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Research Article **Angiogenesis**

Human noncollagenous domain 1 of the α1 chain of type IV collagen [α1(IV)NC1], or arresten, is derived from the carboxy terminal of type IV collagen. It was shown to inhibit angiogenesis and tumor growth in vivo; however, the mechanisms involved are not known. In the present study we demonstrate that human α1(IV)NC1 binds to α1β1 integrin, competes with type IV collagen binding to α1β1 integrin, and inhibits migration, proliferation, and tube formation by ECs. Also, α1(IV)NC1 pretreatment inhibited FAK/c-Raf/MEK/ERK1/2/p38 MAPK activation in ECs but had no effect on the PI3K/Akt pathway. In contrast, α1(IV)NC1 did not affect proliferation, migration, or the activation of FAK/c-Raf/MEK1/2/p38/ERK1 MAPK pathway in α1 integrin receptor knockout ECs. Consistent with this, α1(IV)NC1 elicited significant antiangiogenic effects and tumor growth inhibition in vivo but failed to do the same in α1 integrin receptor knockout mice. This suggests a highly specific, α1β1 integrin–dependent antiangiogenic activity of α1(IV)NC1. In addition, α1(IV)NC1 inhibited hypoxia-induced expression of hypoxia-inducible factor 1α and VEGF in ECs cultured on type IV collagen by inhibiting ERK1/2 and p38 activation. This unravels a hitherto unknown function of human α1(IV)NC1 and suggests a critical role for integrins […]

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Human α1 type IV collagen NC1 domain exhibits distinct antiangiogenic activity mediated by α1β1 integrin

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Human noncollagenous domain 1 of the α1 chain of type IV collagen [α1(IV)NC1], or arresten, is derived from the carboxy terminal of type IV collagen. It was shown to inhibit angiogenesis and tumor growth in vivo; however, the mechanisms involved are not known. In the present study we demonstrate that human α1(IV)NC1 binds to α1β1 integrin, competes with type IV collagen binding to α1β1 integrin, and inhibits migration, proliferation, and tube formation by ECs. Also, α1(IV)NC1 pretreatment inhibited FAK/c-Raf/MEK/ERK1/2/p38 MAPK activation in ECs but had no effect on the PI3K/Akt pathway. In contrast, α1(IV)NC1 did not affect proliferation, migration, or the activation of FAK/c-Raf/MEK1/2/p38/ERK1 MAPK pathway in α1 integrin receptor knockout ECs. Consistent with this, α1(IV)NC1 elicited significant antiangiogenic effects and tumor growth inhibition in vivo but failed to do the same in α1 integrin receptor knockout mice. This suggests a highly specific, α1β1 integrin–dependent antiangiogenic activity of α1(IV)NC1. In addition, α1(IV)NC1 inhibited hypoxia-induced expression of hypoxia-inducible factor 1α and VEGF in ECs cultured on type IV collagen by inhibiting ERK1/2 and p38 activation. This unravels a hitherto unknown function of human α1(IV)NC1 and suggests a critical role for integrins in hypoxia and hypoxia-induced angiogenesis. Collectively, the above data indicate that α1(IV)NC1 is a potential therapeutic candidate for targeting tumor angiogenesis.

Introduction

Angiogenesis is a complex process that involves ECM remodeling, EC migration, proliferation, and the functional maturation of new ECs into mature blood vessels (1, 2). Angiogenesis includes 6 sequential steps: detachment of preexisting mural pericytes, ECM degradation by endothelial proteases, migration of ECs, proliferation of ECs, tube formation by ECs, and reattachment of pericytes or vascular stabilization (3, 4).

The major component of vascular basement membrane (VBM) is type IV collagen. There are 6 distinct gene products, α1–α6, for type IV collagen in VBM (5). Type IV collagen plays a crucial role in angiogenesis (6–8). VBM constitute an important component of blood vessels (7). Remodeling of VBM can provide crucial angiogenic and antiangiogenic molecules to control the formation of new capillaries (8–10). Such antiangiogenic molecules of VBM include endostatin and noncollagenous domain 1 (NC1) domains of α1, α2, α3, and α6 chains of type IV collagen (11–16). Other molecules such as angiostatin and thrombospondins 1 and 2 have also been identified as endogenous inhibitors of angiogenesis (17, 18).

The constituents of VBM contain binding sites for cell surface integrins for cellular attachment (19). Integrins modulate changes in cell shape and signal-transduction events in the absence of growth factors and also play an important role in the response of the cell to growth factors either directly or indirectly through modulation of focal adhesions (20, 21). Integrin α1β1 regulates EC migration, proliferation, cell survival, and VEGF expression, and its antagonists inhibit VEGF-driven angiogenesis in cancers and other important pathologies (2, 22).
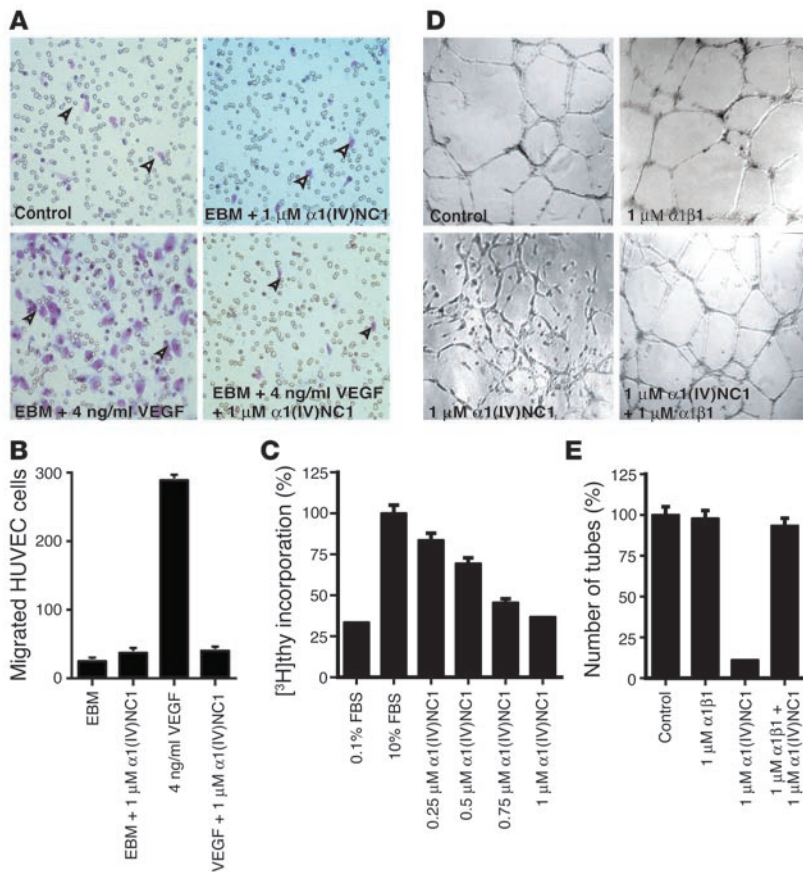
Hypoxia-inducible factor 1α (HIF-1α) is a key transcription factor that regulates cellular responses to physiological, pathological hypoxia including cell proliferation and survival and also plays a role in many diseases (23). HIF-1α regulates expression of VEGF in ECs, and deletion results in the lack of VEGF secretion, suppression of angiogenesis, and reduced solid tumor growth (24–28). Under hypoxic conditions HIF-1α is directly phosphorylated by p42/p44 MAPKs, an action that enhances HIF-1α–dependent transcriptional activation of VEGF (29).

We have identified noncollagenous domain 1 of the α1 chain of type IV collagen [α1(IV)NC1], or arresten, as an antitumorigenic type IV collagen domain of VBM. Here we report that α1(IV)NC1

Figure 1
In vitro antiangiogenic activities of α1(IV)NC1. (**A**) Migration assays. Number of HUVECs migrated (arrowheads) in endothelial growth medium (EGM), EGM with α1(IV)NC1, EGM with VEGF, or EGM with VEGF and α1(IV)NC1, using a light microscope and representative fields (×100 magnification), is shown. Photographs of the underside of Boyden chamber membrane HUVECs are shown. (**B**) Migrated HUVECs. The graph displays the average number of cells migrated in 3 independent experiments (86.7% inhibition at 1 μM). (**C**) Proliferation assays. The graph summarizes the relative [³H]thymidine incorporation inhibition in HUVECs upon treatment with different concentrations of α1(IV)NC1 compared with the 10% FCS control in 3 independent experiments. (**D**) Tube formation assay. HUVECs were plated on Matrigel-coated plates in media control, α1(IV)NC1, soluble α1β1 protein, or both α1(IV)NC1 and soluble α1β1 protein. Tube formation was evaluated after 24 hours using a light microscope, and representative fields (×100 magnification) are shown. (**E**) Number of tubes. The graph displays the average percentage of tubes formed in 3 independent experiments. EBM, endothelial cell basal medium.

binds to the α1β1 integrin and inhibits specific integrin signaling pathways in vascular ECs to induce distinct effects on angiogenesis in vitro and in vivo. We also demonstrate that α1(IV)NC1 regulates HIF-1α and VEGF expression, presumably by inhibiting the MAPK signaling cascade. These observations contribute significantly toward understanding of the therapeutic potential of the α1(IV)NC1 molecule.

Results

Distinct antiangiogenic activities of α1(IV)NC1 on human umbilical vein ECs. α1(IV)NC1 was discovered as an antiangiogenic molecule with significant antitumor activity (16, 30). α1(IV)NC1 was liberated from the NC1 α1 chain of type IV collagen by MMP-9 (31). Current studies have aimed at understanding the molecular mechanisms underlying the angiogenesis inhibition by α1(IV)NC1 in vivo and in vitro and its implications in the treatment of cancer.

We conducted a series of angiogenesis experiments to define the antiangiogenic activity of α1(IV)NC1 using human umbilical vein ECs (HUVECs), mouse lung ECs (MLECs), or *α1 integrin⁻/⁻* MLECs. First, we tested the antiangiogenic activity of α1(IV)NC1 by migration assay. Migration of ECs has been shown to have an important early role in neovascularization (32, 33). Migration of HUVECs through a type IV collagen–coated membrane toward VEGF in a Boyden chamber was significantly inhibited by α1(IV)NC1 (Figure 1, A and B). Next, the antiproliferative effect of α1(IV)NC1 was examined using [³H]thymidine incorporation assays. Proliferation of HUVECs was significantly inhibited by α1(IV)NC1 in a concentration-dependent manner, and the graph summarizes the relative [³H]thymidine incorporation

inhibition (16.41% at 0.25 μM, 30.69% at 0.5 μM, 54.5% at 0.75 μM, and 63.29% at 1 μM) (Figure 1C). Finally, we confirmed the antiangiogenic activity of α1(IV)NC1 by the functional assay of tube formation. Tube formation involves EC migration, proliferation, and survival (34). Addition of α1(IV)NC1 to the cell culture media significantly inhibited HUVEC tube formation on Matrigel Matrix [Figure 1D, α1(IV)NC1]. Preincubation of cells with soluble α1β1 integrin protein had no effect on tube formation (Figure 1D, α1β1). Preincubation of HUVECs with equimolar mixture of soluble α1β1 integrin and α1(IV)NC1 protein reversed the antiangiogenic action of α1(IV)NC1 [Figure 1D, α1(IV)NC1 + α1β1]. Such reversal of inhibition was not observed with soluble α5β1 or αVβ3 integrin proteins in tube formation (data not shown). The number of tubes formed in 3 independent experiments is shown in the graph (Figure 1E).

α1β1 integrin is a functional receptor for α1(IV)NC1. Next, experiments were carried out to trace the binding characteristics and the role of α1β1 integrin in mediating the distinct antiangiogenic properties of α1(IV)NC1. Binding of HUVECs to α1(IV)NC1 is inhibited by blocking antibodies specific for α1β1 integrin and the antiproliferative action of α1(IV)NC1 is mediated by α1β1 integrin, suggesting that α1β1 integrin is a functional receptor for α1(IV)NC1 (30). We have confirmed that soluble α1β1 integrin protein could bind to α1(IV)NC1-precoated culture plates and subsequently inhibit attachment of HUVECs to α1(IV)NC1. Soluble α5β1 or αVβ3 integrin proteins had no effect (Figure 2A). This effect was reversed by capturing of the soluble α1β1 integrin protein with soluble α1(IV)NC1 protein (Figure 2A). These experiments confirm that α1β1 integrin serves as a func-
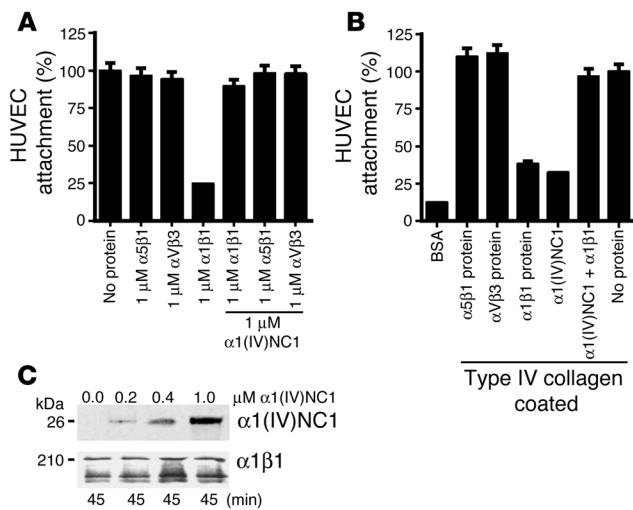
Figure 2
$\alpha1\beta1$ integrin is a functional receptor for $\alpha1(IV)NC1$. (**A**) Cell attachment assay. Attachment of overnight serum-starved HUVECs on $\alpha1(IV)NC1$-coated plates was significantly inhibited (75.14%) by incubation with soluble $\alpha1\beta1$ protein, whereas $\alpha5\beta1$ or $\alpha V\beta3$ integrin had no significant effect. $\alpha1\beta1$ integrin–induced cell attachment inhibition was reversed by addition of soluble $\alpha1(IV)NC1$ protein to the culture media. (**B**) Cell adhesion assay. Serum-starved HUVEC attachment to type IV collagen–coated plates was significantly inhibited in the presence of soluble $\alpha1\beta1$ integrin (61.7%), or $\alpha1(IV)NC1$ protein (67.14%), whereas $\alpha5\beta1$ or $\alpha V\beta3$ integrin had no significant effect. $\alpha1\beta1$ integrin–induced cell attachment inhibition was reversed by addition of equimolar soluble $\alpha1(IV)NC1$ protein to the HUVEC culture media. The graph summarizes the average results of 3 independent experiments. (**C**) Immunoprecipitation and immunoblot. HUVECs treated with $\alpha1(IV)NC1$ for 45 minutes were lysed and immunoprecipitated with $\alpha1\beta1$ (bottom panel). Western blot analysis of the precipitate for bound $\alpha1(IV)NC1$ is shown (top panel). A specific band at 26 kDa was produced in a dose-dependent manner after 45 minutes of incubation with $\alpha1(IV)NC1$ and was absent in lysate of untreated cells.

tional receptor for the $\alpha1(IV)NC1$ molecule. Binding of HUVECs to $\alpha1(IV)NC1$-coated plates was significantly inhibited by $\alpha1\beta1$ integrin antibody, whereas $\alpha5\beta1$ or $\alpha V\beta3$ integrin antibodies had no effect (data not shown). Preincubation of HUVECs with $\alpha1(IV)NC1$ significantly decreased attachment of HUVECs to type IV collagen, the major ligand for $\alpha1\beta1$ integrin (Figure 2B). In the same experiment, addition of soluble $\alpha1\beta1$ integrin protein captured $\alpha1(IV)NC1$ and reversed the impaired binding of HUVECs to type IV collagen. This suggests that $\alpha1(IV)NC1$ competes with type IV collagen for binding to $\alpha1\beta1$ integrin (Figure 2B). We further confirmed that $\alpha1(IV)NC1$ binds to $\alpha1\beta1$ integrin by immunoprecipitating $\alpha1(IV)NC1$ with an antibody to $\alpha1\beta1$ integrin from the HUVEC lysates treated with various concentrations of $\alpha1(IV)NC1$ (Figure 2C).

$\alpha1(IV)NC1$ inhibits proliferation and focal adhesion kinase phosphorylation in MLECs but not in $\alpha1$ integrin$^{-/-}$ MLECs. We investigated the role of $\alpha1\beta1$ integrin and its effector kinase, focal adhesion kinase (FAK), in $\alpha1(IV)NC1$ function and the effect of $\alpha1(IV)NC1$ on EC proliferation using primary ECs isolated from WT and $\alpha1\beta1$ integrin knockout mice (Figure 3, A and B). We observed that $\alpha1(IV)NC1$

treatment leads to inhibition of sustained FAK phosphorylation on type IV collagen matrix (Figure 3C). In contrast, FAK activation in *$\alpha1$ integrin$^{-/-}$* MLECs was not affected by $\alpha1(IV)NC1$ treatment (Figure 3D). Similarly, $\alpha1(IV)NC1$ significantly blocked serum-induced proliferation of MLECs in a dose-dependent manner, with no effect on *$\alpha1$ integrin$^{-/-}$* MLECs (Figure 3, A and B), whereas another collagen NC1–derived molecule, $\alpha3(IV)NC1$, inhibited the proliferation of both MLECs and *$\alpha1$ integrin$^{-/-}$* MLECs equally (Figure 3, A and B). These observations clearly suggest that $\alpha1\beta1$ integrin is essential for $\alpha1(IV)NC1$ function in ECs.

Effect of $\alpha1(IV)NC1$ on MAPK signaling. Integrins transduce biochemical signals across the cell membrane (outside-in signaling) via activation of intracellular signaling pathways, which include ERK1/2, Ras, MEK1/2, Raf, and p38 MAPK family members (35). Activation of cytosolic kinases by integrin-linked transmembrane signaling leads to activation of nuclear transcription factors to regulate gene expression and regulate cell proliferation and migration for cell survival (14). In this study, we examined the role of the MAPK signaling cascade in $\alpha1(IV)NC1$-mediated inhibition of cell migration on type IV collagen matrix.

Figure 3
Cell proliferation and phosphorylation assays. (**A** and **B**) Proliferation. Proliferation was assessed by [³H]thymidine incorporation in WT MLECs treated with different concentrations of $\alpha1(IV)NC1$ (proliferation inhibits 60.11% at 1 μM, 48.33% at 0.75 μM, and 31.39% at 0.5 μM). In *$\alpha1$ integrin$^{-/-}$* MLECs upon treatment with different concentrations of $\alpha1(IV)NC1$, using 20% FCS or 1 μM $\alpha3(IV)NC1$ (proliferation inhibits 62.22% and 63.71% in WT and *$\alpha1$ integrin$^{-/-}$* MLECs) as controls. The results are shown as the mean of 3 independent experiments. (**C** and **D**) FAK phosphorylation. Serum-starved WT MLECs or *$\alpha1$ integrin$^{-/-}$* MLECs were plated on type IV collagen–coated dishes in incomplete medium supplemented with or without $\alpha1(IV)NC1$ for 0–60 minutes as shown in the figure, and lysates were analyzed by Western blot. Immunoblots of phosphorylated FAK (top blot) and total signaling FAK protein (bottom blot) in **C** and **D** were performed as described previously (35).
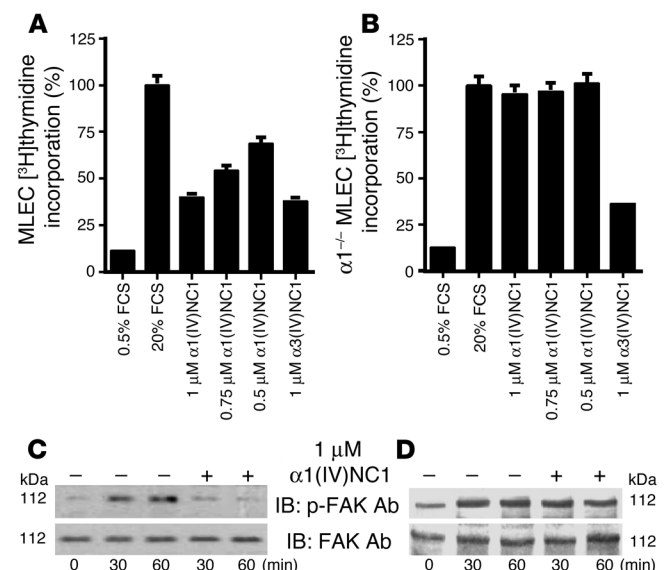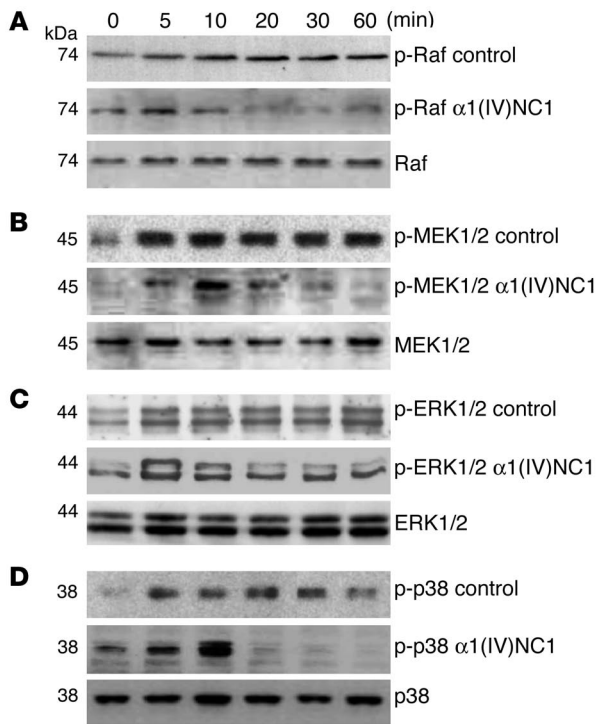
Figure 4
Effect of α1(IV)NC1 on ERK and p38 MAPK signal transduction pathways. Serum-starved WT MLECs were pretreated 10 minutes with α1(IV)NC1, plated on type IV collagen–coated plates in incomplete medium, and lysed at the indicated times (0–60 minutes). The levels of phosphorylated proteins after incubation with α1(IV)NC1 (middle panels) were compared with untreated control (top panels) and the total signaling protein levels in α1(IV)NC1-treated WT MLECs (bottom panels) by immunoblot. (**A**) Raf phosphorylation. Immunoblots for phospho-Raf indicate that sustained phosphorylation of Raf (**A**, top panel) was inhibited by treatment with α1(IV)NC1 (**A**, middle panel) and total Raf protein (**A**, bottom panel). (**B**) MEK1/2 phosphorylation. Immunoblots for phospho-MEK1/2 indicate that sustained phosphorylation of MEK (**B**, top panel) was inhibited by treatment with α1(IV)NC1 (**B**, middle panel) and total MEK1/2 protein (**B**, bottom panel). (**C**) ERK1/2 phosphorylation. Immunoblots for phospho-ERK1/2 indicate that sustained phosphorylation of ERK (**C**, top panel) was inhibited by treatment with α1(IV)NC1 (**C**, middle panel) and total ERK1/2 protein (**C**, bottom panel). Treatment with α1(IV)NC1 inhibited phosphorylation of ERK1 but had no significant effect on ERK2 phosphorylation (**C**, middle panel). (**D**) p38 phosphorylation. Immunoblots for phospho-p38 indicate that sustained phosphorylation of p38 (**D**, top panel) was inhibited by treatment with α1(IV)NC1 (**D**, middle panel) and total p38 protein (**D**, bottom panel).

The MAPK pathway is an important downstream target of FAK that is critical for the regulation of EC migration (36). Mice deficient in FAK display severely impaired vasculogenesis and cell migration (32, 33). Overexpression of FAK significantly increases the migratory capacity of cells via activation of the ERK pathway (37). These findings suggest that the type IV collagen/α1β1 integrin/FAK/ERK pathway may play a major role in regulation of ECM-mediated migration. Attachment of MLECs to type IV collagen via α1β1 integrin activated the c-Raf/MEK/ERK1/2/p38 pathway (Figure 4, A–D, top panels). Pretreatment of MLECs with α1(IV)NC1 did not inhibit the activation but significantly blocked sustained phosphorylation of Raf (Figure 4A, middle panel), MEK (Figure 4B, middle panel), and ERK1 in MLECs (Figure 4C, middle panel). However, α1(IV)NC1 treatment had no effect on ERK2 phosphorylation (Figure 4C, middle panel, bottom bands). It was reported recently that ERK1 is involved in matrix-mediated migration (38). These data support a role for the MAPK pathway in α1β1 integrin–mediated inhibition of EC migration on type IV collagen matrix.

In ECs VEGF-induced migration and cytoskeletal reorganization is mediated by the ERK pathway, whereas the expression of integrins and proteases is regulated by the p38 pathway. This suggests that these 2 pathways regulate EC migration in a coordinated manner (39, 40). p38 phosphorylation in MLECs was significantly inhibited by treatment with α1(IV)NC1 (Figure 4D, middle panel). In contrast, treatment of MLECs prepared from the *α1 integrin*−/− mouse had no effect on phosphorylation of c-Raf, MEK1/2, ERK1, and p38 on type IV collagen matrix, suggesting that α1(IV)NC1-induced signaling is mediated through α1β1 integrin (Figure 5, A–D, top panels).

Effect of α1(IV)NC1 on hypoxia-induced HIF-1α and VEGF expression in ECs. Hypoxia in tumors is known to induce HIF-1α expression, which in turn induces VEGF expression and promotes angiogenesis. Here we investigated the role of α1β1 integrin and the ability of α1(IV)NC1 to modulate HIF-1α and VEGF expression, using WT and *α1 integrin*−/− knockout ECs. We observed that α1(IV)NC1 inhibited hypoxia-induced ERK1/2 and p38 activation (Figure 6, A and B). We also observed that α1(IV)NC1 blocked HIF-1α and VEGF expression in hypoxic ECs in a time-dependent manner (Figure 6C), but it had no effect on hypoxia-induced HIF-1α and VEGF expression in hypoxic *α1 integrin*−/− MLECs (Figure 6D). In addition, consistent with previous reports (41–43), we observed that hypoxia-induced HIF-1α and VEGF expression was inhibited by MEK1/2 (PD98059) and p38 (SB203580) inhibitors (Figure 6G). Combination of PD98059 and SB203580 was much more effective than each individual treatment (Figure 6, E–G). This suggests that α1(IV)NC1 inhibits HIF-1α and VEGF expression by inhibiting ERK1/2 and p38 activation in hypoxic cells.

Effect of α1(IV)NC1 on tumor growth and tumor angiogenesis in WT and α1 integrin knockout mice. We further evaluated the integrin-specific effect of α1(IV)NC1 on tumor growth and angiogenesis in vivo using SCC-PSA1 cell xenografts in WT and α1 integrin knockout 129/Sv mice. SCC-PSA1 cells (1 × 10⁶) were implanted s.c. on the backs of mice. Tumors appeared about 1 week after implantation. We started administering α1(IV)NC1 (30 μg/mouse) i.v. once a day to the 10 tumor-bearing mice (5 WT and 5 α1 integrin knockout) to check the effect of the α1(IV)NC1 on tumor regression. The control mice (5 WT and 5 α1 integrin knockout) were injected with only sterile PBS in equal volume, every day.

The WT mice demonstrated an increased rate of tumor growth and, consistent with tumor growth, displayed increased numbers of CD31-positive blood vessels (Figure 7, A and B, WT). In contrast, the α1(IV)NC1-treated WT mice demonstrated a clear regression of tumor growth and a decreased number of CD31-positive blood vessels [Figure 7, A and B, WT + α1(IV)NC1]. As expected, α1(IV)NC1 treatment did not affect the tumor growth nor CD31-positive blood vessels in *α1 integrin*−/− mice. This clearly demon-
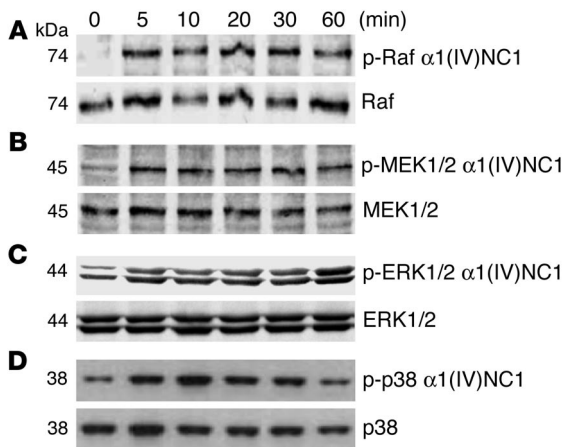
Figure 5
Effect of α1(IV)NC1 on ERK and p38 MAPK signal transduction pathways in *α1 integrin*⁻/⁻ MLECs. Serum-starved *α1 integrin*⁻/⁻ MLECs were pretreated 10 minutes with α1(IV)NC1 and plated on type IV collagen–coated plates, similar to Figure 4. (**A**) Raf phosphorylation. Immunoblots for phospho-Raf indicate that sustained phosphorylation of Raf was not inhibited by treatment with α1(IV)NC1 (**A**, top panel) and total Raf protein (**A**, bottom panel). (**B**) MEK1/2 phosphorylation. Immunoblots for phospho-MEK1/2 indicate that sustained phosphorylation of MEK was not inhibited by treatment with α1(IV)NC1 (**B**, top panel) and total MEK1/2 protein (**B**, bottom panel). (**C**) ERK1/2 phosphorylation. Immunoblots for phospho-ERK1/2 indicate that sustained phosphorylation of ERK was not inhibited by treatment with α1(IV)NC1 (**C**, top panel) and total ERK1/2 protein (**C**, bottom panel). (**D**) p38 phosphorylation. Immunoblots for phospho-p38 indicate that sustained phosphorylation of p38 was not inhibited by treatment with α1(IV)NC1 (**D**, top panel) and total p38 protein (**D**, bottom panel).

strates the role of α1β1 integrin in α1(IV)NC1's mechanism of action [Figure 7A, *α1 integrin*⁻/⁻ vs. *α1 integrin*⁻/⁻ + α1(IV)NC1]. Consistent with earlier studies, we did observe a slower growth rate of tumors in α1 integrin knockout mice (Figure 7A) (39, 40).

In addition to quantitation of tumor vascularity, frozen sections from the tumors of all 4 groups of mice were analyzed for CD31-positive tumor vasculature, by immunofluorescence (Figure 7B). Consistent with our in vitro data, we observed that WT tumor-bearing mice with α1(IV)NC1 treatment showed a significant inhibition of CD31-positive tumor vasculature and VEGFR2-positive circulating ECs [Figure 7, B and C, WT vs. WT + α1(IV)NC1]. Further supporting our in vitro observations, α1(IV)NC1 treatment did not affect CD31-positive tumor vasculature in tumors from

*α1 integrin*⁻/⁻ mice or circulating VEGFR2-positive ECs [Figure 7B, *α1 integrin*⁻/⁻ vs. *α1 integrin*⁻/⁻ + α1(IV)NC1, and Figure 7C, *α1 integrin*⁻/⁻ vs. *α1 integrin*⁻/⁻ + α1(IV)NC1]. As described in previous reports, percentage of CD31-positive vasculature in tumor sections was reduced by approximately 50% in the α1-null tumors (39, 40). However, systemic administration of α1(IV)NC1 did not alter tumor vasculature in any way in α1-null mice (Figure 7A, *α1 integrin*⁻/⁻ vs. WT). These in vivo observations strongly support the potential antiangiogenic use of α1(IV)NC1 molecule (Figure 8).

Discussion

Several noncollagenous domains of type IV collagen were identified as novel inhibitors of angiogenesis and tumor growth
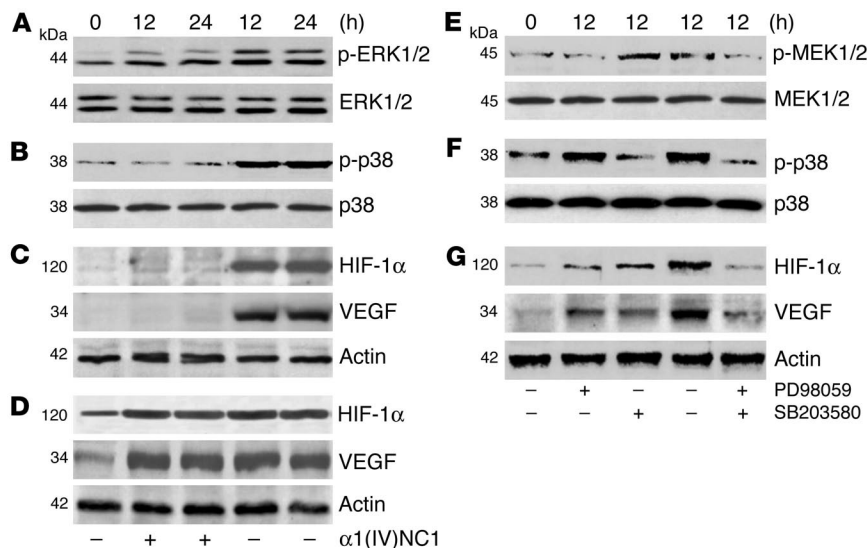
Figure 6
Effect of α1(IV)NC1 on hypoxia-induced HIF-1α and VEGF expression. Serum-starved WT MLECs were treated with α1(IV)NC1 or PD98059/SB203580 in complete medium, plated on type IV collagen–coated plates, and then exposed to hypoxic conditions for 12 and 24 hours. Total cytosolic cell extracts were prepared and analyzed by SDS-PAGE and Western blot with different antibodies shown in the figure. (**A**) Hypoxia-induced ERK1/2 phosphorylation upon α1(IV)NC1 treatment (**A**, top panel) and total ERK1/2 protein (**A**, bottom panel). (**B**) Hypoxia-induced p38 phosphorylation upon α1(IV)NC1 treatment (**B**, top panel) and total p38 protein (**B**, bottom panel). (**C**) Hypoxia-induced expression of HIF-1α and VEGF proteins upon α1(IV)NC1 treatment (**C**, top and middle panels); actin levels are shown as an internal control (**C**, bottom panel). (**D**) Hypoxia-induced expression of HIF-1α and VEGF proteins upon α1(IV)NC1 treatment in *α1 integrin*⁻/⁻ MLECs (**D**, top and middle panels); actin levels are shown as an internal control (**D**, bottom panel). (**E**–**G**) Hypoxia-induced MEK1/2 and p38 phosphorylation and HIF-1α and VEGF proteins upon PD98059 (50 μM)/SB203580 (20 μM) treatment, similarly as shown in **A**–**C**.
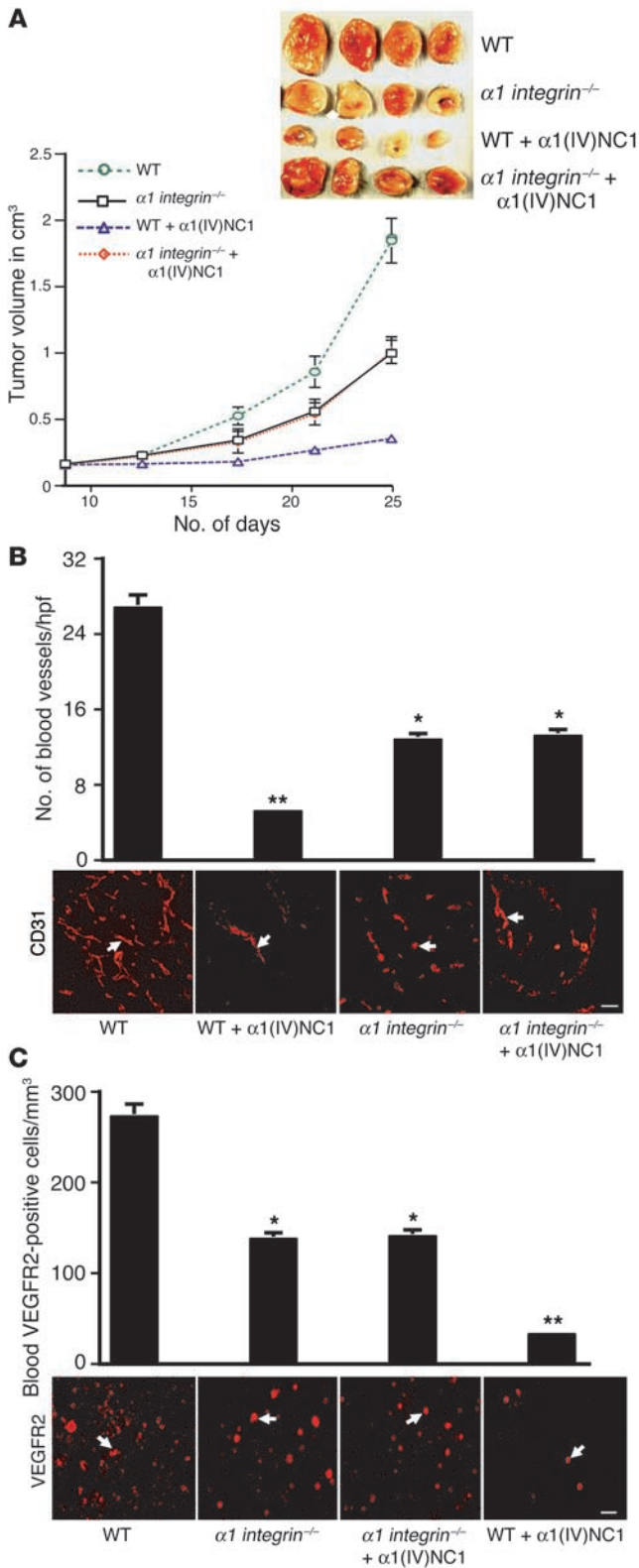
Figure 7
Tumor burden studies using WT and α1 integrin−/− mice. (**A**) SCC-PSA1 tumor growth in WT and α1 integrin−/− mice. Ten male mice in each group, age-matched WT and α1 integrin−/− mice, were used in this experiment. Recombinant α1(IV)NC1 protein was injected i.v. into 5 WT and 5 α1 integrin−/− mice daily (30 μg) for 14 days in sterile PBS, while only PBS was injected into the other 5 WT and 5 α1 integrin−/− mice. Data are representative of 3 such independent experiments. The results are shown as the mean ± SEM; $P < 0.001$ compared with WT mice with and without injection. (**B**) Frozen sections (4 μm) from tumor tissue were stained with anti-CD31 antibody, and the numbers of CD31-positive blood vessels (arrows) were counted in 6 fields at ×200 magnification. Scale bar: 50 μm. The blood vessel quantification results are shown as the mean ± SEM. $*P$ = NS versus α1 integrin−/− mice with and without treatment. $**P < 0.001$ versus WT mice with and without treatment. (**C**) VEGFR2-positive circulating ECs from tumor-bearing mouse peripheral blood were stained with VEGFR2 antibody, and the numbers of positive cells (arrows) were counted. Scale bar: 50 μm. The results are shown as the mean ± SEM. $*P$ = NS between α1 integrin−/− mice with and without treatment. $**P < 0.001$ versus WT mice with and without injection.

proliferation, and migration. This is consistent with the previous studies showing α1(IV)NC1-mediated inhibition of EC proliferation and tumor growth (16, 30). α1 Integrin–null mice display halted pathological angiogenesis and tumor growth (39, 40). However, α1(IV)NC1 had no effect in α1 integrin–null MLECs. In contrast, it significantly inhibited proliferation of WT MLECs. This confirms the significance of integrin-mediated signaling in α1(IV)NC1 function (Figure 8).

In ECs, ligand binding to integrins induces phosphorylation of FAK (35, 46), which serves as a platform for different downstream signals (47). α1(IV)NC1 inhibits phosphorylation of FAK when ECs are plated on type IV collagen matrix. Similarly, other collagen NC1 domains, tumstatin or endostatin, inhibit phosphorylation of FAK on vitronectin or fibronectin (35). Similar inhibition of FAK phosphorylation was not observed in α1(IV)NC1-treated α1 integrin−/− MLECs. Downstream of FAK, protein kinase B (Akt/PKB) plays an important role in EC survival signaling (35, 48). Interestingly α1(IV)NC1 does not inhibit Akt or PI3K phosphorylation. These results suggest that α1(IV)NC1 regulates migration of ECs in an Akt-independent manner similar to that of another collagen-derived antiangiogenesis inhibitor, endostatin (35, 49).

Classical integrin ligation is known to initiate intracellular signaling pathways, whereas these signaling events are inhibited by α1(IV)NC1 occupancy of α1β1 integrin. αVβ3 integrin antagonists disrupt vascular development in the embryo and block pathological angiogenesis in animal models, thereby making integrin an attractive target for therapeutic intervention. Here, our results demonstrate regression of SCC-PSA1 tumors in 129/Sv mice upon α1(IV)NC1 treatment as well as inhibition of tumor vasculature in these mice. These results provide further evidence for the potent tumor-suppressive action of α1(IV)NC1.

HIF-1α is an oxygen-dependent transcriptional activator, which plays crucial roles in the angiogenesis of tumors (50, 51). It regulates cellular responses to physiological and pathological hypoxia, and studies have demonstrated that it is a potential target for tumor angiogenesis (52, 53). HIF-1α transcriptionally regulates VEGF expression in hypoxic cells and inhibits angiogenesis in solid tumors (28, 54, 55). Based on these findings, HIF-1α could be a prime target for anticancer therapies (56–58).

(11–16, 30, 44, 45). Understanding the mechanism(s) of action is crucial for their potential therapeutic use. We determined that α1(IV)NC1 binds to α1β1 integrin in a type IV collagen manner, mediates all of its antiangiogenic functions through α1β1 integrin, and inhibits angiogenesis by inhibiting EC tube formation,
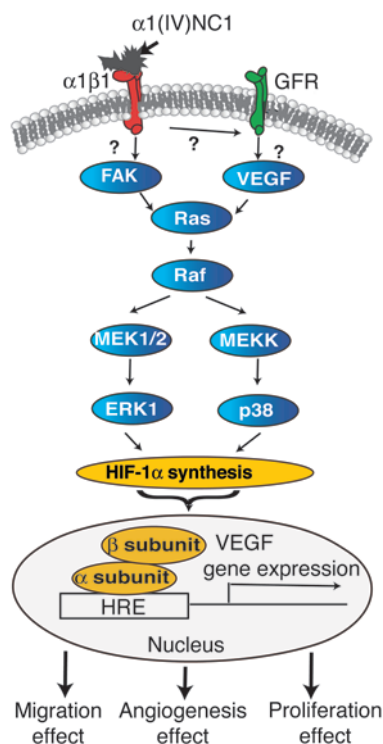
Figure 8
Schematic illustration of signaling pathway mediated by α1(IV)NC1. α1(IV)NC1 binds to α1β1 integrin and inhibits phosphorylation of FAK. Inhibition of FAK activation leads to inhibition of Raf/MEK/ERK1/2/p38 MAPK pathways that leads to inhibition of HIF-1α and VEGF expression, resulting in inhibition of EC migration, proliferation, and tube formation in proliferating ECs. GFR, growth factor receptor; HRE, hypoxia response element.

HIF-1α protein expression was inhibited by treatment of ECs with α1(IV)NC1. The HIF-1α subunit is rapidly degraded in normoxic conditions via the von Hippel-Lindau tumor suppressor gene product–mediated (pVHL-mediated) ubiquitin-proteasome pathway. Our results strongly demonstrate that when ECs are treated with α1(IV)NC1, it binds to α1β1 integrin, and inhibits MAPK signaling, resulting in inhibition of HIF-1α expression. Based on these observations, α1(IV)NC1 has the ability to inhibit angiogenic factors and inhibit tumor vasculature, which leads to regression of tumor growth. These results support earlier observations (16, 30). Recent studies have suggested that increases in the circulating VEGFR2-positive ECs correlate directly with increase in tumor angiogenesis and can serve as in vivo indicators of tumor angiogenesis (16, 30). Consistent with recent studies, WT mouse tumors treated with α1(IV)NC1 had decreased circulating VEGFR2-positive ECs as compared with WT control tumors. No change was observed in VEGFR2–positive circulating ECs in α1 integrin$^{-/-}$ mice with or without α1(IV)NC1 treatment. In this study, we have shown that proliferation, migration, and tube formation in ECs was inhibited by α1(IV)NC1. HIF-1α and VEGF are likely to be the primary factors mediating inhibition of hypoxia-induced angiogenesis by α1(IV)NC1.

The α1(IV)NC1-induced inhibition of cell survival mechanisms may contribute to the inhibition of hypoxia-induced angiogenesis by inhibiting the MAPK signaling pathway. We have compared the signaling mechanisms of different collagen-derived antiangiogenic NC1 domains in Table 1. Our data provide a rationale for the use of α1(IV)NC1 as an inhibitor of HIF-1α and VEGF in hypoxic ECs. This inhibitory activity might be exploited for antiangiogenic therapy in the treatment of cancer.

Methods

Primary HUVECs were purchased from Cambrex Corp. SCC-PSA1 or pluripotent teratocarcinoma tumor cells were obtained from ATCC. Recombinant human VEGF was from R&D Systems, and protein A-Sepharose CL-4B beads were from Amersham Pharmacia Biotech Inc. Commercially available antibodies specific for FAK (Santa Cruz Biotechnology Inc.), phosphorylated FAK (Tyr397; BioSource International), Akt, phosphorylated Akt (Ser473; New England Biolabs Inc.), phospho-p42/p44 MAPK (Thr202/Tyr204), control MAPK, phospho-Raf (Ser 2590RSK), phospho-MEK1/2 (Ser217/Ser221), control MEK1/2 (Ser217/Ser221), p38, and phospho-p38 (Cell Signaling Technology), and anti-VEGFR2 antibody (Santa Cruz Biotechnology Inc.), rbc lysis solution (PUREGENE; Gentra Systems Inc.), and mouse mAb to human α1β1, α5β1, and αVβ3 integrins and their proteins, were purchased from Chemicon International. HRP-labeled secondary antibodies, type IV collagen, glutamine, and penicillin/streptomycin were purchased from Sigma-Aldrich. BD Matrigel Matrix (14.6 mg/ml) was purchased from BD Biosciences — Discovery Labware. CD31 antibody was purchased from BD Biosciences — Pharmingen. ICAM-2 (BD Biosciences — Pharmingen) was conjugated to magnetic beads (Dynabeads M-450; Dynal Biotech). Ham's F-12, DMEM/LOW Glucose, FBS, heparin, and endothelial mitogen were from Biomedical Technologies Inc. FBS was purchased from Fisher Scientific International Co. PD98059 and SB203580 were from Calbiochem-Novabiochem Corp.

Cell culture. HUVECs were maintained in endothelial growth medium-2 (EGM-2), and SCC-PSA1 or pluripotent teratocarcinoma tumor cells were maintained in DMEM containing 10% FCS with penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a humidified 5% CO_2 atmosphere (59). Passages 2–6 of HUVECs were used for experiments.

Preparation of primary MLECs. Primary MLECs were isolated from 10- to 14-week-old WT or *α1 integrin$^{-/-}$* 129/Sv mice (Dominic Cosgrove's laboratory). Briefly, ICAM-2–expressing MLECs were enriched using rat anti–mouse ICAM-2 conjugated to magnetic beads. MLECs were maintained in 40% Ham's F-12, 40% DMEM/LOW Glucose, 20% FBS supplemented

Table 1
Distinct signaling mechanisms mediated by different collagen-derived angiogenesis inhibitors

	Human α1(IV)NC1	Human tumstatin	Human endostatin
Origin	*α1 Type IV collagen*	*α3 Type IV collagen*	*α1 Type XIII collagen*
Tube formation	Inhibition	Inhibition	Inhibition
Proliferation	Inhibition	Inhibition	No effect
Receptor	α1β1	αVβ3	α5β1
Signaling pathway	FAK, Ras, c-Raf, EK1/2, M p38, ERK1/2, HIF-1α	FAK, Akt, PI3K, mTOR, eIF-4E/4E-BP1, ?	FAK, Ras, c-Raf, MEK1/2, p38, ERK1/2, ?

with heparin, endothelial mitogen (Biomedical Technologies Inc.), glutamine, and penicillin/streptomycin. WT and *α1 integrin*^{−/−} MLECs were positive for expression of the endothelial-specific marker VE-cadherin at cell junctions and contact points, and both cell lines were able to take up 1,1′-dioctadecyl-3,3,3′,3′ tetramethyl indocarbocyanine perchlorate acetylated LDL (DiI-Ac-LDL) (60).

Production of recombinant α1(IV)NC1 using baculovirus insect cell system. The sequence encoding α1(IV)NC1 was amplified by PCR from the pET28b (+) vector (30) using a forward primer (5′-TATATAGAATTCTCTGTT-GATCACGGCTTCCT-3′) and a reverse primer (5′-TTAATTTCTAGAT-TATGTTCTTCTCATACAGACTTG-3′). The resulting cDNA fragment was digested with EcoRI and BglII and ligated into predigested pAcHLT-A transfer vector (BD Biosciences − Pharmingen). The resulting recombinant vector, pAcHLT-A/α1(IV)NC1, was cotransfected into Sf9 cells with Bsu361-digested linearized BaculoGold (catalog no. 21100D) viral DNA to obtain an infectious complete viral genome according to Baculovirus Expression Vector System manual (BD Biosciences − Pharmingen) (61).

We amplified recombinant viral stock and infected Sf9 cells to monitor the kinetics of recombinant protein expression in insect cells at different time points (62). At 60 hours after infection the expression of recombinant α1(IV)NC1 protein was maximum, and at that time point cell extracts were prepared for purification of recombinant protein. Recombinant α1(IV)NC1 6XHis fusion protein is expressed in Sf9 cells, and the total cell lysate was mixed with the appropriate affinity matrix (Ni-NTA Agarose; BD Biosciences − Pharmingen). After centrifugation, the supernatant containing all untagged protein is dissolved, and the 6XHis-tagged protein was eluted from the affinity matrix. The fusion tag can be proteolytically cleaved from the recombinant protein at the thrombin cleavage site downstream of the 6XHis sequence recombinant protein that is eluted from the affinity matrix. SDS-PAGE analysis revealed a monomeric band at 26 kDa, which was detected by Western blotting using anti-α1(IV)NC1 antibody (data not shown).

Migration assay. Ten thousand cells per well were seeded in incomplete medium containing recombinant α1(IV)NC1. Medium containing 10% FCS and 4 ng/ml VEGF was placed into the bottom wells of the Boyden chamber, and the chamber was incubated for 6 hours at 37°C with 5% CO₂. The number of cells that migrated and attached to the bottom side of the membrane was counted as described (35).

Proliferation assay. A suspension of HUVECs, WT MLECs, or α1 integrin–deficient MLECs (20,000 cells per well, passages 2–6) was used in proliferation assay. Five hundred microliters per well of MLEC medium containing 0.5% FBS supplemented with heparin, endothelial mitogen, glutamine, and penicillin/streptomycin was added to 24-well plates precoated with type IV collagen (10 μg/ml). After 24 hours, medium was replaced with MLEC medium containing 20% FCS and different concentrations of α1(IV)NC1 (0.25–1.0 μM), and after 24 hours, 1 μCuri of [³H]thymidine was added into each well. After 48 hours, [³H]thymidine incorporation was measured using a scintillation counter described earlier (35).

Endothelial tube assay. Two hundred fifty microliters Matrigel was added to each well of a 24-well plate and allowed to polymerize 20 minutes at 37°C. A suspension of 50,000 HUVECs in EGM-2 medium without antibiotic was plated on top of the Matrigel-coated wells. The cells were treated with α1(IV)NC1, α1β1 integrin, α1(IV)NC1 plus α1β1 integrin, or PBS. All assays were performed in triplicate. Cells were incubated for 48 hours at 37°C and viewed using a CK2 Olympus Corp. microscope described previously (35). The average number of tubes formed in 3 independent experiments is shown in the graph [88% inhibition at 1 μM α1(IV)NC1].

Cell attachment assay. This assay was performed as previously described (35). Briefly, 96-well plates were coated overnight with 100 μl/well 10 μg/ml of human α1(IV)NC1 or type IV collagen. Plates were blocked with 100 μl/well 100 μg/ml of BSA for 2 hours. HUVECs (1.5 × 10⁵ cells/ml) were

incubated with 1 μM α1β1, αVβ3, or α5β1 integrin or α1(IV)NC1 for 15 minutes. Then, 100 μl of the cell suspension was added to each well and incubated for 2 hours at 37°C. After washing twice with PBS, the number of attached cells was determined after methylene blue staining.

Immunoprecipitation and immunoblotting analysis. Cell extracts were prepared in RIPA-DOC buffer (50 mM Tris-HCl buffer, pH 8.0, containing 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 10 mM EDTA, 10 μg/ml aprotinin, 1 μg/μl pepstatin A, 1 μg/ml aminoethylbenzene sulfonyl fluoride, 1 mM sodium orthovanadate, and 10 mM sodium fluoride), and immunoprecipitation was performed as described previously (35, 63).

Cell signaling experiments. For cell signaling experiments, 1 × 10⁶ HUVECs or WT or *α1 integrin*^{−/−} MLECs were seeded into 10-cm² dishes coated overnight with type IV collagen (10 μg/ml). According to the experimental protocol, the cells were preincubated with α1(IV)NC1 for 15 minutes. The cells were lysed and the cell extracts analyzed by SDS-PAGE and immunoblotting using antibodies specific to phosphorylated and unphosphorylated proteins as described previously (35, 60).

Hypoxia experiments. HUVECs or WT or *α1 integrin*^{−/−} MLECs were cultured up to 70% confluence and trypsinized, and 1 × 10⁶ cells were seeded onto 10-cm type IV collagen–coated plates. Cells were treated in complete medium with 1 μM α1(IV)NC1 or PD98059 (50 μM)/SB203580 (20 μM) and exposed to hypoxic conditions using a modular incubator chamber (Billups-Rothenberg Inc.). The hypoxia chamber oxygen concentration (0–1%) was determined before and after incubation using an Oxygen Analyzer (Vascular Technology). The hypoxia chamber containing cell culture dishes was transferred to a cell culture incubator for 12–24 hours. Cells were lysed into RIPA buffer, and cell extracts were analyzed by SDS-PAGE and immunoblotting using antibodies specific to phosphorylated (p) ERK1/2, p-MEK1/2, p-p38, HIF-1α, and VEGF.

Western blotting. Thirty micrograms per lane of cell extracts were mixed with 5× sample buffer supplemented with 10% β-mercaptoethanol and boiled 5 minutes. Samples were run on a 10% SDS-PAGE gel, and proteins were transferred onto nitrocellulose membranes previously described (35).

In vivo tumor studies using 129/Sv mice. Age- and sex-matched *α1 integrin*^{−/−} and WT (129/Sv) mice were used for these studies. All animal studies were conducted under the approval of the Institutional Animal Care and Use Committee of Boys Town National Research Hospital. Every effort was made to minimize pain and distress. Twenty male 129/Sv mice (10 WT and 10 *α1 integrin*^{−/−}) were used for this study. The mice's backs were shaved, and 1 × 10⁶ SCC-PSA1 (teratocarcinoma) cells were injected s.c. into the back of each mouse. The tumors were measured using Traceable Digital Calipers (Fisher Scientific) 1 week after implantation, and the volume was calculated using a standard formula (width² × length × 0.52) (44). Ten days after the SCC-PSA1 cells were implanted, the mice were divided into 4 groups [10 WT mice, 5 for control and 5 for α1(IV)NC1 treatment; and 10 *α1 integrin*^{−/−} mice, 5 for control and 5 for α1(IV)NC1 treatment]. For the experimental set, α1(IV)NC1 was injected i.v. daily at 1 mg/kg body weight or 30 μg/mouse, while the same volume of sterile PBS was injected into the control mice. When control tumors reached 3.0 cm³, mice were sacrificed and the tumors and other organs were frozen for histological analysis.

Immunohistochemical staining. Immunohistochemical staining was performed as previously described (44). Briefly, 4-μm frozen sections were fixed in 100% acetone for 3 minutes at −20°C and air-dried. The sections were incubated with rat anti–mouse CD31 antibody at room temperature for 2 hours. Subsequently, the sections were washed 3 times in PBS and incubated with tetramethyl rhodamine–conjugated secondary antibodies at room temperature for 1 hour. After 3 washes with PBS, VECTA-SHIELD anti-fade mounting medium (Vector Laboratories) was applied, and sections were covered with cover slips and imaged. In each group, difference in vascularity and number of CD31-positive structures per tumor

microscopic field were determined for each section at ×400 magnification in a blinded fashion.

Measurement of circulating ECs. Four hundred microliters of blood was collected from each tumor-bearing mouse in EDTA- and heparin-containing tubes and examined for the number of circulating ECs. Briefly, plasma was separated from blood, and 250 μl of DMEM supplemented with 10% FBS was added to each tube. Red blood cells were removed with rbc lysis solution, and the mixture was placed on 8-chamber slides. After a 6-hour incubation that allowed ECs to attach to the slide, the attached cells were stained with anti-VEGFR2 antibody. The VEGFR2-positive cells were counted under the fluorescence microscope in different fields at a magnification of ×200 as described previously (44).

Statistics. Statistical differences between control and α1(IV)NC1-treated tumor groups were calculated using 2-tailed Student's *t* test or Welch's *t* test. ANOVA was used to determine statistical differences among the groups. As needed, further analysis was carried out using *t* test with conferring correction to identify significant differences. A *P* value less than 0.001 was considered statistically significant.

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