1999]. Collectively, these observations suggest that αvβ6 integrin may play a substantial role in proteolytic events associated with tumor progression, invasion, and metastasis.

Integrin signaling may occur via several different pathways of which p42/44 MAPK is one of the best characterized. Integrin-mediated MAPK signaling leads to the activation of transcription factors (e.g., ets, elf1, fos, AP-1, NFκB, etc.) that respond in part by regulating transcription of a number of target genes critical for extracellular matrix degradation [Aplin et al., 2001]. Among these are the matrix metalloproteinases (MMPs) [Gum et al., 1997] and a serine protease uPA that converts plasminogen to plasmin [Ghosh et al., 2000]. It has been shown that uPA and type IV collagenase with its two subtypes, MMP-2 and MMP-9, play an important role in ovarian cancer progression and metastasis [Moser et al., 1994; Young et al., 1994]. In this study, we show that ovarian cancer cells expressing αvβ6 integrin have elevated p42/44 MAPK activity and generate a series of proteolytic activities that involve HMW-uPA and pro-collagenase IV. We show that inhibition of the activation of p42/44 MAPK cascade inhibits HMW-uPA and pro-collagenase IV expression. This results in a complete abrogation of plasminogen-dependent extracellular matrix degradation. Inhibition of plasminogen-dependent matrix degradation can also be achieved by using antibodies against uPA or MMP-9 or αvβ6 integrin but not by antibodies against αvβ3 or αvβ5. These data indicate that in ovarian cancer uPA-mediated plasmin-dependent basement membrane degradation may be regulated by αvβ6 integrin and sustained activation of p42/44 MAPK pathway. These observations represent a promising target for pharmacological intervention in ovarian cancer.

MATERIALS AND METHODS

Antibodies and Reagents

The monoclonal antibodies against integrin α1 (FB12), α2 (PIE6), α3 (PIB5), α4 (PIH4), α5 (PID6), α6 (CLB-701), αv (LM 142), β1 (P5D2), αvβ3 (LM609), αvβ5 (P1F6), and αvβ6 (E7P6, 10D5) were from Chemicon International (Temecula, CA). Monoclonal antibody against uPA (394) and uPAR (3936) from American Diagnostic (Greenwich) and anti-phospho Erk from New England Biolabs (Beverly, MA). Phycoerythrin-conjugated goat anti-mouse IgG was obtained from Chemicon International and peroxidase-conjugated goat anti-mouse antibody from Bio-Rad (Hercules, CA). Amiloride, 1,10-phenanthroline, genistein, and *p*-aminophenylmercuric acid (APMA) were purchased from Sigma (St Louis, MO). The MEK 1 inhibitor UO126 was from Promega (Madison, WI).

Cell Lines

Epithelial ovarian cancer cell lines OVCA 429 and OVCA 433 were obtained from Dr. Robert Bast, MD, Anderson Centre, Houston, USA;

clear cell carcinoma cell line OVHS1 from Dr. Hideki Sakamoto, Nihon University School of Medicine, Japan, and primary human ovarian surface epithelial cell lines (clones HOSE 17.1) immortalized by a retroviral vector (LXSN-16E6E7) expressing HPV-E6E7 open reading frame were from Dr. Samuel Mok, Harvard Medical School, Boston, MA. OVCA 429, OVCA 433, and OVHS 1 cell lines were maintained in MEM medium, and HOSE cell lines were maintained in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10 mM HEPES, 100 μg/ml penicillin, 100 μg/ml at 37°C in 5% CO_2. Viability was checked routinely by the Trypan blue exclusion method.

Flowcytometric Analyses

Monolayer cultures of ovarian cancer cell lines were washed twice with PBS, harvested with trypsin-versene (CSL Biosciences, Australia), and 10^6 cells incubated with primary antibody for 20 min at 4°C and washed twice with PBS. Cells were stained with secondary antibody conjugated with phycoerythrin for 20 min at 4°C, washed twice with PBS, and then resuspended in 0.5 ml PBS prior to FACScan analysis (Becton Dickinson, NJ).

p42/44 MAPK Assay

Cells were collected, sonicated, and MAPK assay performed as described in the manufacturer's instructions (BIOTRAN Assay kit Amersham, UK).

Preparation of Conditioned Medium

Cells were seeded at 2×10^6 cells/25-cm^2 flask and allowed to grow in medium in the presence of FBS for 24 h. After 24 h, adherent cells were washed three times with medium lacking FBS and maintained in their FBS free medium for 48 h after which conditioned medium was collected, cleared of cells, and debris by centrifugation at 2,000*g* for 10 min. Conditioned medium was concentrated 20–25-fold using Biomax Ultrafree Centrifugal Filter Unit (Millipore, Bedford, MA) with a 10-kDa pore diameter cut-off. To ensure equal loading, protein estimation on the conditioned medium was performed using the BCA protein assay kit (Pierce, Rockford, IL).

Western Blotting

Conditioned medium and cell lysates containing equal amounts of protein were electrophoresed on 10% SDS–PAGE gels under non-reducing conditions and transferred to nitrocellulose membranes. Membranes were probed with primary antibody followed by peroxidase-labeled secondary antibody and visualized by the ECL (Amersham, UK) detection system, according to the manufacturer's instructions.

Zymography

Pro-MMP-2 and pro-MMP-9 expression in conditioned medium were analyzed using 10% SDS–gelatin (1 mg/ml final concentration) zymography under non-reducing conditions. Conditioned medium were mixed with substrate gel sample buffer (0.5 M Tris pH 6.8, 5% SDS, 20% glycerol, and 1% bromophenol blue) at 1:1 ratio. Five micrograms of protein were loaded onto the gel without prior boiling. Following electrophoresis, gels were washed in 2.5% Triton X-100 and then without Triton at room temperature to remove SDS. Gels were incubated at 37°C overnight in the incubation buffer (50 mM Tris-HCl and 5mM $CaCl_2$, pH 8.0), stained with 0.15% Coomassie Blue R250 at room temperature and de-stained in the same solution without Coomassie Blue. Gelatin-degrading enzymes were identified as clear bands against a blue background. Gelatinolytic activity attributed by MMP-2 and MMP-9 was abolished by incubating zymograms with 2mM 1:10 phenanthroline and EDTA (data not shown). Pro-MMP-2 and pro-MMP-9 activation was obtained by incubating conditioned medium with APMA (2 mM) for 4 h prior to zymography (data not shown). BHK-cell conditioned medium was used as a standard in each case.

Preparation and Degradation of Collagen Type IV Basement Membrane

Collagen type IV (5 mg/100 ml) dissolved in 0.25% acetic acid and labeled with 10 μCi of [^{3}H]-collagen type IV was seeded onto 24-well plates at 37°C overnight. Plates were rinsed gently with PBS three times and allowed to dry in room temperature. Fresh plates were prepared for each experiment.

Ovarian cancer cell lines (OVCA 433, OVCA 429, OVHS 1, and HOSE) were plated directly onto the isotopically labeled extracellular matrix at a density of 1×10^5 cells/well in 0.3 ml serum free medium. After 24 h, the conditioned medium was removed, centrifuged in a Beckman microfuge for 5 min, and counted in a β-scintillation counter to monitor release

of soluble [^{3}H]-collagen fragments from the insoluble extracellular matrix. In studies involving inhibition of plasminogen activation, uPA and MMP activity, cells were treated with inhibitors and antibodies 30 min prior to plating onto the [^{3}H]-collagen labeled extracellular matrix.

RESULTS

αvβ6 Integrin Is Expressed in Ovarian Cancer Cell Lines, But Not in Normal Ovarian Surface Epithelial Cell Line

The pattern of cell surface expression of α integrin and associated β subunit was assessed by flow cytometry in ovarian cancer cell lines (OVCA 429, OVCA 433, and OVHS 1) and in a HOSE cell line using a panel of integrin-specific antibodies, with the result presented in Tables I, II, and Figure 1. All ovarian cell lines expressed αv, α2, α3, α5, α6, β1, β3, and β5 integrin subunits. α1 and α5 were present only in OVCA 429 and OVHS 1 cells. Among the α subunits, the immunoreactivity was stronger for α2, α3, and αv integrin (Table I). On the other hand, cell surface expression of αvβ6 integrin was found only in OVCA 429, OVCA 433, while no such expression was observed in HOSE or OVHS 1 cell lines.

αvβ6 Expressing Ovarian Cancer Cells Have Elevated p42/44 MAPK Activity

As constitutive activation of p42/44 MAPK has been implicated in several human tumor types [Hoshino et al., 2000], the possibility that p42/44 MAPK activity might play a role in the regulation of αvβ6 integrin expression was analyzed. In vitro kinase assay, specific for p42/44 MAPK activity, showed a 3.5-fold higher MAPK activity in αvβ6 expressing OVCA 429 and OVCA 433 cell lines than those that do not express αvβ6 integrin (HOSE and OVHS 1 cell lines) (Fig. 2a). Immunoblotting of cell lysates with an antibody, which crossreacts with phospho-p42/44 MAPK, indicated the presence of immunoreactive protein indistinguishable in size from recombinant phospho-p42/44 MAPK (Fig. 2b). Furthermore, treatment of OVCA 433 cells with the broad spectrum tyrosine kinase inhibitor (genistein) and the specific inhibitor of MEK 1 (U0126) caused a 60–80% inhibition in the activity of p42/44 MAPK (Fig. 2c). Similar results were obtained by immunoblotting of the inhibitor-treated cell lysates with anti-phospho p42/44 MAPK (Fig. 2d).

Expression of αvβ6 Integrin Correlates With Higher Cell Surface Expression of uPA/uPAR and Elevated Secretion of HMW-uPA, pro-MMP-2, and pro-MMP-9 Into the Conditioned Medium

In order to evaluate a potential role of αvβ6 integrin in the regulation of proteolytic systems, cell surface expression of uPA and uPAR were analyzed by flow cytometry and conditioned medium from all cell lines examined for the expression of HMW-uPA, pro-MMP-2, and pro-MMP-9. Both uPA and uPAR expression were 2–3-fold higher in cells expressing high αvβ6 integrin (OVCA 429 and OVCA 433) compared to cells that lack the integrin (OVHS 1 and HOSE) (Table III).

Western blotting of conditioned medium with equal protein loading showed a 4–5-fold higher expression of HMW-uPA in conditioned medium of cells expressing αvβ6 integrin (OVCA 429 and OVCA 433) and a very low to nondetecable levels of HMW-uPA in cell lines that do not express αvβ6 integrin (HOSE and OVHS 1) (Fig. 3). Gelatin zymography on conditioned medium revealed a similar pattern where αvβ6 integrin expressing cell lines (OVCA 429 and OVCA 433) expressed high levels of both pro-MMP-9 and pro-MMP-2 expression than either HOSE and OVHS 1 cell lines, which showed no pro-MMP-9 expression and 1.6–6-fold lower pro-MMP-2 expression (Fig. 4). To confirm that the gelatinolytic activity seen at the 72 and 92 kDa positions represented MMP-2 and MMP-9

TABLE I. α Integrin Subunit Expression in Normal Ovarian and Cancer Cell Lines

Cell type	α1 integrin MIF	α2 integrin MIF	α3 integrin MIF	α4 integrin MIF	α5 integrin MIF	α6 integrin MIF	αv integrin MIF
OVCA 429	30	47	27	13	20	13	640
OVCA 433	8	210	388	6	15	50	1263
OVHS 1	85	53	67	9	32	30	1625
HOSE	8	415	452	4	18	58	1170

MIF, median intensity of fluorescence (arbitrary unit, log scale).
Data is representative of three different experiments.

TABLE II. β Integrin Subunit Expression in Normal Ovarian and Cancer Cell Lines

Cell type	β1 integrin MIF	αvβ3 integrin MIF	αvβ5 integrin MIF	αvβ6 integrinMIF
OVCA 429	2502	18	42	590
OVCA 433	2090	30	68	460
HOSE	4216	300	140	5
OVHS 1	1046	73	84	4

MIF, median intensity of fluorescence (arbitrary unit, log scale).
Data is representative of three different experiments.

proenzymes, conditioned medium from OVCA 433-cell line was incubated with APMA prior to zymography resulting in decreased degradation at the 92-kDa position (uncleaved proenzyme) and increased gelatinolytic activity at the 84-kDa position (cleaved, activated form of MMP-9) (data not shown). When conditioned medium was exposed to 1,10-phenanthroline or EDTA (2 mM) complete loss of gelatinase activity was seen (data not shown). Collectively, these data demonstrate that in ovarian cancer cells expression of αvβ6 integrin strongly correlates with elevated expression of the cell surface proteases.

Expression of αVβ6 Integrin Regulates Enhanced Plasminogen-Dependent [^{3}H]-Collagen IV Degradation

To assess the possible correlation of αvβ6 integrin expression with higher levels of extracellular matrix proteolysis, [^{3}H]-collagen IV substrate was chosen, as this molecule is the predominant collagen type in the basement membrane and is the substrate for both type IV collagenases (MMP-2 and MMP-9).

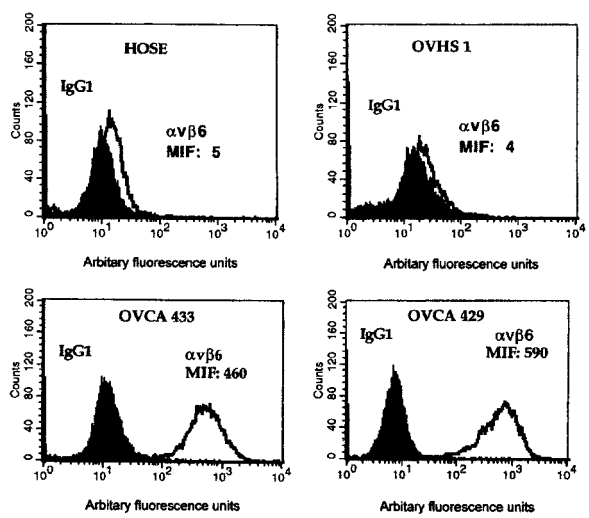


Fig. 1. Flow-cytometric analyses of αvβ6 integrin in OVCA 429, OVCA 433, OVHS 1, and HOSE cell lines. Cells were incubated with either control IgG or primary αvβ6 monoclonal antibody followed by secondary goat anti-mouse IgG conjugated with phycoerythrin. The median intensity of fluorescence (MIF, arbitary units, log scale) was measured. Results are representative of three independent experiments.

Degradation of basement membrane was monitored by the release of soluble [^{3}H]-fragments from [^{3}H]-collagen IV labeled basement membrane in the absence or presence of purified human plasminogen. Experiments showed that the plasminogen-dependent process was complete within the initial 24 h of incubation and the most optimal concentration of purified plasminogen was 10–20 μg/ml (Fig. 5a). Moreover, exposure of [^{3}H]-collagen IV to serum-free culture medium does not result in tritium release either in the presence or absence of plasminogen (data not shown).

To assess if αvβ6 integrin expression correlated high expression of HMW-uPA and pro-MMP's led to elevated rates of plasminogen-dependent matrix degradation, αvβ6 expressing and non-expressing ovarian cell lines were compared. Even though, the basal matrix degradation capacity in the absence of plasminogen for all four ovarian cell lines was approximately the same, both αvβ6 integrin expressing ovarian cancer cell lines had a 2-fold enhanced capacity to degrade [^{3}H]-labeled collagen IV when plasminogen was present (Fig. 5b). In contrast, the addition of plasminogen to HOSE and OVHS 1 cell lines produced no increase in degradation of basement membrane. In addition, the 2-fold enhanced plasminogen-dependent collagen IV degradation seen with αvβ6-expressing cell line (OVCA 433) was abolished by the addition of either uPA inhibitor amiloride, the zinc chelator 1,10-phenanthroline and antibodies directed against either uPA or MMP-9 or αvβ6 (Fig. 5c). However, no such effect was observed with antibodies against αvβ3 or αvβ5 (Fig. 5c). These data strongly suggest that in ovarian cancer, uPA-mediated plasmin-dependent serine and metalloproteolytic activity requisite for matrix degradation coincides with high αvβ6-integrin expression

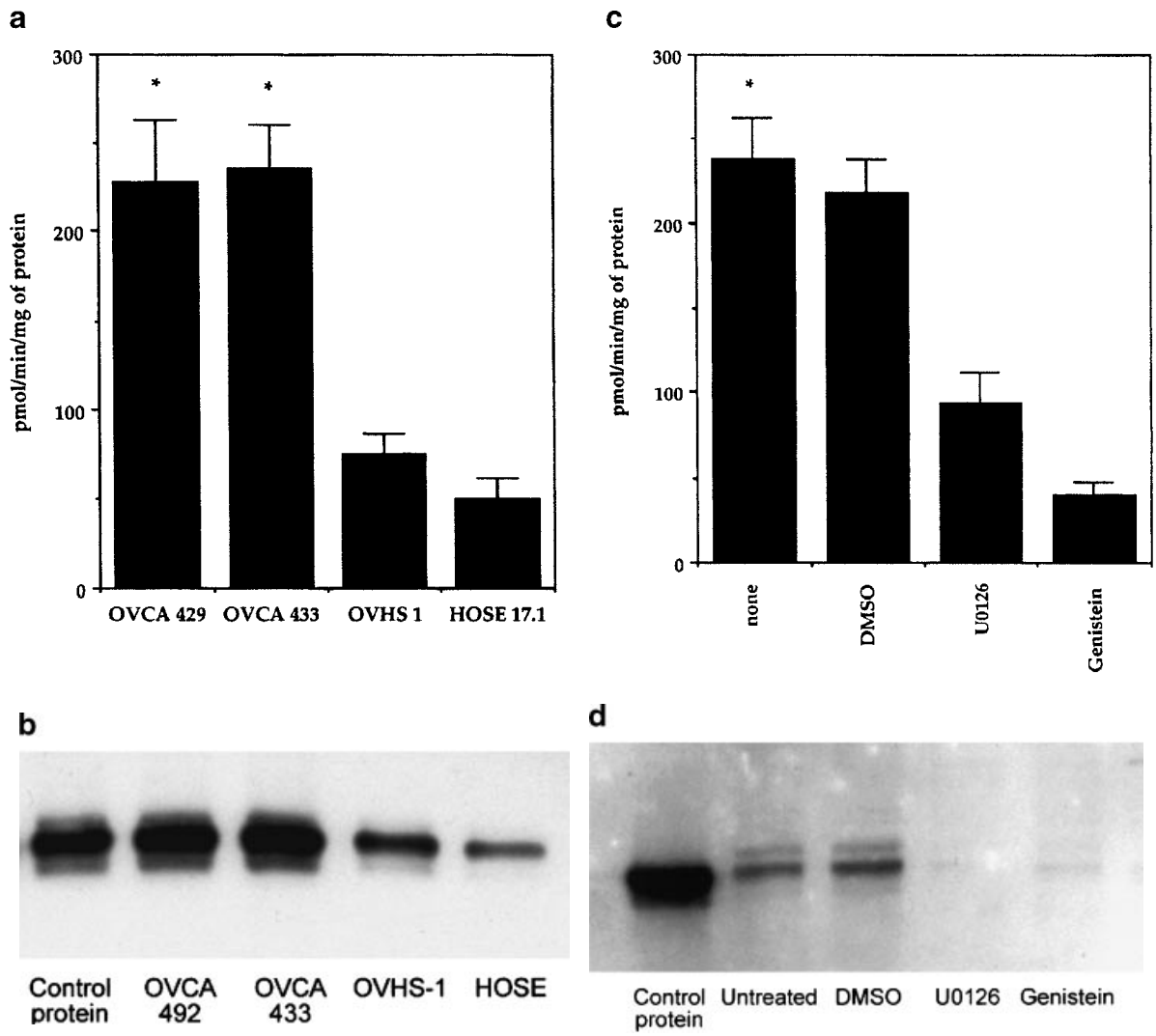


Fig. 2. p42/44 MAPK activity in OVCA 429, OVCA 433, OVHS 1, and HOSE cell lines. **a**: In vitro kinase assay was performed on cell lysates prepared from OVCA 429, OVCA 433, OVHS 1, and HOSE cell lines as described in Materials and Methods. Results are shown as mean ± SEM of three different experiments performed in triplicate; (*$P < 0.001$, compared to OVHS 1 and HOSE cell lines). **b**: Cell lysates used for in vitro kinase assay were analyzed by equal protein loading on 10% SDS–PAGE electrophoresis (under non-reducing conditions) followed by Western blotting using antibodies against phosphorylated p42/44 MAPK (E10 antibody, New England Biolabs, Beverly, MA). **d**: In vitro kinase assay was performed on cell lysates prepared by treating OVCA 433 cells for 24 h with genistein (0.2 mM) and U0126 (5 μM). Results are shown as mean ± SEM of three different experiments performed in triplicate; (*$P < 0.001$, compared to genistein and U0126-treated cells). **e**: Cell lysates used for in vitro kinase assay were analyzed by equal protein loading on 10% SDS–PAGE electrophoresis (under non-reducing conditions) followed by Western blotting using antibodies against phosphorylated p42/44 MAPK (E10 antibody, New England Biolabs).

and can be inhibited by blocking αvβ6 integrin function.

Effect of Tyrosine Kinase Inhibitor and Specific MEK 1 Inhibitor on the Expression of HMW-uPA pro-MMPs and Plasminogen Dependent [^{3}H]-Labeled Collagen IV Degradation

OVCA 433 cells were treated for 48 h with genistein (0.2 mM) and U0126 (5 μM), at concentrations known to inhibit p42/44 MAPK activity by 60-80% without affecting the viability of cells. There was 2–20-fold diminution of HMW-uPA and 2–3-fold reduced secretion of MMP-9 (Fig. 6a,b), while the secretion of MMP-2 was not substantially affected by U0126 (Fig. 6b).

OVCA 433 cells treated with genistein and U0126 were subsequently analyzed for their

TABLE III. uPA and uPAR Expression in Normal Ovarian and Cancer Cell Lines

Cell type	uPA MIF	uPAR MIF
OVCA 429	40	20
OVCA 433	42	20
HOSE	15	10
OVHS 1	15	10

MIF, Median intensity of fluorescence (arbitrary unit, log scale) Data is representative of three different experiments.

ability to degrade [^{3}H]-labeled collagen IV. Plasminogen was added at 20 μg/ml and the cells incubated for 24 h. The 2-fold increase in collagen IV degradation by OVCA 433 cells in the presence of plasminogen was totally abolished by pre-treatment with either genistein or U0126 (Fig. 6c). In conclusion, treatment of OVCA 433 cells with MAPK inhibitors reduced expression of HMW-uPA and pro-MMP-9 secretion and totally abrogated plasminogen-dependent collagen IV degradation.

Statistics

MAPK activity on normal proliferating cells and the effect of plasminogen on collagen IV degradation by normal ovarian and cancer cell lines were analyzed by nonparametric anaslysis of variance (Mann–Whitney U test). Statistical significance was indicated by $P < 0.05$. Data are presented as means ± SEM.

DISCUSSION

Altered expression of integrins and/or activation of oncogenes and signal transducing proteins have been linked with cancer [Jones et al., 1997; Petitclerc et al., 1999; Esteller et al., 2001]. Even though, MEK and p42/44 MAPK were not identified as oncogene products,

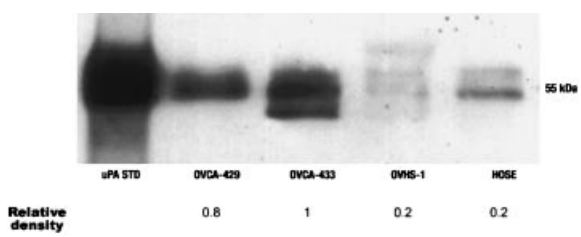


Fig. 3. Expression of HMW-uPA in conditioned medium of ovarian cancer and HOSE cell lines. Conditioned medium was prepared as described in the Materials and Methods section. The samples were analyzed by equal protein loading on 10% SDS–PAGE electrophoresis (under non-reducing conditions) followed by Western blotting using monoclonal anti-uPA antibody. Ukidan was used as standard reference uPA (Serono, Australia). Results are representative of three experiments.

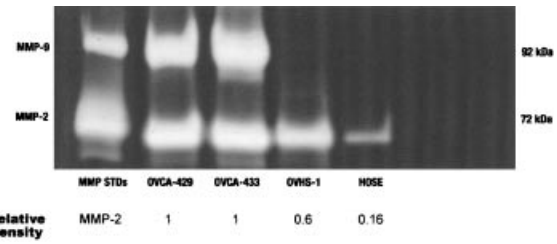
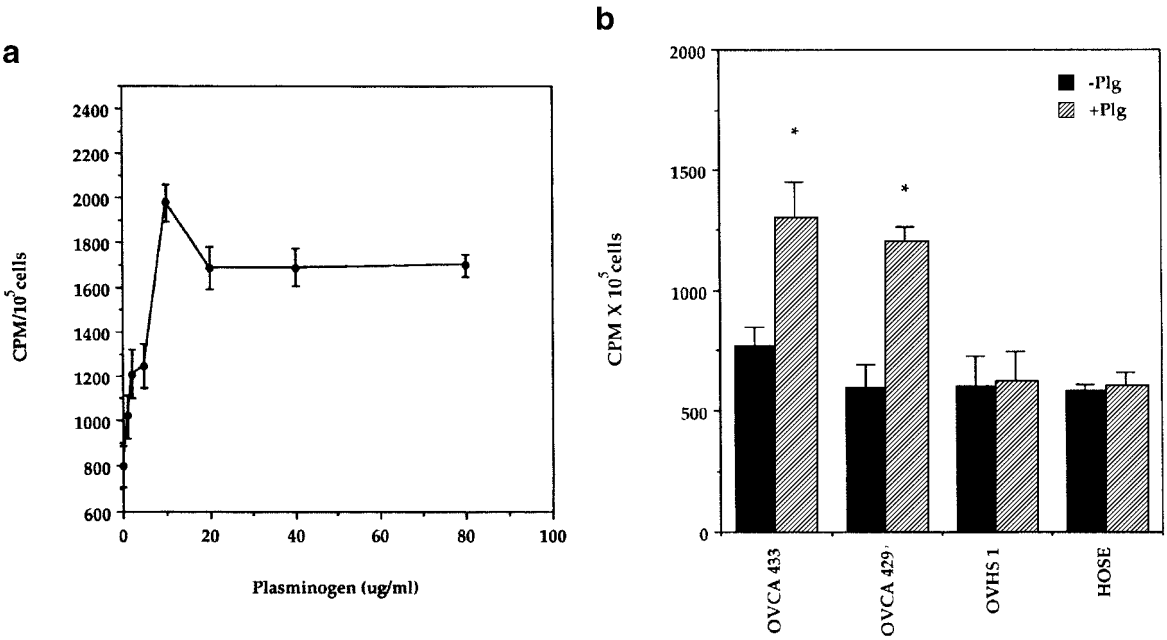


Fig. 4. Gelatin zymography showing the amounts of pro-MMP-2 and pro-MMP-9 secreted in conditioned medium (concentrated 20-fold) from ovarian cell lines. The positions of purified pro-MMP-2 and pro-MMP-9 are shown on the left. Results are representative of three experiments.

activation of MEK 1 has been shown to transform mammalian cells [Blalock et al., 2000], but few studies have examined disorders of the MAP kinase cascade in human tumors. In this study, we demonstrate that the sustained activation of p42/44 MAPK coincides with the expression of αvβ6 integrin in ovarian cancer and that p42/44 MAPK at multiple levels is associated with the neoplastic phenotype of ovarian cancer cells.

Although, p42/44 MAPK cascade is an essential mitogenic signaling pathway in many types of cells [Campbell et al., 1995], inappropriate activation of the cascade has been linked to the carcinogenesis of several tissues such as colon, lung, ovary, and kidney [Hoshino and Kohno, 2000]. Recently it has been shown that blockade of p42/44 MAPK cascade suppresses growth of colon tumors in vivo [Sebolt-Leopold et al., 1999]. Cross-linking of integrins is known to activate various signaling molecules including *ras* and focal adhesion kinase (FAK) contributing to the activation of p42/44 MAPK [Lin et al., 1997]. These studies, however, did not address the possibility that specificity may exit among integrin subunits for their ability to modulate p42/44 MAPK activity. Recently, however, this possibility was substantiated by the finding that only a subset of integrins (α1β1, α5β1, and αvβ3) can activate p42/44 MAPK through the phosphorylation of Shc [Wary et al., 1996]. Other integrins (α2β1, α3β1, and α6β1) were unable to induce MAPK activation under similar conditions. Our study extends these findings and demonstrates that the sustained elevation of p42/44 MAPK activity may be required for maintaining the expression of αvβ6 integrin and proteolytic activity of ovarian cancer cells. In our study, αvβ6 integrin expressing OVCA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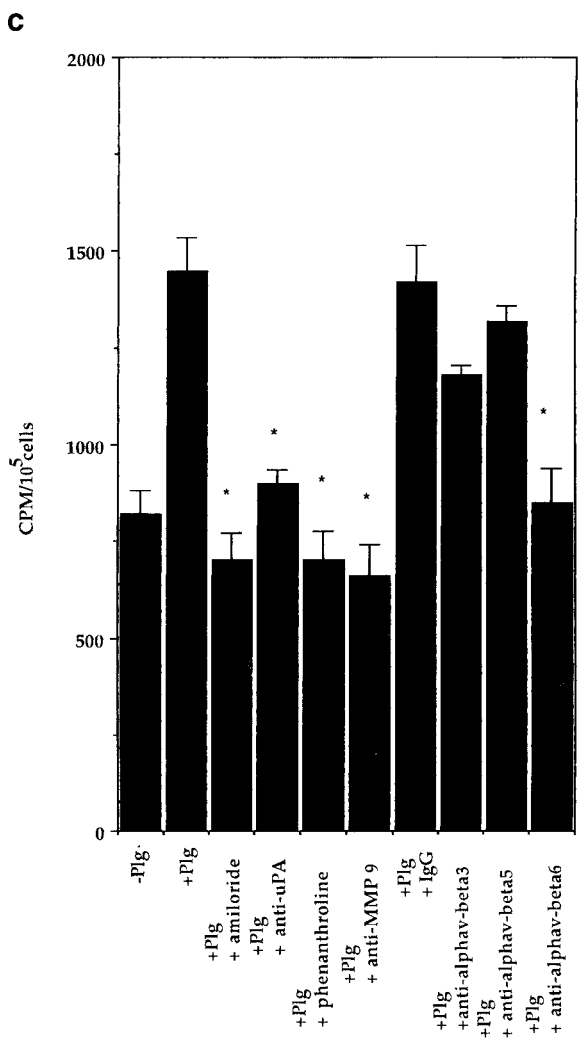
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+Plg + anti-alphav-beta5
+Plg + anti-alphav-beta6

433 and OVCA 429 cells demonstrated elevated p42/44 MAPK activation and expression of cell surface bound uPA/uPAR. Our results are consistent with that of Patton co-workers, who have demonstrated similar correlation of the activation of p42/44 MAPK pathway with uPA expression in OVCA 429 and OVCA 433 cells [Patton et al., 1998]. Compared to HOSE and OVHS 1 cell lines, αvβ6 integrin expressing OVCA 433 and OVCA 429 also had high secretion of HMW-uPA, pro-MMP-2, and pro-MMP-9 in conditioned medium. As uPAR occupancy of uPA has been shown to activate p42/44 MAPK signaling cascade [Lengyel et al., 1997; Santibanez et al., 2000], requisite for plasmin generation, activation of MMPs [Crowe and Shuler, 1999], and subsequent matrix degradation, the presence of an αvβ6 integrin-mediated potential autocrine loop can be suggested in ovarian cancer cells. The fact that inhibition of p42/44 MAPK activity by U0126 and genistein not only reduces pro-MMP-9 and HMW-uPA expression in tumor conditioned medium but also completely abrogates plasminogen-dependent matrix degradation indicates a link between p42/44 MAPK activity, uPA and MMP-9 secretion and activity. The fact that plasminogen-dependent matrix-degradation was completely abolished by inhibitors and antibodies of uPA or MMP-9 or αvβ6 integrin suggest a functional role of this integrin in uPA and MMP mediated proteolysis of ovarian cancer. Even though, there was equal plasminogen-independent degradation of basement membrane in all the ovarian cancer cell lines studied, it was only in αvβ6 expressing cell lines that the addition of plasminogen produced substantial increases in basement membrane degradation. Previous studies have shown activation of MMPs by plasminogen through uPA-dependent plasmin generation [Ramos-DeSimone et al., 1999]. uPA can trigger several biological responses in cells including proliferation, migration, invasion, etc [Aguirre Ghiso et al., 1999]. The fact that high expression of αvβ6 integrin correlates with high secretion of uPA in the medium suggest that uPA in a paracrine fashion, in the presence of plasminogen, may activate the proteolytic mechanism(s) of αvβ6 expressing ovarian cancer cells resulting in enhanced basement membrane degradation. Since the process can be blocked by the inhibitory antibody of αvβ6 integrin suggest that this molecule may regulate ovarian cancer progression by modulating uPA-mediated processes.

The coordinate expression of some integrins with uPA and MMPs in invasive tumors has been shown previously. Ligation of αvβ3 integrin with vitronectin has been shown to regulate expression of uPA in melanoma cells [Khatib et al., 2001]. On the other hand, treatment of human keratinocytes with anti-α3 or anti-β1 monoclonal antibodies induces expression of MMP-2 [Larjava et al., 1993]. De novo expression of αvβ6 integrin has been shown to modulate several processess in colon carcinoma cells including cell adhesion and spreading on fibronectin, proliferation within collagen gels, and MMP-9 regulation [Agrez et al., 1994, 1999; Thomas et al., 2001b]. αvβ6 integrin plays a critical role in keratinocyte migration on fibronectin and that effect is enhanced by protein kinase C activation [Huang et al., 1996]. Inhibitory antibodies to αvβ6 integrin block keratinocyte migration [Thomas et al., 2001a] and fibronectin-dependent MMP-9 activation [Thomas et al., 2001b]. This study provides further evidence of a link between αvβ6 integrin and uPA-mediated proteolysis leading to enhanced matrix-degradation in ovarian cancer. It is interesting to speculate that αvβ6 integrin targets proteases through p42/44 MAPK signaling cascade to bring about localized changes in protease concentration at sites of distant metastasis. Our findings, however, do not exclude the

Fig. 5. **a**: Effect of αvβ6 integrin expression on the degradation of [^{3}H]-labeled collagen IV. OVCA 433 cells were incubated for 24 h in the absence and presence of plasminogen (0–80 μg/ml) in 24-well plates coated with [^{3}H]-labeled basement membrane. Basement membrane degradation was measured by the release of tritium into the fluid phase. Results are shown as mean ± SEM of three different experiments performed in triplicate. **b**: OVCA 429, OVCA 433, OVHS 1, and HOSE cells were incubated for 24 h in the absence and presence of plasminogen (20 μg/ml), and basement membrane degradation was measured as described above. Results are shown as mean ± SEM of three different experiments performed in triplicate; (*$P < 0.001$, compared to control cells in the absence of plasminogen). **c**: OVCA 433 cells in the presence of plasminogen (10 μg/ml) were exposed either to amiloride (anti-uPA 2mM), 1,10-phenanthroline (anti-MMPs 2mM) or monoclonal antibodies against uPA or MMP-9 or αvβ6 (10D5, Chemicon, Temecula, CA, USA) for the duration of the experiment. Results are shown as mean ± SEM of three different experiments performed in triplicate (*$P < 0.001$, compared to control cells in the absence of plasminogen).

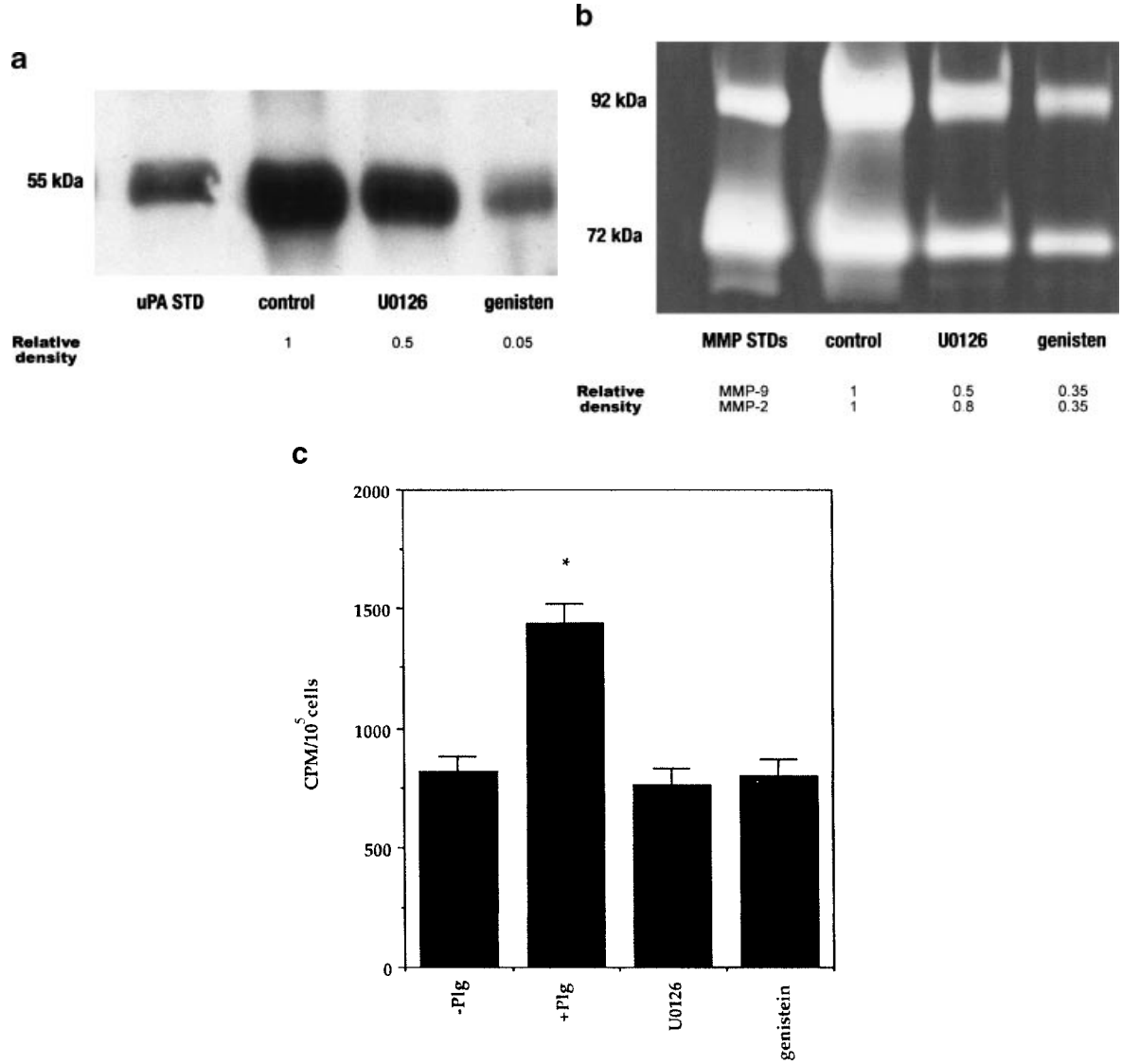

Fig. 6. **a**: Expression of HMW-uPA in tumor-conditioned medium of OVCA 433 cells in the absence and presence of genistein (0.2 mM) and U0126 (5 μM). Conditioned medium was collected 48 h after inhibitor treatment. Samples were analyzed by SDS–PAGE and Western blotting as described previously. **b**: The same inhibitor treated samples were analyzed by zymography for pro-MMP-2 and pro-MMP-9 expression. **c**: OVCA 433 cells in the presence of plasminogen (20 μg/ml) were exposed either to genistein (0.2 mM) or U0126 (5 μM) for 24 h, and collagen IV degradation was analyzed as described above. Results are shown as mean ± SEM of three different experiments performed in triplicate (*$P<0.001$, compared to control cells in the absence of plasminogen).

possibility that expression of αvβ6 integrin also effects signaling upstream of p42/44 MAPK. The *ras* oncogene, which is mutated in approximately 50% of ovarian cancer, has been shown to be activated in αvβ6 integrin expressing OVCA 433 and OVCA 429 cells [Patton et al., 1998]. Co-expression of the dominant-negative ras-Asn^{17} cDNA gene abrogated activity of uPA element in OVCA 433 and OVCA 429 cells [Patton et al., 1998] indicating that activated *ras*-dependent MAPK pathway may be operational in αvβ6 integrin expressing ovarian cancer cells. The full scope of ovarian tumor types, which rely most heavily on p42/44 MAPK activation for their proliferation and invasive properties, is yet to be known. Yet, our study clearly demonstrates in vitro an association between p42/44 MAPK activation, expression of αvβ6 integrin, and proteolytic activity of ovarian cancer cells.

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