Regulation of COX-2–mediated signaling by α3 type IV noncollagenous domain in tumor angiogenesis

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Human α3 chain, a noncollagenous domain of type IV collagen [α3(IV)NC1], inhibits angiogenesis and tumor growth. These biologic functions are partly attributed to the binding of α3(IV)NC1 to αVβ3 and α3β1 integrins. α3(IV)NC1 binds αVβ3 integrin, leading to translation inhibition by inhibiting focal adhesion kinase/phosphatidylinositol 3-kinase/Akt/mTOR/4E-BP1 pathways. In the present study, we evaluated the role of α3β1 and αVβ3 integrins in tube formation and regulation of cyclooxygenase-2 (COX-2) on α3(IV)NC1 stimulation. We found that although both integrins were required for the inhibition of tube formation by α3(IV)NC1 in endothelial cells, only α3β1 integrin was sufficient to regulate COX-2 in hypoxic endothelial cells. We show that binding of α3(IV)NC1 to α3β1 integrin leads to inhibition of COX-2–mediated pro-angiogenic factors, vascular endothelial growth factor, and basic fibroblast growth factor by regulating IκBα/NFκB axis, and is independent of αVβ3 integrin. Furthermore, β3 integrin–null endothelial cells, when treated with α3(IV)NC1, inhibited hypoxia-mediated COX-2 expression, whereas COX-2 inhibition was not observed in α3 integrin–null endothelial cells, indicating that regulation of COX-2 by α3(IV)NC1 is mediated by integrin α3β1. Our in vitro and in vivo findings demonstrate that α3β1 integrin is critical for α3(IV)NC1-mediated inhibition of COX-2–dependent angiogenic signaling and inhibition of tumor progression. (Blood. 2007;110:1168-1177)

Introduction

Tumor angiogenesis is a complex process consisting of endothelial cell (EC) proliferation, migration, vascular basement membrane reorganization, and new lumen (tube) formation.[1-3] It is also required for a variety of physiopathologic processes, including development and wound-tissue regeneration.[4,5] Because angiogenesis plays a predominant role in tumor growth and invasion, antiangiogenic molecules may have therapeutic potential in cancer.[6,7] In the past decade, several antiangiogenic molecules have been identified from the vascular basement membrane and proteins such as angiostatin of plasminogen, which are circulating endogenously and may inhibit tumor growth.[8-10] In addition, researchers identified that several new functions of the type IV collagen noncollagenous 1 domains (NC1) of certain α-chains display antiangiogenic and antitumorogenic activity.[11] The capacity of the exogenously supplemented α1(IV)NC1 and α2(IV)NC1 domains to inhibit tissue development in vivo was first described in *Hydra vulgaris*.[12] The antiangiogenic and antitumorogenic activities of type IV collagen NC1 domains appear to be mediated by binding to integrins in ECs.[11,13-16] These NC1 domains exert their antiangiogenic effects by direct binding to newly formed tumor vasculature or proliferating ECs, where they induce apoptosis or inhibit EC signaling.[11,14,15,17-21]

The mechanism of action of several of these NC1 domains is attributed to their specific interactions with different cell surface integrins.[11,14-17,19,21-23] For example, α1(IV)NC1 binds to integrin α1β1 and regulates hypoxia-associated factors in ECs.[15,24] α2(IV)NC1 binds to α1β1, αVβ3, and αVβ5 integrins, and regulates antiangiogenic action by inhibiting PI3-K and promoting apoptosis.[11,18-20] α3(IV)NC1 binds to integrins αVβ3 and α3β1, and regulates PI3-K/4E-BP1 pathway.[11,14,17,21] α6(IV)NC1 regulates antiangiogenic actions by binding to integrin αVβ3.[11] Among all these type IV collagen NC1 domains, the α3(IV)NC1 domain is the best characterized with regard to its potent antiangiogenic properties. The signaling mechanisms by which these molecules regulate antitumorogenic activities in the hypoxic tumor bed are not known.

In this study we have identified that recombinant α3(IV)NC1 protein binds to integrins αVβ3 and α3β1, and its antiangiogenic functions appear to be mediated by these 2 integrins. It was recently identified that integrin α3β1, a nonclassical collagen-binding integrin, is a novel functional receptor for soluble α3(IV)NC1 and transdominantly inhibits the activation of αVβ3 integrin in ECs.[21] Similarly, integrin α3β1 has been demonstrated to alter the functions of other integrins and also play a crucial role in kidney and lung organogenesis, and to regulate hair follicle development.[25-28]

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Inhibitors of cyclooxygenase enzymes (COX-2) are known to block angiogenesis in models of tissue repair and in several solid tumor models.[29-31] Because hypoxia-regulated COX-2 is a major stimulus for angiogenesis, the aim of this study was to determine the molecular mechanism(s) of α3(IV)NC1-mediated inhibition of hypoxia-induced COX-2 in mouse lung ECs (MLECs). Here we show that α3(IV)NC1 regulates expression of hypoxia-mediated COX-2 and its associated effector molecules in vitro and in vivo. We also show that α3β1 (and not αVβ3) integrin receptor binds to the α3(IV)NC1 domain and regulates COX-2–mediated signaling. Inhibition of COX-2 expression is observed in integrin β3-null MLECs, and not in α3-null MLECs, when treated with α3(IV)NC1, supporting the hypothesis that this inhibition is mediated through integrin α3β1. Thus, while both integrin α3β1 and αVβ3 are involved in the inhibition of tube formation mediated by α3(IV)NC1, integrin α3β1 appears to play a key role in mediating the regulation of COX-2–mediated antitumorogenic activity of α3(IV)NC1 domain.

Materials and methods

The Institutional Animal Care and Use Committee at Boys Town National Research Hospital approved all procedures involving animals.

Primary cow pulmonary artery ECs were purchased from Clonetech, San Diego, CA. SCC-PSA1/teratocarcinoma tumor cells were obtained from the ATCC (Manassas, VA). Anti-integrin antibodies antimouse αVβ3, αV, α3, β1, β3, and recombinant human vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) were purchased from R&D Systems (Minneapolis, MN). VEGF, bFGF, and COX-2 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Protein A Sepharose CL-4B beads were from Pharmacia. Antihuman αVβ3, α3β1, and integrin proteins were purchased from Chemicon (Temecula, CA). FAK (Santa Cruz Biotechnology), phosphorylated FAK (Tyr 397; Biosource), Akt, phosphorylated Akt (Ser473; New England Biolabs, Ipswich, MA) were also purchased. Celecoxib (Celebrex) was purchased from Pfizer (New York, NY). NFκB and IκB-α antibodies were purchased from Cell Signaling Technology (Danvers, MA). Phosphorylated IκB-α (Tyr42) antibody was purchased from ECM Biosciences (Versailles, KY). HRP-labeled secondary antibodies, IFN-α, penicillin/streptomycin, and fibronectin (FN) were purchased from Sigma-Aldrich (St. Louis, MO). BD Martigel Martix (14.6 mg/mL) was purchased from BD Biosciences (San Jose, CA). Intracellular adhesion molecule-2 and rat antimouse CD31 were from PharMingen, San Diego, CA. Magnetic beads, Dynabeads M-450, were from Dynal, Oslo, Norway. Ham F-12, DME—Low-Glucose, heparin (Pierce, Rockford, IL), and endothelial mitogen were from Biomedical Technologies (Stoughton, MA). Affinity matrix (Ni-NTA Agarose) was from Qiagen (Valencia, CA). Fetal bovine serum was purchased from Fisher Scientifics (Houston, TX). ECL Kit was from Amersham Biosciences (Buckingham, United Kingdom). Tetramethyl rhodamin–conjugated secondary antibodies were from Jackson Laboratory (Bar Harbor, ME). Red blood cell lysis solution (pure gene), 8-chamber slides, and transwell were from Nalgene Nunc International, Naperville, IL. Vectashield antifade mounting medium was purchased from Vector Laboratories (Burlingame, CA).

Cell culture

Wild-type or β3 integrin–null MLECs were maintained in 40% Ham F-12, 40% DME–Low-Glucose, 20% fetal bovine serum supplemented with heparin, endothelial mitogen (Biomedical Technologies), glutamine, and penicillin/streptomycin (100 units/mL each). α3 integrin-null–immortalized ECs were maintained similar to MLECs with 20 U/mL of murine IFN-γ and cultured at 33°C for expansion, but required a shift to 37°C approximately 48 hours without IFN-γ for experimentation. Cow pulmonary artery ECs and SCC-PSA1 cells were maintained in Delbecco modified Eagle medium (DMEM) containing 10% fetal calf serum with penicillin and streptomycin (100 μg/mL each) at 37°C under a humidified mixture of air and CO_2 (95%/5% v/v). Passages 2 to 6 of MLECs were used for experiments.

Preparation of primary mouse lung ECs

MLECs were isolated from 10- to 14-week-old wild-type or β3 integrin–deficient mice. α3 integrin-null–immortalized ECs generated from newborn mice, which are SV40 large T-antigen–positive. Briefly, intracellular adhesion molecule-2 expressing MLECs were enriched using rat antimouse intracellular adhesion molecule-2 conjugated to magnetic beads. Primary MLECs were positive for the expression of endothelial-specific marker; VE-cadherin was at cell junctions as reported previously.[15,32]

Expression of recombinant α3(IV)NC1

The sequence encoding human α3(IV)NC1 was polymerase chain reaction-amplified using total RNA isolated from human placenta and Super Script One-Step (Invitrogen, Carlsbad, CA) reverse-transcription polymerase chain reaction system supplemented with 5 units of *Pfu* polymerase per reaction. The forward primer (5′-CGCCATATGCCGTGGAGACAGTG-GATC-3′) and reverse primer (5′-GCGAGATCTTCAGTGTCTTTTCT-TCATGCACA-3′) sequences were modified to incorporate *Nde*I and *Bgl*II restriction sites and were used to amplify a 720-bp piece of DNA encoding 240 amino acids of a noncollagenous protein domain from α3 type IV collagen.[33] Polymerase chain reaction amplification was performed in a PTC-100 Programmable Thermal Controller from MJ Research (Waltham, MA). Amplification was performed according to the instructions in reverse-transcription polymerase chain reaction manual and the resulting amplicon was first cloned into pBSIISKP vector at *Eco*RV site and the recombinant clones were identified by blue-white selection. The recombinant clones were digested with *Nde*I and *Bgl*II to release the coding sequence for α3(IV)NC1, which was ligated into pAcHLT-A transfer vector (BD Biosciences PharMingen, San Diego, CA) predigested with the same restriction enzymes and the resulting recombinant transfer vector, pAcHLT-A/α3(IV)NC1, was cotransfected into Sf-9 cells as previously reported.[15,24,34,35]

Cell adhesion assay

Briefly, 96-well plates were coated with α3(IV)NC1 (10 μg/mL) overnight at 4°C. After 12 hours, nonspecific binding sites were blocked with 5% bovine serum albumin at 37°C for 2 hours. MLECs (1.5×10^5 cells/mL) were preincubated with indicated integrin antibodies (10 μg/mL) for 15 minutes and 100 μL of cell suspension were added to each well and incubated at 37°C for approximately 2 hours. The attached cells were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, and lysed with 10% acetic acid. Cell adhesion was quantified by reading the plates at 595 nm with a microtiter plate reader as described previously.[14-16,36]

Proliferation assay

A suspension of 7000 MLECs/well in a 96-well plate was used in proliferation assay. Cells were grown overnight in a 96-well plate precoated with fibronectin (10 μg/mL) under 0.5% fetal calf serum with penicillin/streptomycin. After 24 hours, the medium was replaced with medium containing 20% fetal calf serum with different integrin proteins (1 μM) with and without α3(IV)NC1 (1 μM). After 48 hours the cells were washed and stained with methylene blue as reported previously.[8]

Tube formation assay

A suspension of 50 000 MLECs in EGM-2 medium without antibiotic was plated on top of the matrigel-coated wells. The cells were treated with or without α3(IV)NC1 or with and without α3β1 or αVβ3 and α3(IV)NC1 proteins (1.0 μM), as indicated in Figure 3. Phosphate-buffered saline in triplicate wells was used as control. Cells were incubated for 48 hours at 37°C and viewed using a Leitz Fluovert microscope as described previously.[14,15] The average number of tubes formed in 3 independent experiments was showed.

Cell lysis, immunoprecipitation, and immunoblotting

MLECs were lysed for 30 minutes in ice-cold RIPA lysis buffer. After centrifugation, cleared supernatants were incubated for 2 hours at 4°C with continuous mixing with different integrin antibodies, or IgG coupled to protein A-Sepharose as reported previously.[14,15,37] For immunoblotting, samples were separated using SDS-PAGE, transferred to nitrocellulose, blocked with 5% nonfat dry milk in Tris-buffered saline buffer, and probed with primary antibodies. Antibody binding was detected using peroxidase-labeled second antibody and enhanced luminescence (ECL) kit as described.[15]

Cell-signaling experiments

For cell-signaling experiments, 10^6 MLECs were seeded into 10-cm^2 dishes coated overnight with FN (10 μg/mL). According to the experimental protocol, the cells were preincubated with α3(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.[14,15]

Cytology experiments

MLECs were grown to 70% confluence, serum-starved, and stimulated with 5 ng/mL VEGF, 10 ng/mL bFGF, and seeded on FN-coated 8-chamber slides. The slides were exposed to hypoxia in the presence of α3(IV)NC1 (1 μM) for 60 minutes and fixed in −20°C acetone. Fixed cells were incubated with NFκB p65 antibody for 60 minutes at room temperature, followed by incubation with secondary antibody. Nuclear translocations of NFκB determined using a fluorescence confocal microscope (100× magnifications).

Northern blot

MLECs/cow pulmonary artery ECs were serum-starved for 24 hours, stimulated with 5 ng/mL VEGF, 10 ng/mL bFGF, treated with α3(IV)NC1 (1 μM), and seeded on FN-coated plates; cells were then exposed to hypoxia for 24 hours. Total RNA was isolated, and Northern transfer was performed according to manufacturer's instructions and probed with COX-2 and bFGF, as reported previously.[38,39]

Hypoxia experiments

Wild-type, β3, or α3 integrin-null ECs or cow pulmonary artery ECs (10^6) were serum-starved, trypsinized, and seeded on 10-cm FN-coated plates. Cells were exposed to hypoxia (oxygen concentration 0%-1%) using a modular incubator chamber (Billumps-Rothenberg; Del Mar, CA) in the presence of α3(IV)NC1 (1 μM), IFN-α (50 units/mL), or COX-2 inhibitor celecoxib (100 μM) for 12 to 24 hours in complete medium. Total cellular RNA and cytosolic extracts were prepared as previously reported.[15,38]

Immunohistochemical staining

Briefly, 4-μm frozen tumor sections were fixed in 100% acetone for 3 minutes at −20°C and air-dried. The sections were incubated with primary antibodies (ie, rat antimouse CD31 and rat antimouse COX-2 antibodies) at room temperature for 60 minutes. The sections were subsequently washed with phosphate-buffered saline and incubated with tetramethyl rhodamine–conjugated secondary antibody at room temperature for 60 minutes. The staining was analyzed using a fluorescence microscope; Zeiss AX10 (Carl Zeiss, Sheerin Scientific, Shawnee, KS); after 60 minutes. In each group, the numbers of CD31-positive blood vessels were counted in 10 to 15 fields at 100× magnification in a blinded fashion as previously described.[15]

In vivo study of angiogenesis using matrigel plug assay

Angiogenesis was measured in matrigel plugs (500 μL) containing heparin with and without bFGF or VEGF, and α3(IV)NC1 proteins were injected subcutaneously into the right and left sides of 12-week-old male 129/Sv mice at sites lateral to the abdominal midline. As a negative control, matrigel with heparin alone was injected in a similar manner. Animals were killed 6 days after matrigel injection. The matrigel plugs were recovered, and half of the control and the α3(IV)NC1–treated plugs from each group were fixed in 4% paraformaldehyde or 10% formalin. The matrigel was embedded in paraffin and sectioned and stained with hematoxylin and eosin. The other matrigel plugs were dispersed in phosphate-buffered saline and incubated at 4°C overnight. Hemoglobin levels were determined with Drabkin solution according to the instructions of the manufacturer. This assay was performed as previously described.[40]

In vivo tumor studies using 129/Sv mice

Twenty male 6-month-old mice were used for this study. Mouse backs were shaved and 0.5×10^6 SCC-PSA1/teratocarcinoma cells were injected subcutaneously into the back of each mouse; 10 days after the injection, the mice were divided into 2 groups (10 each). For the experimental mice, α3(IV)NC1 was intravenously injected daily at 1 mg/kg per body weight or 30 μg per mouse, while only sterile phosphate-buffered saline was injected

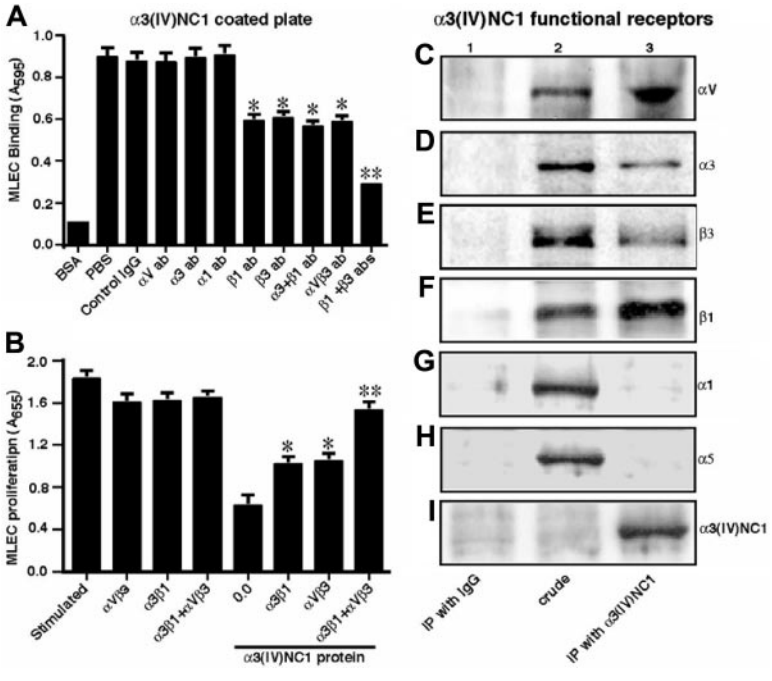

Figure 1. Blocking of integrin β1 and β3 inhibits adhesion to α3(IV)NC1 domain. (A) Cell adhesion assay. MLECs were seeded onto a 96-well plate coated with α3(IV)NC1 in the presence of the indicated integrin antibodies and cell adhesion was evaluated. Values are means (± the standard error of the mean [SEM]) of triplicate wells. Differences between 3 independent experiments control IgG and various integrin antibodies treated cells binding were significant. $^*P < .05$ and $^{**}P < .01$. (B) Proliferation assay. Similar to panel A, cells were preincubated with indicated integrin proteins with and without α3(IV)NC1 and cell proliferation was evaluated. The results are shown as mean (± the standard error of the mean [SEM]) $^*P < .05$, α3(IV)NC1 without vs with α3β1 and αVβ3 integrins. $^{**}P < .008$, α3(IV)NC1 without vs with α3β1 + αVβ3 integrins together. (C-I) Identification of α3(IV)NC1 functional binding integrins. MLECs were treated with α3(IV)NC1 for approximately 6 hours and extracts were immunoprecipitated with anti-α3(IV)NC1 antibody or control IgG. Immunoprecipitates were fractionated by SDS-PAGE and immunoblotted with anti-α3(IV)NC1, αV, α3, β3, β1, α1, and α5 antibodies. Crude cell lysate was used as a positive control.

into the control mice. When control tumors reached 3.0 cm^3, mice were killed and the tumor and other organs were frozen for histologic analysis as described previously.[15,41]

Measurement of circulating ECs

Mouse blood was collected (400 to 500 μL) in EDTA (ethylenediaminetetraacetic acid)/heparin into microcentrifuge tubes. Plasma was separated and 300 μL of DMEM supplemented with 10% fetal bovine serum was added to each tube. Red blood cells were removed with red blood cell lysis solution and the mixture was placed on 8-chamber slides. After a 6-hour incubation at 37°C, the attached ECs were stained with anti-VEGFR2 or CD31 antibody. The positive cells were counted under the fluorescence microscope in 10 fields at a magnification of 200× as described previously.[15]

Statistical analysis

Statistical differences between control and α3(IV)NC1-treated tumor groups were calculated using Student *t* test or Welch *t* test. ANOVA was used to determine statistical differences among the groups. As needed, further analysis was performed using *t* test with conferring correction to identify significant differences. *P* less than .001 was considered statistically significant.

Results

Identification of α3β1 and αVβ3 as functional integrin receptors for α3(IV)NC1

α3(IV)NC1 was shown to be an antiangiogenic molecule with significant antitumor activity.[11] α3(IV)NC1 interacts with several integrins on ECs, including αVβ3, CD47/integrin-associated protein, α5β1, αVβ5, and α3β1, and it has been postulated that these interactions may mediate its antiangiogenic activity.[11,16,21,42,43] We therefore performed integrin-binding experiments to characterize the functional roles of αVβ3 and α3β1 integrins in mediating the distinct antiangiogenic/antitumorogenic properties of α3(IV)NC1 in ECs. Binding of ECs to α3(IV)NC1-coated plates was inhibited by blocking with antibodies specific for β1, β3, α3+β1, αVβ3, or β1+β3 integrins, whereas no significant affect was observed using blocking antibodies specific for αV, α3, and α1, confirming that α3(IV)NC1 is not binding to these integrin subunits (Figure 1A). We have further confirmed that soluble α3β1 and αVβ3 integrin proteins could bind to α3(IV)NC1 precoated culture plates and subsequently inhibit attachment of ECs to α3(IV)NC1 (data not shown). These experiments confirm that integrins α3β1 and αVβ3 may serve as functional receptors for the α3(IV)NC1 molecule. Binding of ECs to α3(IV)NC1-coated plates was significantly inhibited by α3+β1 and αVβ3 integrin antibodies, whereas α5β1 or α1β1 or α2β1 integrin antibodies had no significant effect (data not shown). Preincubation of ECs with α3β1, αVβ3, or α3β1+αVβ3 integrin proteins has no significant effect on proliferation, whereas preincubation of ECs with α3(IV)NC1 significantly decreased proliferation of ECs (Figure 1B). In the same experiment, addition of equimolar concentrations of soluble α3β1, αVβ3, or α3β1+αVβ3 integrin proteins captured α3(IV)NC1 and reversed the inhibition of ECs proliferation (Figure 1B). These results support the hypothesis that the antiproliferative action of α3(IV)NC1 is mediated by α3β1 and αVβ3 integrins, suggesting that α3β1 and αVβ3 integrins are functional receptors for α3(IV)NC1. α3(IV)NC1 binding to α3β1 and αVβ3 integrins was further confirmed by coimmunoprecipitation experiments (Figure 1C-I).

α3(IV)NC1 binds to α3β1 and αVβ3 integrins and regulates tube formation in ECs cultured on matrigel

We tested the antiangiogenic activity of α3(IV)NC1 by tube formation assay using ECs cultured on matrigel. Tube formation

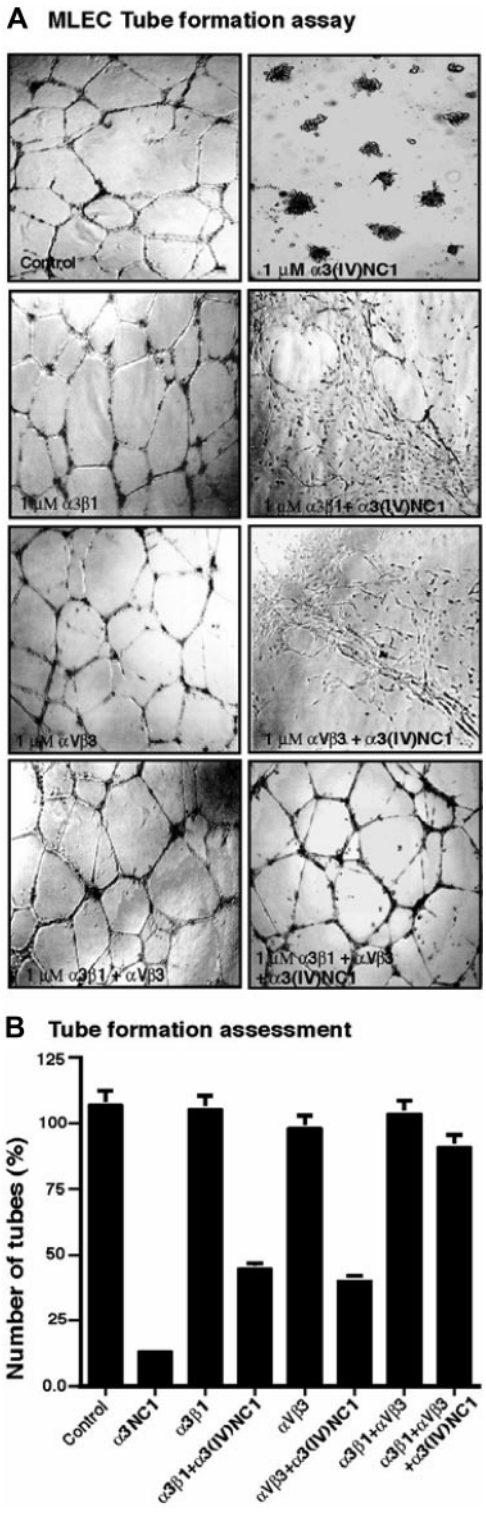

Figure 2. Tube formation assays. (A) Tube formation assay on matrigel was studied with or without α3(IV)NC1 and with different integrins (α3β1, αVβ3, or α3β1+αVβ3), with and without α3(IV)NC1 protein. Tube formation was evaluated after 48 hours using a Leitz Fluovert microscope (100×/1.25 NA), and representative fields were shown. Images were captured using a Flex Digital IEEE-1394 camera (Sheerin Scientific) and processed using SPOT advanced imaging software version 3.1.0 (Diagnostic Instruments, Sterling Heights, MI) and Adobe Photoshop 7.0 (Adobe, Redmond, WA). (B) Tube assay graphical representation. Average number of tubes formed (% values, with error bars indicating SEM) in 3 independent experiments is shown in the graph.

involves EC migration, proliferation, and survival.[44] Addition of α3(IV)NC1 significantly inhibited ECs tube formation on matrigel matrix (Figure 2A; α3(IV)NC1). Preincubation of cells with α3β1-integrin protein had no effect on tube formation (Figure 2A α3β1). Preincubation of ECs with equimolar mixture of α3β1 and α3(IV)NC1 protein, reversed the inhibitory affect of α3(IV)NC1 by 50% (Figure 2A α3(IV)NC1+α3β1). Preincubation of ECs with αVβ3-integrin protein had no effect, whereas preincubation of ECs with equimolar mixtures of αVβ3 integrin and α3(IV)NC1 protein reversed the inhibition of tube formation action of α3(IV)NC1 by 45% (Figure 2A α3(IV)NC1+αVβ3). These results confirm that the antiangiogenic/antitumorogenic action of α3(IV)NC1 may be mediated through αVβ3 and α3β1 integrins. To further confirm this observation, ECs were preincubated with equimolar mixtures of αVβ3 and α3β1-integrin proteins, which had no effect on tube formation, whereas preincubation of ECs with equimolar mixture of α3β1+αVβ3 integrins and α3(IV)NC1 protein reversed the tube formation inhibitory action of α3(IV)NC1 by 90%, suggesting that the 2 integrins function additively in the tube formation assay (Figure 2A; α3β1+αVβ3+α3(IV)NC1). Such reversal of inhibition is not observed with soluble α5β1 integrin and α3(IV)NC1 proteins in tube formation (data not shown). The number of tubes formed in 3 independent experiments are shown in the graph (Figure 2B).

αVβ3 and α3β1 integrin-dependent regulation of FAK and Akt phosphorylation by α3(IV)NC1

We investigated the role of α3β1/αVβ3 integrin and its effector kinase, focal adhesion kinase (FAK)/Akt, in α3(IV)NC1-mediated antiangiogenic functions in ECs. We observed that α3(IV)NC1 treatment leads to inhibition of sustained FAK/Akt phosphorylation on FN matrix (Figure 3A,B, lanes 3 and 5). Preincubation of ECs with equimolar mixtures of α3β1 and α3(IV)NC1 proteins on a FN matrix reversed the inhibitory action of α3(IV)NC1 on sustained FAK phosphorylation by 45% to 50% (Figure 3C, lanes 4 and 5). Preincubation of ECs with equimolar mixtures of αVβ3 integrin and α3(IV)NC1 protein on a FN matrix did not reverse the sustained inhibitory action of α3(IV)NC1 on phosphorylation of FAK completely (Figure 3D, lane 4 and 5). To further test whether these 2 integrins are acting together in the regulation of α3(IV)NC1 antiangiogenic action, we preincubated ECs with equimolar mixtures of soluble αVβ3+α3β1 integrin proteins with α3(IV)NC1 and seeded them on FN-coated plates for 30 to 60 minutes. We found that the presence of soluble integrins as competitive inhibitors lead to complete reversal of the inhibition of sustained FAK phosphorylation on FN matrix (Figure 3E, lanes 4 and 5). In contrast, sustained activation of FAK in ECs was not affected by αVβ3 or α3β1, or αVβ3+α3β1 (data not shown). These results suggest that α3β1 and αVβ3 integrins are essential for α3(IV)NC1 function in ECs. Similar results of Akt and phosphatidylinositol 3-kinase phosphorylation inhibition reversal was observed when cells were treated with α3β1+αVβ3+α3(IV)NC1 (data not shown). These results suggest that α3(IV)NC1 binding to α3β1 and αVβ3 integrins inhibits FAK downstream signaling.

Effect of α3(IV)NC1 on NFκB and COX-2–mediated cell signaling

Integrins transduce biochemical signals across the cell membrane (outside-in signaling) via activation of intracellular signaling pathways, which include phosphatidylinositol 3-kinase or mitogen-activated protein kinase family members.[14,15] Activation of cytosolic kinases by integrin-linked transmembrane signaling leads to activation of NFκB to regulate gene expression and cell survival.[45] Here, we examined the role of the NFκB signaling cascade in α3(IV)NC1-mediated inhibition of cellular functions when cells were cultured on FN matrices in hypoxic conditions. Attachment of MLECs to FN via α3β1/αVβ3 integrins activated the FAK/Akt

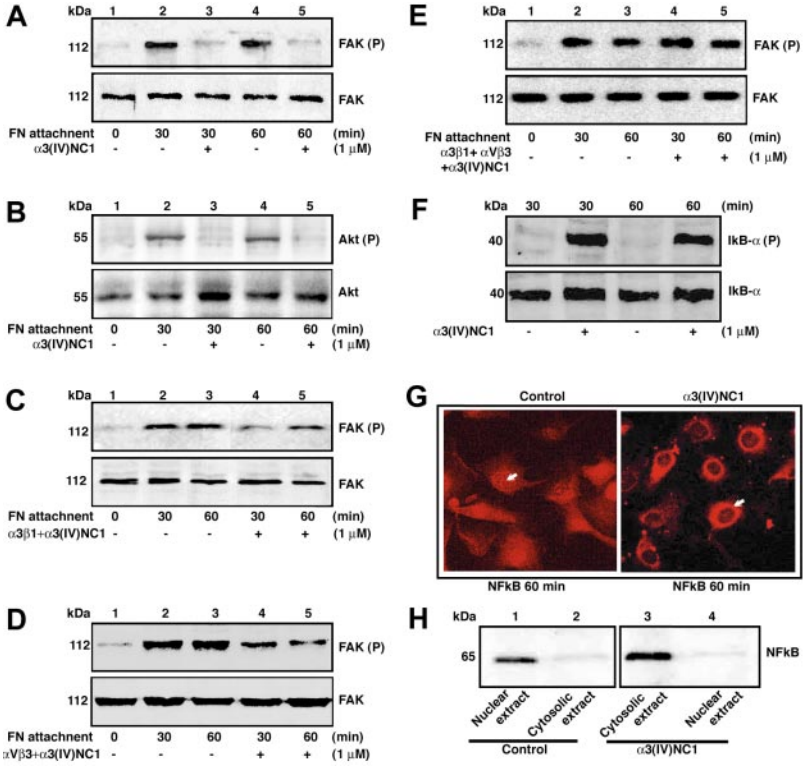

Figure 3. FAK and AKT phosphorylation. Serum-starved wild-type MLECs were plated on FN-coated dishes in incomplete medium (ICM) supplemented with and without α3(IV)NC1 or α3β1+α3(IV)NC1 or αVβ3+α3(IV)NC1, or α3β1+αVβ3+α3(IV)NC1, for 0 to 60 minutes and cytosolic extracts were analyzed by Western immunoblotting. (A) Immunoblots of phosphorylated FAK (p-FAK, upper blot) and total signaling FAK protein (FAK, lower blot). (B) Phosphorylated AKT (p-AKT, upper blot) and total signaling AKT protein (AKT lower blot). (C-E) Similar to panels A and B but with and without α3(IV)NC1 or α3β1+α3(IV)NC1, or αVβ3+α3(IV)NC1, or α3β1+αVβ3+α3(IV)NC1. (F-H) Regulation of IκB-α and NFκB in ECs by α3(IV)NC1. (F) Hypoxic cell extracts were immunoblotted with phosphorylated IκB-α (p-IκB-α, upper blot) and total IκB-α protein (lower blot). (G) Nuclear translocations of NFκB staining in MLECs were determined using a Zeiss LSM 510 Meta Confocal microscope (Carl Zeiss) with a Plan-Neo 63×/1.4 NA objective lens. Images were captured using a Flex Digital IEEE-1394 camera (Sheerin Scientific), and processed using SPOT advanced imaging software version 3.1.0 (Diagnostic Instruments) and Adobe Photoshop 7.0 (Adobe). (H) Immunoblots of cytosolic and nuclear extracts showing the NFκB translocation.

pathway. Pretreatment of MLECs with α3(IV)NC1 before plating on FN matrix inhibited the sustained phosphorylation of IκB-1α (Figure 3F top panel). ECs exposed to hypoxia in the presence of α3(IV)NC1 (1 μM) for 60 minutes inhibited NFκB nuclear translocation (Figure 3G,H; α3(IV)NC1).

Additional experiments were designed to address whether regulation of NFκB activation by α3(IV)NC1 regulates other hypoxia factors such as COX-2, bFGF, and VEGF, which are key players in tumor angiogenesis. Cultured ECs, when treated with α3(IV)NC1 under hypoxic conditions, showed inhibition of COX-2 mRNA expression (Figure 4A). Similarly, Western blot analysis of cytosolic extracts revealed that α3(IV)NC1 treatment inhibited COX-2 protein expression in hypoxic ECs (Figure 4B). Surprisingly, α3(IV)NC1 inhibits COX-2 expression in β3 integrin-null MLECs, suggesting that α3(IV)NC1 regulation of COX-2 expression is independent of αVβ3 integrin (Figure 4C). These results were further confirmed by immunohistochemical staining of α3(IV)NC1 binding to β3 integrin-null MLECs and inhibiting COX-2 expression (Figure 4D). Furthermore, α3(IV)NC1 inhibited upregulation of bFGF mRNA and protein levels in response to hypoxia in cow pulmonary artery ECs (Figure 4E,F). COX-2 mediated upregulation of VEGF expression in hypoxic ECs was also modulated by α3(IV)NC1 (Figure 4G). To further conform that the regulation of COX-2 expression depends on α3β1 integrin, α3 integrin-null ECs were treated with α3(IV)NC1, and it was observed that COX-2 expression was not affected (Figure 4H; 12 and 24 hours treated with α3(IV)NC1). These results confirm that COX-2 expression is mediated by α3(IV)NC1 through α3β1 integrin.

Regulation of VEGF-induced and bFGF-induced neovascularization by α3(IV)NC1

We evaluated the effects of α3(IV)NC1-regulated VEGF-mediated and bFGF-mediated angiogenesis in vivo using matrigel matrix plugs in 129/Sv mice. In vivo matrigel plugs containing VEGF and bFGF were used to assess the role of α3(IV)NC1 in inhibiting growth factor-induced neovascularization. α3(IV)NC1 significantly inhibited (nearly 88%) VEGF-induced and bFGF-induced neovascularization in the matrigel plugs in mice (Figure 5A). The number of blood vessels were as follows: VEGF+α3(IV)NC1, 6.45 (± 0.35); bFGF+α1(IV)NC1, 7.25 (± 0.25); and controls, VEGF, 30.5 (± 3); bFGF, 32 (± 1.5, Figure 5B). The hemoglobin contents in VEGF control were 8.5 g/dL (± 1.3 g/dL, n = 6) or bFGF control 7.8 g/dL (± 1.5 g/dL,n = 6; Figure 5C). In contrast, the hemoglobin contents of VEGF+α3(IV)NC1 treated was 1.95 g/dL (± 0.15 g/dL, n = 6) and bFGF+α3(IV)NC1 treated was 1.75 g/dL (± 0.28 g/dL, n = 6; Figure 5C). These results suggest that α3(IV)NC1 inhibits VEGF-mediated and bFGF-mediated neovascularization.

Regulation of tumor growth by α3(IV)NC1in 129/Sv mice

Previously, several researchers reported that α3(IV)NC1 reduces the rate of tumor growth and angiogenesis in vivo.[11,23,46] Here we

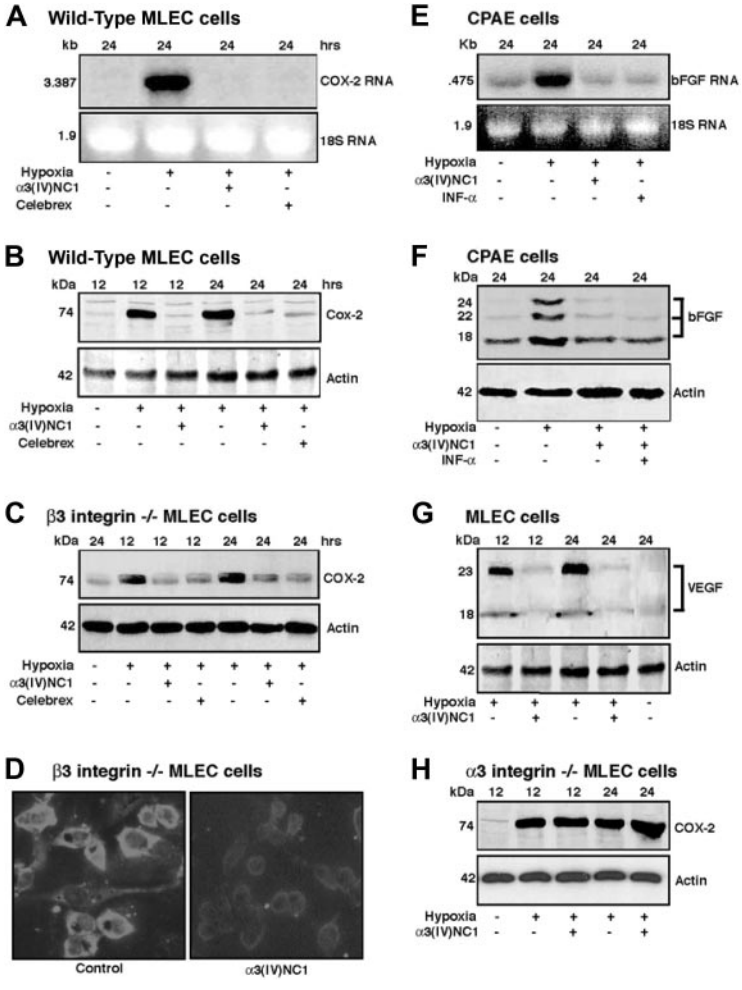

Figure 4. Regulation of COX-2–mediated signaling by α3(IV)NC1 in hypoxic ECs. (A) Autoradiogram showing expression of COX-2 mRNA on α3(IV)NC1 treatment. Celecoxib was used as a positive control. (B) Western immunoblot of MLEC extracts using antibodies specific to COX-2. (C) β3 integrin-null MLEC extracts were analyzed by Western blotting using antibodies specific to COX-2. (D) MLECs treated with and without α3(IV)NC1 were exposed to hypoxia for approximately 12 hours and stained with COX-2 antibody. Images were viewed using a Zeiss AX10 microscope (Carl Zeiss) with a 40×/0.75 NA objective lens, captured using a Flex Digital IEEE-1394 camera (Sheerin Scientific), and processed using SPOT advanced imaging software version 3.1.0 (Diagnostic Instruments) and Adobe Photoshop 7.0 (Adobe). (E) Autoradiogram showing expression of bFGF mRNA on α3(IV)NC1 treatment in cow pulmonary artery ECs. The top half of the autoradiogram (0.475 bFGF probe) shows inhibition of expression of bFGF mRNA on treatment with α3(IV)NC1 or IFN-α (positive control). (F,G) Similar to panel B, cow pulmonary artery ECs and MLEC extracts were immunoblotted with antibodies specific for bFGF and VEGF. (H) α3 integrin-null ECs were treated with α3(IV)NC1 and exposed to hypoxic conditions and cell extracts were immunoblotted with COX-2 antibody. 18S RNA (A,E) and actin (B,C,F-H) levels were shown as loading controls.

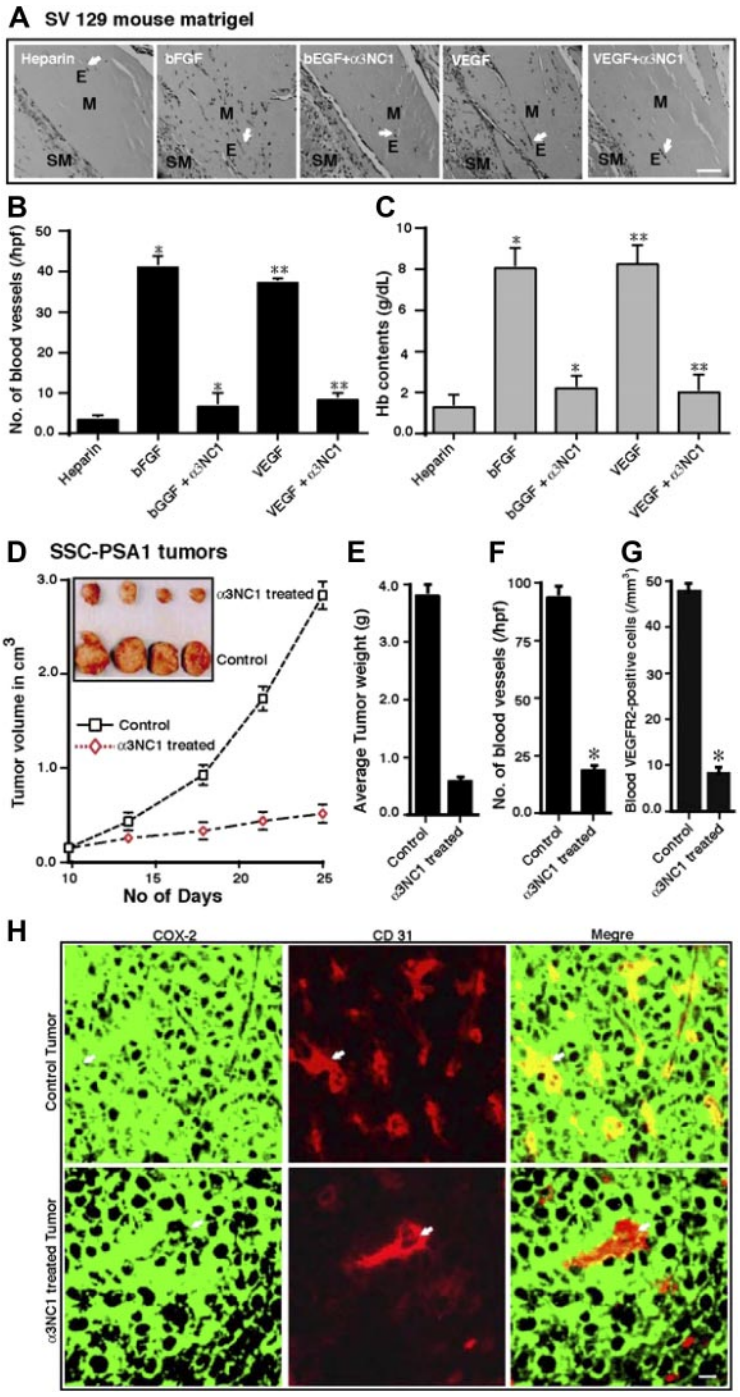

Figure 5. In vivo matrigel plug and tumor angiogenesis in 129/Sv mice. (A) From left to right, control, bFGF, bFGF+α3(IV)NC1, VEGF, VEGF+α3(IV)NC1. E indicates ECs; M, matrigel; SM, smooth muscle. Scale bar: 40 μm. Arrows point to the blood vessels. The number of blood vessels in the matrigel plugs was counted in 10 fields at ×200 magnification. Images were viewed using a Zeiss AX10 camera (Carl Zeiss) with a 100×/1.4 NA objective lens, captured using a Flex Digital IEEE-1394 camera (Sheerin Scientific), and processed using SPOT advanced imaging software 3.1.0 (Diagnostic Instruments) and Adobe Photoshop 7.0 (Adobe). (B,C) Number of blood vessels and Hb content quantification from panel A. The mean (± SEM) are shown. $*P < .01$, VEGF with vs without α3(IV)NC1. $**P < .01$, bFGF with vs without α3(IV)NC1. (D) The graph of the growth of mice tumors with and without α3(IV)NC1 injections. The results are shown as mean (± SEM). $P < .005$, tumor mice without α3(IV)NC1 injection as control group. (E) The average tumor weights of different groups shown in panel D. (F) Frozen sections (4-μm) from different tumor tissues were stained with anti-CD31 antibody and the number of CD31-positive blood vessels were counted. Blood vessel quantification results are shown as the mean (± SEM). $*P < .005$, mice with vs without α3(IV)NC1 treatment. (G) The circulating VEGFR2-positive cells in the blood of tumor-bearing mice, quantification results are shown as the mean (± SEM). $*P < .005$, mice with vs without α3(IV)NC1 treatment. (H) Frozen sections from different tumor tissues were stained with anti–COX-2 and CD31 antibodies, followed by FITC rhodamine-conjugated secondary antibodies. CD31 and COX-2 merged positive blood vessels were shown (arrow) in 6 fields at 200× magnification. Images were viewed, captured, and processed as described for panel A. Scale bar: 50 μm.

have examined the effect of COX-2 expression on tumor angiogenesis on α3(IV)NC1 treatment in tumor-bearing mice. Unlike earlier studies, in this study tumors were allowed to reach 150 mm^3, and then α3(IV)NC1 was administered (30 μg/mouse) intravenously once per day. The control (untreated) mouse group demonstrated an increased rate of tumor growth, numbers of CD31-positive blood vessels, whereas the α3(IV)NC1-treated tumor mice demonstrated a regression of tumor growth and numbers of CD31-positive blood vessels (Figure 5D-H). Further circulating VEGFR2-positive ECs were also measured, and resulted in inhibition of circulating ECs on α3(IV)NC1 treatment (Figure 5G; α3(IV)NC1 treated). The possible integrin-mediated signaling regulated by α3(IV)NC1 in hypoxia-induced angiogenesis, affecting NFκB activation and downregulating COX-2 and VEGF/bFGF expressions are shown in the illustration (Figure 6).

Discussion

Collagen type IV α3 chain, noncollagenous domain (α3(IV)NC1), was identified as an endogenous potent inhibitor of angiogenesis and tumor growth.[11] Later researchers identified other domains from type IV collagen noncollagenous such as α1, α2, α3, and α6 that were also inhibitors of tumor angiogenesis.[13,15,18-21,46,47] Understanding the mechanisms of action of these molecules is crucial for their potential therapeutic use. We identified that α3(IV)NC1 binds

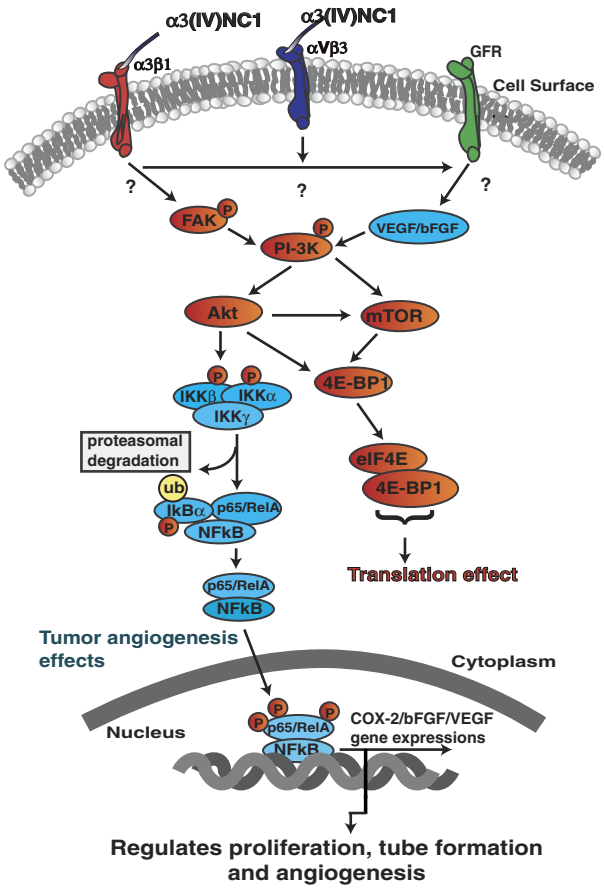

Figure 6. Schematic illustration of different signaling pathway mediated by α3(IV)NC1. α3(IV)NC1 binds to αVβ3 and α3β1 integrins and inhibit phosphorylation of FAK. Inhibition of FAK activation leads to inhibition of FAK/phosphatidylinositol 3-kinase/eIF4E/4E-BP1. In addition, α3(IV)NC1 also inhibits NFκB-mediated signaling in hypoxic conditions leads to inhibition of COX-2/VEGF/bFGF expressions, resulting in inhibition of hypoxic tumor angiogenesis.

to 2 distinct integrins, αVβ3 and α3β1, in an FN-dependent manner and mediates its antiangiogenic activities by inhibiting EC tube formation and proliferation. This is consistent with the previous studies demonstrating that the antiangiogenic activity of α3(IV)NC1 is through αVβ3 integrin-mediated inhibition of protein synthesis, specifically in ECs.[15,17] Recently published evidence strongly supports that the antitumorigenic affects of the α3(IV)NC1 domain may also be mediated via integrin α3β1, and that this integrin might alter αVβ3-dependent cell function by transdominant activation or inhibition.[21]

In ECs, ligand binding to integrins induces phosphorylation of FAK, which serves as a platform for different downstream signals.[14,15,48-50] α3(IV)NC1 inhibits phosphorylation of FAK when ECs are plated on FN matrix.[14] Similarly, we previously reported other collagen NC1 domain α1(IV)NC1 inhibiting phosphorylation of FAK on type IV collagen.[15] Here we show inhibition of FAK phosphorylation was not completely reversed when α3(IV)NC1 was mixed with either purified αVβ3 or α3β1 integrin proteins and plated on FN matrix. Interestingly, inhibition of FAK phosphorylation was completely reversed when both αVβ3 and α3β1 integrins were mixed with α3(IV)NC1. These results suggest that both integrins are involved in α3(IV)NC1-mediated signaling through FAK.

Downstream of FAK, Akt/PKB plays an important role in EC survival signaling.[14,15,51] Therefore, it appears that the antiangiogenic activity of α3(IV)NC1 is regulated via 2 distinct integrin-dependent signaling mechanisms in ECs. The first was previously reported and involves EC-specific protein synthesis inhibition by α3(IV)NC1 binding to αVβ3 integrin.[14,17] This mechanism has since been implicated to contribute to inhibition of tumor growth for several different tumor cell lines, including CT26 (colon adenocarcinoma), LLC (Lewis lung carcinoma), renal cell carcinoma (786-O), prostate carcinoma (PC3), human prostate cancer (DU145), human lung cancer (H1299), and human fibrosarcoma (HT1080), by inhibiting tumor angiogenesis.[11,23,46] The findings reported here suggest that inhibition of tumor growth by α3(IV)NC1 may also be mediated by regulating hypoxic COX-2.

COX-2 is known to play a key role in the tumor angiogenesis.[29,52] COX-2 upregulation is a hallmark of inflammation as well.[53] Moreover, several investigators have demonstrated that blockade of the COX-2–mediated pathway provides therapeutic benefit in different cancer models.[54] COX-2 regulates cellular responses to pathologic conditions, and studies have demonstrated that COX-2 is a potential target for tumor angiogenesis.[54] COX-2–mediated signaling regulates expression of several inflammatory factors in hypoxic cells and enhances angiogenesis in solid tumors.[55] In the present study, we have demonstrated for the first time that α3(IV)NC1 inhibits hypoxia-induced COX-2 expression in ECs via FAK/Akt/NFκB pathway, which in turn leads to decreased tumor angiogenesis and tumor growth. In addition to COX-2 inhibition, VEGF and bFGF protein expression were also inhibited on α3(IV)NC1 treatment in ECs.

Our results suggest that when ECs are treated with α3(IV)NC1, it binds to α3β1 integrin and inhibits NFκB signaling, resulting in inhibition of COX-2–mediated signaling. This was further supported by our observation that α3(IV)NC1 inhibits COX-2 expression in β3 integrin-deficient ECs, demonstrating that COX-2–mediated signaling is regulated through α3β1, and not by αVβ3 integrin. To confirm the regulation of α3β1 integrin-dependent COX-2 expression on α3(IV)NC1 treatment, α3 integrin–null ECs were treated with α3(IV)NC1 protein under hypoxic conditions. COX-2 expression was not affected when α3(IV)NC1 was treated with α3 integrin–null ECs. This is consistent with earlier observations that α3(IV)NC1 binds to α3β1 and transdominantly inhibits αVβ3 integrin.[21] These findings strongly suggest that α3(IV)NC1 can inhibit proinflammatory factor COX-2, and inhibit tumor vasculature, which leads to regression of tumor growth by binding to α3β1 integrin.

Here we showed regression of SCC-PSA1 tumors in 129/Sv mice on α3(IV)NC1 treatment. Regression was associated with reduced tumor vasculature, reduced COX-2 expression, and reduced circulating VEGFR2-positive ECs compared with control mice. We also observed inhibition of VEGF- and bFGF-stimulated matrigel angiogenesis and hemoglobin content on α3(IV)NC1 treatment in 129/Sv mice. These data provide further evidence that the tumor suppressive action of α3(IV)NC1 is also mediated through α3β1 integrin by regulating COX-2 expression. 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.[15]

The antitumorogenic activity of α3(IV)NC1 under hypoxic conditions in solid tumors was not clearly understood. Our studies shed light on this mechanism by demonstrating that α3(IV)NC1 binds to α3β1 integrin, which inhibits COX-2 expression in in vitro and in vivo. It is clear that inhibition of hypoxia-induced angiogenesis by α3(IV)NC1 is a complex process requiring further investigation. Based on our work here, there may be several targets for the

inhibitory effects of α3(IV)NC1 on tumor angiogenesis, including or in addition to COX-2, VEGF, and bFGF.

In summary, the in vitro and in vivo observations support αVβ3 and α3β1 integrins role in antiangiogenic activity of α3(IV)NC1. While both of these integrins mediate tube formation by cultured ECs, α3β1 integrin-mediated signaling influences downstream effects on COX-2 expression, which appears central to the mechanism of α3(IV)NC1 antitumor activities. The study also demonstrates that α3(IV)NC1 inhibits hypoxia-induced angiogenesis by inhibiting NFκB activation, leading to inhibition of COX-2 expression, which in turn results in downregulation of hypoxia-induced VEGF/bFGF expression. Our findings have potential implications of α3(IV)NC1 for treatment of solid tumor growth, which depend critically on hypoxic angiogenesis. The decrease in COX-2 expression under hypoxia that results in decreased VEGF/bFGF expression probably represents a primary molecular mechanism by which α3(IV)NC1 inhibits the pathologic angiogenesis essential to the growth of tumors.

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Authorship

Contribution: C.S.B. and A.P.M. designed and performed all the studies. D.C. contributed vital reagents and tools. R.S. and K.M.H.D. generated α3 integrin-null immortalized ECs. V.G.K. performed design and analysis of signaling experiments. A.S. was responsible for conception, design, execution, and analysis of the studies and wrote the manuscript.

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