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Contortrostatin, a Homodimeric Disintegrin, Binds to Integrin $\alpha v\beta 5$[1]

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Contortrostatin is a homodimeric disintegrin from snake venom. We have shown that contortrostatin binds to integrins αIIbβ3, α5β1, and $\alpha v\beta$3. We now use several criteria to demonstrate the binding of contortrostatin to $\alpha v\beta$5. First, incubation of T24 cells, which express $\alpha v\beta$3 and $\alpha v\beta$5, with antibody against $\alpha v\beta$3 failed to completely inhibit adhesion of cells to vitronectin. However, pretreatment of the cells with contortrostatin or the combination of antibodies against $\alpha v\beta$3 and $\alpha v\beta$5 completely blocked adhesion to vitronectin. By contrast, either anti-$\alpha v\beta$5 alone or contortrostatin blocked adhesion of an $\alpha v\beta$3-negative T24 subline. Second, contortrostatin as well as anti-$\alpha v\beta$5 inhibits invasion of OVCAR-5, which express only $\alpha v\beta$5. Third, contortrostatin binds to purified $\alpha v\beta$5 in a saturable manner. Finally, radioligand binding assays yielded a K_d value of 24 nM for [^{125}I]contortrostatin binding to $\alpha v\beta$5. This investigation identifies $\alpha v\beta$5 as a binding site for contortrostatin. Blockage of $\alpha v\beta$5 by contortrostatin inhibits $\alpha v\beta$5-mediated adhesion and invasion.

Abbreviations used: bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; ECM, extracellular matrix; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PKC, protein kinase C; R/KGD, Arg/Lys-Gly-Asp; VEGF, vascular endothelial growth factor.

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Disintegrins, the most potent known inhibitors of integrin function, are a class of cystine-rich peptides isolated from the venom of the *Viperidae* and *Crotalidae* families of snakes (1, 2). The sequence K/RGD (Lys/Arg-Gly-Asp) is conserved in all of these peptides (1, 2). Disintegrins bind to the fibrinogen receptor αIIbβ3, which results in the inhibition of fibrinogen-dependent platelet aggregation (3). Except for barbourin, a KGD-containing disintegrin which is a relatively specific antagonist for αIIbβ3 (4), other disintegrins are rather nonspecific and can block function of other β3 integrins, as well as β1 integrins (3, 5).

Integrins are important α/β heterodimeric cell surface glycoproteins that are involved in cell-cell and cell–matrix interactions (6). The diversity of subunit combinations results in a range of specificities toward distinct extracellular matrix (ECM) proteins (6). Integrin $\alpha v\beta$5 has been found to play a role in angiogenesis and cancer metastasis (7–11). It has been reported that an anti-$\alpha v\beta$5 antibody blocks vascular endothelial growth factor (VEGF) induced angiogenesis (12). There is evidence that $\alpha v\beta$5 is involved in cell adhesion (13). Recent studies have shown that $\alpha v\beta$5 requires activation of insulin-like growth factor-1 (IGF-1) to mediate cancer cell migration (10, 11).

Contortrostatin is a disintegrin isolated from *Agkistrodon contortrix contortrix* (southern copperhead) venom (14). Unlike other disintegrins, contortrostatin is a homodimer with a mass of 13,505 for the intact molecule and 6750 for the reduced peptide as shown by mass spectrometry (14). The dimeric structure of contortrostatin imbues the protein with special reactivity in platelet protein phosphorylation compared to a monomeric disintegrin (15). In addition to its platelet aggregation inhibitory activity through binding to αIIbβ3, contortrostatin inhibits adhesion of human melanoma cells (16) and breast cancer cells (17) to vitronectin by binding to vitronectin receptor(s). Contortrostatin is also a potent inhibitor of osteoclast at-

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tachment (18). Contortrostatin binds to $\alpha v\beta 3$ (17). Other disintegrins have been reported to inhibit angiogenesis by antagonizing $\alpha v\beta 3$ on vascular endothelial cells (19–21). In the chick chorioallantoic membrane angiogenesis assay, we demonstrated that contortrostatin inhibits VEGF-induced angiogenesis, a pathway reportedly regulated via $\alpha v\beta 5$-mediated signal transduction (12). Based on this observation, we hypothesized that contortrostatin binds directly to $\alpha v\beta 5$. In the present study, we report several observations confirming our hypothesis. This is the first observation that a disintegrin binds to $\alpha v\beta 5$, an important integrin in tumor metastasis and angiogenesis.

MATERIALS AND METHODS

Materials. Venom of *A. contortrix contortrix* was purchased from Biotoxins, Inc. (St. Cloud, FL). Contortrostatin was purified according to an established protocol (14). Vitronectin and Matrigel were purchased from Becton–Dickinson (Bedford, MA). Purified 7E3, a monoclonal antibody (mAb) against $\alpha IIb\beta 3$ (22), but which also cross-reacts with $\alpha v\beta 3$ (23), was kindly provided by Centocor (Malvern, PA). P1F6 (mAb against $\alpha v\beta 5$) and P4C10 (mAb against $\beta 1$) were purchased from Chemicon International, Inc. (Temecula, CA) and Gibco BRL Life Technologies (Rockville, MD), respectively. Soluble recombinant integrin $\alpha v\beta 5$ (24) was kindly provided by Dr. Glen Nemerow, Scripps Institute (La Jolla, CA). Antiserum against contortrostatin, obtained by immunization of rabbits with native contortrostatin, was generated by Alpha Diagnostic International (San Antonio, TX). Goat anti-rabbit IgG conjugated with alkaline phosphatase was purchased from Jackson ImmunoResearch (West Grove, PA).

Cell culture. Human bladder carcinoma T24 cells[4] were purchased from ATCC (Manassas, VA), and are grown in RPMI 1640 medium containing 5% fetal bovine serum (FBS). 293β5, human embryonic kidney carcinoma 293 cells expressing exogenous $\alpha v\beta 5$ (25) were kindly provided by Dr. Jeffrey Smith (The Burnham Institute, La Jolla, CA). Expression of $\alpha v\beta 5$ by the cells was confirmed by flow cytometry analysis using anti-$\alpha v\beta 5$ mAb P1F6. The cells were maintained in DMEM containing 250 μg/ml G418. The human ovarian cancer cell line, OVCAR-5 (26), and HT1080 fibrosarcoma cells were generous gifts from Dr. Thomas Hamilton (Fox Chase Cancer Institute, Philadelphia, PA) and Dr. Walter Laug (Children's Hospital of Los Angeles, CA), respectively, and were grown in RPMI 1640 medium containing 10% FBS.

Flow cytometry assay. Cells were resuspended in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) at a density of 1×10^7/ml. Aliquots were incubated with different concentrations of contortrostatin at room temperature for 30 min, followed by addition of mAb against specific integrins (5 μg/ml). Incubations were continued for another 30 min. The cells were washed and resuspended in 1% BSA/PBS. Goat anti-mouse IgG conjugated with FITC was added to the suspension at a final dilution of 1:200. After 30 min incubation at room temperature in darkness, cells were washed and analyzed using flow cytometry (FACScan, Becton–Dickinson, Bedford, MA). Tests were performed in duplicate.

[4] These cells, including those selected for $\beta 3$ deficiency, were originally purchased from ATCC as ECV304, human vascular endothelial cells. Recently, ATCC announced that the cell line was misidentified from a population of human bladder tumor cells (T24) prior to being deposited with ATCC.

Cell adhesion and invasion assay. The method for adhesion assay was described in detail elsewhere (16). Modified Boyden chambers were employed for invasion assay (27). Transwell chambers with 12-μm pores (Corning Costar, Cambridge, MA) were coated with 1:50 dilution of Matrigel in serum free medium (SFM). Cells (2.5×10^5 in 200 μl) pretreated with contortrostatin, antibodies against different integrins, or vehicle, were applied to the upper wells. HT1080 conditioned medium was added to the bottom well. Cells were incubated at 37°C for 8 h. Method for quantitation of invaded cells was described elsewhere (27). Each inhibitor concentration was tested in duplicate, and experiments were repeated three times to confirm results.

Detection of contortrostatin binding to $\alpha v\beta 5$. One hundred nanograms of soluble $\alpha v\beta 5$ was immobilized on wells of a 96-well plate at 4°C overnight. Contortrostatin at various concentrations was allowed to bind to the coated plate at room temperature for 1 h. Bound contortrostatin was detected with a 1:1000 dilution of antiserum against contortrostatin. Goat anti-rabbit antibody conjugated with alkaline phosphatase was used as a secondary antibody. The bound antibodies were quantitated by adding disodium *p*-nitrophenyl phosphate (pNPP) and determining the absorbance at 405 nm. Background is determined from the binding of contortrostatin in the absence of $\alpha v\beta 5$. Specific binding is obtained by subtracting the background from the total binding. Analysis of each concentration of ligand was performed in triplicate.

Radioligand binding assay. Contortrostatin was labeled with [^{125}I] using the chloroamine-T meethod (28). The specific activity of [^{125}I]contortrostatin was approximately 27 Ci/mmol. The biological activity of [^{125}I]contortrostatin was confirmed by platelet aggregation assay (14). $\beta 5$ transfected 293 cells (293$\beta 5$) were suspended in adhesion buffer containing 1× Hanks' balanced salt solution lacking divalent cations, 50 mM Hepes (pH 7.4), 1% BSA, 0.5 mM Ca^{2+} and 0.5 mM Mg^{2+}. The cell density was adjusted to 5×10^5 cells/ml. Two hundred microliters of the cell suspension was used for each binding reaction. Prior to addition of the radiolabeled ligand, anti-$\beta 1$ integrin mAb P4C10 (1:500 dilution) was added into the cell suspension to block binding of contortrostatin to $\beta 1$ integrins. Different concentrations of [^{125}I]contortrostatin were added into the suspension and incubated at room temperature for 2 h. Triplicate samples were examined at each concentration. Unbound ligand was washed off with ice-cold adhesion buffer using a vacuum separation method. Amount of bound ligand was determined with a γ-counter. Nonspecific binding was measured after treating the cells with 50 mM EDTA for 30 min prior to the addition of [^{125}I]contortrostatin. Data was processed using GraphPad Prism (GraphPad Software, Inc., San Diego, CA). Nonspecific binding obtained by values predicted from a linear regression were subtracted from each total binding count to determine specific binding. A one-site binding nonlinear regression was performed using the specific binding data. The equation used is $Y = B_{max} * X/(K_d + X)$. After nonlinear regression analysis, the data were transformed and displayed as a Scatchard plot.

RESULTS

Contortrostatin inhibits bladder carcinoma cell adhesion to vitronectin mediated by integrins $\alpha v\beta 3$ and $\alpha v\beta 5$. It was previously demonstrated that contortrostatin inhibits adhesion of human melanoma cells (M24 met) to vitronectin (16). In the present study contortrostatin inhibited adhesion of human bladder carcinoma (T24) cells to immobilized vitronectin in a dose-dependent manner, with 100% inhibition at a concentration of less than 10 μg/ml (Fig. 1A). Flow cytometry indicates that T24 cells express both $\alpha v\beta 3$ and $\alpha v\beta 5$ (not shown). While the maximum inhibitory ef-

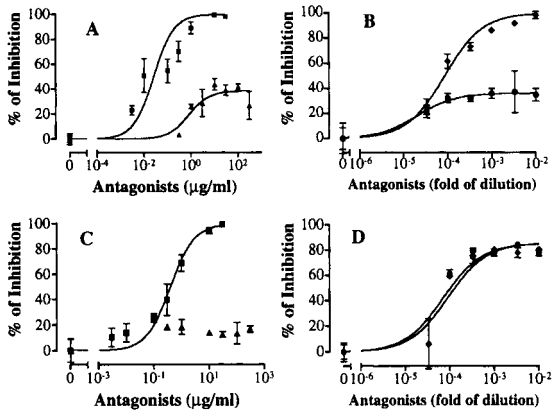

FIG. 1. Inhibition of adhesion of T24 and T24$\beta3^-$ cells to vitronectin by different antagonists. (A) Contortrostatin inhibits adhesion of T24 completely (■), whereas 7E3 only partially inhibits adhesion (▲). (B) P1F6 alone (●) only partially inhibits adhesion of T24, but the combination of 7E3 (10 μg/ml) and P1F6 (◆) inhibits adhesion completely. (C) Contortrostatin inhibits adhesion of T24$\beta3^-$ completely (■), whereas 7E3 has no effect on adhesion (▲). (D) P1F6 alone (●) significantly inhibits adhesion of T24$\beta3^-$; the presence of 7E3 (10 μg/ml) does not enhance the inhibitory ability of P1F6 (◆).

fect of anti-$\alpha v\beta3$ (7E3) alone was no higher than 40% at concentrations up to 100 μg/ml (Fig. 1A), the inhibitory effect of anti-$\alpha v\beta5$ (P1F6) alone on adhesion was below 40% at 1:100 dilution (Fig. 1B). However, the combination of 7E3 (constant concentration of 10 μg/ml) and increasing concentrations of P1F6 showed dose-dependent inhibition with nearly 100% inhibition at 1:100 dilution of P1F6. These results suggest that both $\alpha v\beta3$ and $\alpha v\beta5$ mediate the adhesion of T24 cells to vitronectin. The inhibition resulting from contortrostatin treatment is comparable to the combination of both antibodies, indicating that contortrostatin binds to both integrins.

To further distinguish functional blockage of the two integrins by contortrostatin, $\alpha v\beta3$-negative T24 cells (T24$\beta3^-$) (29) were employed for the adhesion assay. T24$\beta3^-$ only expresses $\alpha v\beta5$, as indicated by flow cytometry assay. Although inhibition by 7E3 was negligible, contortrostatin completely inhibited adhesion of the cell line to vitronectin (Fig. 1C). The adhesion of T24$\beta3^-$ cells to vitronectin was predominantly mediated by $\alpha v\beta5$, since the inhibitory effect of P1F6 was approximately 80% (Fig. 1D) in contrast to T24 cells, where maximum inhibition was below 40% (Fig. 1B). The inhibition curve of P1F6 was not altered by addition of 7E3 in $\alpha v\beta3$-negative cells (Fig. 1D).

Contortrostatin inhibits invasion of ovarian carcinoma cells (OVCAR-5) by blocking integrin $\alpha v\beta5$. To test whether the binding of contortrostatin to $\alpha v\beta5$ inhibits invasion of cancer cells, assays were performed with the highly invasive human ovarian carcinoma cell line, OVCAR-5. Flow cytometry analysis showed that OVCAR-5 cells express $\alpha v\beta5$ but not $\alpha v\beta3$ (not shown). During the invasion assay, neither contortrostatin, nor antibodies alone, nor the combination of antibodies to $\alpha v\beta3$ and $\alpha v\beta5$ inhibited attachment and spreading of the OVCAR-5 cells to Matrigel. Matrigel is derived from mouse Engelbreth–Holm–Swarm tumor, and is primarily a laminin matrix. Contortrostatin does not inhibit adhesion of tumor cells to laminin (16). Therefore, the anti-invasive activities of these vitronectin receptor antagonists were independent of their anti-adhesive effect in this system. Figure 2 illustrates that contortrostatin (1000 nM) almost completely inhibited invasion of OVCAR-5 cells. Comparable inhibition was achieved with 100 nM P1F6. However, at the same molar concentration, 7E3 showed no effect on invasion. The results strongly suggest that integrin $\alpha v\beta5$ mediates invasion of OVCAR-5 cells through Matrigel, and that by blocking $\alpha v\beta5$, contortrostatin effectively inhibited invasion of these cells.

Contortrostatin associates with purified $\alpha v\beta5$. Direct evidence that contortrostatin binds to $\alpha v\beta5$ was obtained from solid-phase binding assay using purified $\alpha v\beta5$ and a modified ELISA. Figure 3A shows that contortrostatin binds to $\alpha v\beta5$ in a dose-dependent and saturable manner.

Determination of the dissociation constant of [^{125}I]contortrostatin and $\alpha v\beta5$. To quantitate the affinity of contortrostatin to $\alpha v\beta5$, radioligand binding assay was performed using transfected 293 cells which express exogenous $\alpha v\beta5$. 293 cells express $\alpha v\beta1$, but not $\alpha v\beta3$ or $\alpha v\beta5$. Following transfection with $\beta5$ cDNA, 293 cells also express the $\alpha v\beta5$ heterodimer on the cell surface (25). Integrin profiles were confirmed in our laboratory by flow cytometry. The transfectants do express endogenous $\beta1$ integrin at a low level, there-

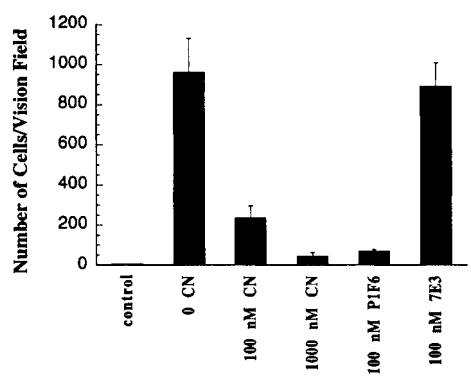

FIG. 2. Contortrostatin inhibits $\alpha v\beta5$-mediated invasion of OVCAR-5 cells. OVCAR-5 cells treated with various antibodies or contortrostatin were applied to a Transwell chamber and allowed to migrate across the Matrigel layer toward HT1080 conditioned medium in the lower chamber for 8 h. A negative control was performed by applying fresh medium in the lower chamber. After noninvaded cells were removed, invaded cells attached on the bottom of the membrane were fixed and stained. The number of cells per high power field were counted and plotted as average ± SD.

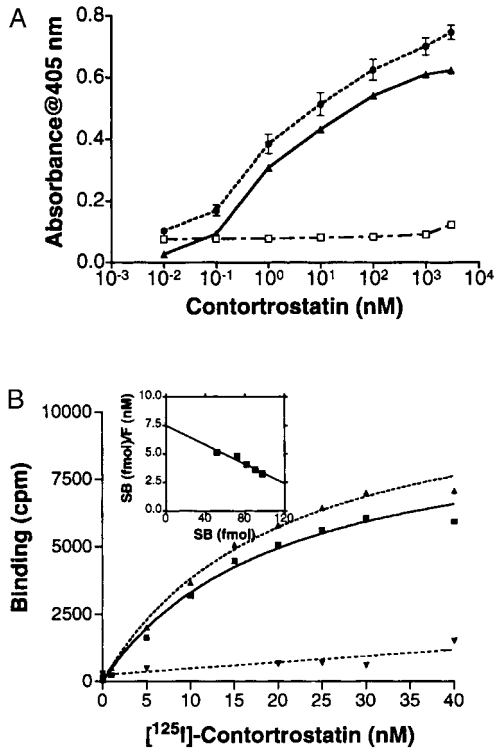

FIG. 3. Contortrostatin binds to $\alpha v\beta 5$ specifically. (A) Purified $\alpha v\beta 5$ (100 ng) was immobilized in individual wells of microtiter plates. Contortrostatin at various concentrations was added to the wells. Total binding (●) was determined using anti-serum against contortrostatin. Contortrostatin bound to bovine serum albumin is defined as the nonspecific binding (□). Specific binding (▲) was calculated by subtracting nonspecific binding from total binding. Each point represents mean absorbance at 405 nm ± SD from triplicate analyses. (B) Isotherms of [^{125}I]contortrostatin binding to $\alpha v\beta 5$ was generated using 293$\beta 5$ cells pre-treated with anti-$\beta 1$ mAb P4C10 (1:500 dilution). Nonspecific binding was measured by treating the cells with 50 mM EDTA for 30 min prior to the addition of [^{125}I]contortrostatin. (▲) Total binding, (▼) nonspecific binding, (■) specific binding. The data were transformed into a Scatchard plot (insert). K_d = 24 nM, B_{max} = 160 fmol/10^6 cells.

fore, anti-$\beta 1$ mAb (P4C10), a functional blocker of the $\beta 1$ integrins, was employed to block contortrostatin binding to this integrin. To measure the relative affinity of [^{125}I]contortrostatin to $\alpha v\beta 5$, binding isotherms were generated across a concentration range of the labeled ligand (Fig. 3B). Nonspecific binding was measured by treating the cells with 50 mM EDTA for 30 min prior to addition of [^{125}I]contortrostatin. Under these conditions, EDTA chelates cations required for integrin function and prevents integrin/ligand association. Nonspecific values predicted from a linear regression were subtracted from total binding to determine specific binding. After analyzing with one site binding nonlinear regression, the data were transformed and displayed as a Scatchard plot (Fig. 3B, inset). The goodness-of-fit of the linear regression (R^2) is 0.930. Both nonlinear regression and Scatchard analysis of the binding isotherms revealed a dissociation constant (K_d) ≅ 24 nM. B_{max} value is approximately 160 fmol/10^6 cells, which indicates that there are about 9.63×10^4 binding sites on each cell when equilibrium is reached. Three independent assays resulted in similar K_d values with deviation less than 10%.

DISCUSSION

It is well established that integrins are ECM protein receptors which transduce cellular signals bidirectionally (6). The promiscuity of integrins allows one ECM protein to have multiple integrin receptors, whereas one integrin can bind to several ECM proteins (6). The redundancy of integrin binding specificity enables cells to interact with complex combinations of ECM proteins. Vitronectin receptors include $\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$ and αIIb$\beta 3$ (30). In our experiments with the T24 cell line, both $\alpha v\beta 3$ and $\alpha v\beta 5$ mediated adhesion of these cells to vitronectin, since blockage of either integrin failed to fully inhibit adhesion. Complete elimination of attachment of T24 cells to vitronectin requires combination of antibodies against both integrins. However, contortrostatin effectively blocks the adhesion of this cell line to vitronectin, strongly supporting our hypothesis that contortrostatin binds to $\alpha v\beta 5$ as well as to $\alpha v\beta 3$. To prove this hypothesis, we employed an $\alpha v\beta 3$ deficient variant of the T24 cells (29), where antibody against $\alpha v\beta 3$ (7E3) had little effect on adhesion of the cells, but pretreatment either with anti-$\alpha v\beta 5$ (P1F6) or contortrostatin completely blocked their adhesion to vitronectin. The hypothesis was confirmed by the finding that contortrostatin binds to purified $\alpha v\beta 5$. The binding affinity between contortrostatin and $\alpha v\beta 5$ was quantitated using radioligand binding assay, and the K_d was determined to be 24 nM. To our knowledge, this is the first report that a disintegrin binds to a $\beta 5$ integrin.

Trikha *et al.* determined the affinity of [^{125}I]contortrostatin to M24met melanoma cells, and found that there was a high affinity binding site (K_d = 3 nM) and a low affinity site (K_d = 60 nM) (16). Although integrin $\alpha v\beta 5$ was detected in this cell line, due to the limitation of method employed, it was impossible to correlate the K_d value with a specific integrin. In addition, the affinity so determined may be influenced by "inside-out" signals from multiple integrins, making interpretation of the data difficult. Transfected 293 cells expressing exogenous integrins provide a better model for quantitation of the ligand/receptor affinity (25). With a similar model, the monomeric disintegrin echistatin was found to bind to $\alpha v\beta 3$ with K_d of 940 nM (31). Although several antiangiogenic disintegrins have been found to bind $\alpha v\beta 3$ (20, 21, 32), quantitative analysis of the affinity has not been reported. The ability of these disintegrins to bind $\alpha v\beta 5$ has not been demonstrated. Osteopontin is an RGD-containing extracellular ma-

trix protein which binds to αv integrins. Using similar methods, Hu *et al.* found that the affinity of recombinant osteopontin to αvβ5 is 48 nM (25). Contortrostatin has two RGD sites, but it is not known if both RGD sites participate in the association with integrins, although it is likely that they do. It was shown by Clark *et al.* that contortrostatin is able to stimulate tyrosine phosphorylation of platelet proteins that are known to be phosphorylated upon αIIbβ3 crosslinking (15). It is also not known if binding of the first RGD site alters the affinity of the interaction.

The function of αvβ5 has been investigated in many studies. Friedlander and colleagues found that blockage of αvβ3 only affects bFGF induced angiogenesis, whereas blockade of αvβ5 exclusively inhibits VEGF induced angiogenesis, suggesting that these growth factors utilize distinct signal transduction pathways which depend on different integrins (12). Interestingly, contortrostatin inhibits angiogenesis induced by both bFGF and VEGF in a chick chorioallantoic membrane model (17). Unlike αvβ3 which mediates cell migration constitutively, αvβ5 only mediates migration following exposure to growth factors such as insulin-like growth factor-1 (IGF-1) (10, 11), epidermal growth factor (EGF) (33), and transforming growth factor-α (TGF-α) (34). Although focal adhesion kinase (FAK) colocalizes with αvβ5 upon adhesion to vitronectin, tyrosine phosphorylation of FAK does not increase until protein kinase C (PKC) is activated, perhaps as a result of upstream tyrosine kinase activation by growth factor receptors (35). A specific PKC inhibitor blocks VEGF-induced angiogenesis (12). In another report, Yebra *et al.* showed that αvβ5-mediated cell migration requires a late activation event involving NFκB-induced *de novo* gene transcription and protein synthesis (34). More recent findings by the same group showed that activation of PKC and the consequent increase of αvβ5-dependent cell migration requires upregulation of the urokinase-type plasminogen activator/urokinase-type plasminogen activator receptor (uPA/uPAR) complex and uPA enzymatic activity (36).

OVCAR-5 is a human ovarian carcinoma cell line which expresses αvβ5 but not αvβ3. In our invasion assay, HT-1080 conditioned medium was used as chemoattractant. The specific cytokines triggering αvβ5-mediated signal transduction in this medium have not been identified. However, OVCAR-5 cells invade through the Matrigel-coated membrane toward the gradient of HT-1080 conditioned medium. It is likely that invasion of this cell line is mediated by αvβ5, since the cells are αvβ3 negative and anti-αvβ3 (7E3) failed to inhibit invasion. Both contortrostatin and anti-αvβ5 (P1F6) prevent invasion of these cells, suggesting that antagonism of αvβ5 is an effective way to block invasion of OVCAR-5 cells.

Despite reports that blockade of αvβ3 prevents angiogenesis, Hynes and his colleagues recently observed that neovascularization in the developing retina of β3-null mice is not affected by the absence of β3-integrin (37). Some cancer cell lines lacking αvβ3, such as OVCAR-5, are also invasive. Functional overlap and redundancy of the integrins implies that targeting a single integrin may result in activation of "alternative routes" of angiogenesis and metastasis. Although the antiangiogenic activity of most disintegrins can be explained by their antagonism of αvβ3 (20, 21, 32), the mechanism of action of contortrostatin is clearly not limited to its αvβ3 specificity. Our findings support the concept that αvβ5 has an important role in mediating cell invasion. Thus, antagonists of αvβ5 may be of significant utility for anti-angiogenic and anti-metastatic therapy.

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