Tumorigenesis and Neoplastic Progression

Recombinant $\alpha 2(\text{IV})\text{NC1}$ Domain Inhibits Tumor Cell-Extracellular Matrix Interactions, Induces Cellular Senescence, and Inhibits Tumor Growth *in Vivo*

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Cellular interaction with the extracellular matrix is thought to be a critical event in controlling angiogenesis and tumor growth. In our previous studies, genetically distinct noncollagenous (NC) domains of type-IV collagen were shown to interact with integrin receptors expressed on the surface of endothelial cells. Moreover, these NC1 domains were shown to inhibit angiogenesis *in vivo*. Here, we provide evidence that a recombinant form of the $\alpha 2(\text{IV})\text{NC1}$ domain of type-IV collagen could bind integrins $\alpha 1\beta 1$ and $\alpha \nu \beta 3$ expressed on melanoma cells and inhibit tumor cell adhesion in a ligand-specific manner. Systemic administration of recombinant $\alpha 2(\text{IV})\text{NC1}$ domain potently inhibited M21 melanoma tumor growth within full thickness human skin and exhibited a dose-dependent inhibition of tumor growth in nude mice. Interestingly, $\alpha 2(\text{IV})\text{NC1}$ domain enhanced cellular senescence in tumor cells *in vitro* and *in vivo*. Taken together, these results suggest that recombinant $\alpha 2(\text{IV})\text{NC1}$ domain is not only a potent anti-angiogenic reagent, but it also directly impacts tumor cell behavior. Thus, $\alpha 2(\text{IV})\text{NC1}$ domain represents a potent inhibitor of tumor growth by impacting both endothelial and tumor cell compartments. (Am J Pathol 2005, 166:901–911)

In recent years a great deal of attention has been focused on understanding the molecular mechanisms by which the extracellular matrix (ECM) regulates angiogenesis and tumor growth.^{1–6} In this regard, the basement membrane is a specialized form of ECM composed of a complex network of collagenous and noncollagenous glycoproteins and proteoglycans. This specialized compartment of the ECM lines epithelial sheets, surrounds blood vessels and nerves, and is composed predominately of col-

lagen type-IV, laminin, and nidogen/entactin. Numerous studies have demonstrated that while basement membranes can provide mechanical support for cells and tissues, it also regulates a great diversity of other cellular processes including adhesion, migration, invasion, differentiation, cell cycle, and gene expression. Importantly, all these events have been implicated in the regulation of angiogenesis and tumor growth and are currently under intensive study in an attempt to gain a more complete understanding of the molecular mechanisms contributing to normal physiology and human disease. In fact, recent studies have identified several endogenous inhibitors of angiogenesis and tumor growth, which are derived from specific domains of ECM components such as collagen.^{1–6}

Collagen type-IV is a major component of the basement membrane and to date at least six genetically distinct collagen type-IV chains [$\alpha 1(\text{IV})$ to $\alpha 6(\text{IV})$] have been identified and characterized.^{7,8} One of the most widely distributed forms of collagen type-IV is composed of two $\alpha 1(\text{IV})$ chains and one $\alpha 2(\text{IV})$ chain organized in a triple helical manner. Collagen type-IV is organized into a number of functional domains including an N-terminal 7S domain, a central triple helical region, and a C-terminal noncollagenous (NC1) domain. Previous studies have identified unique functions for these structural domains, including the regulation of chain selection during the formation of the triple helical molecule, network formation, and regulation of integrin-dependent cell adhesion and migration.^{1–8} In fact, in studies using *Hydra vulgaris*, exogenous addition of purified NC1 domains of collagen type-IV resulted in disruption of ECM formation and inhibition of morphogenesis.⁹ These important early findings led to studies that suggested that recombinant NC1 domains disrupt angiogenesis.¹⁰ Importantly, the recent elucidation of the crystal structure of an NC1 domain

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hexamer will likely provided critical insight into the molecular mechanisms regulating collagen network assembly as well as contribute to a better understanding of the mechanisms by which isolated NC1 domains impact both physiological and pathological processes.¹¹

Cellular interactions with collagen type-IV are facilitated predominately by integrin cell-surface receptors.^{12,13} Integrins are a diverse group of transmembrane receptors composed of noncovalently associated α and β chains that combine to give heterodimers with distinct functional activities. Integrins play critical roles in facilitating bidirectional signal transduction between the local ECM and cells. In addition, studies have indicated that a number of $\beta 1$ -containing integrins can facilitate cellular interactions with triple helical collagen type-IV including $\alpha 1\beta 1$, $\alpha 2\beta 1$, and $\alpha 3\beta 1$.^{12,13} Moreover, evidence has also been provided that specific NC domains can interact with distinct integrins including, $\alpha 1\beta 1$, $\alpha 3\beta 1$, $\alpha 5\beta 1$, $\alpha \nu\beta 3$, and $\alpha \nu\beta 5$.^{14–19} These findings suggest that NC domains may exhibit specificity as to which integrin receptors they interact with, which in turn, may contribute to the regulation of distinct cellular events.

Recent studies from many laboratories have identified unique functions and biological activities for collagen NC1 domains.^{4,7,14} In fact, investigators have demonstrated that recombinant forms of collagen type-IV NC1 domains including $\alpha 1(IV)$ (arrestin), $\alpha 2(IV)$ (canstatin), $\alpha 3(IV)$ (tumstatin), and the $\alpha 6(IV)$ can potentially inhibit angiogenesis and tumor growth *in vivo*.^{14–19} In contrast, little if any anti-angiogenic or anti-tumor activity was observed with similar NC1 domains derived from the $\alpha 4$ and $\alpha 5$ chains of collagen type-IV.¹⁴ Although significant progress has been made in understanding the molecular mechanisms by which $\alpha 3(IV)$ NC1 domain (tumstatin) inhibits angiogenesis and tumor growth, less is known concerning the anti-angiogenic and anti-tumor effects of $\alpha 2(IV)$ NC1 domain (canstatin). To this end, our previous studies suggested that $\alpha 2(IV)$ NC1 domain could interact with integrins $\alpha 3\beta 1$, $\alpha \nu\beta 3$, and $\alpha \nu\beta 5$ on the surface of endothelial cells and potentially inhibit growth factor-induced angiogenesis and tumor growth in the chick embryo.¹⁴

To expand these studies, we examined whether recombinant $\alpha 2(IV)$ NC1 domain could specifically interact with tumor cells and furthermore, whether it could directly effect tumor cell behavior. Here, we provide evidence that human tumor cells such as M21 melanoma and SKOV-3 ovarian carcinoma cells can directly interact with $\alpha 2(IV)$ NC1 domain in an integrin-specific manner that is distinct from that of endothelial cells. In addition, our results suggest for the first time that recombinant $\alpha 2(IV)$ NC1 domain can specifically inhibit tumor cell adhesion to collagen type-IV and induce tumor cell senescence in a ligand-dependent manner. Finally, we demonstrate that $\alpha 2(IV)$ NC1 domain can potentially inhibit tumor angiogenesis and tumor growth in multiple models. Taken together, our findings suggest that $\alpha 2(IV)$ NC1 domain is not only an anti-angiogenic molecule but can impact both the endothelial and tumor cell compartments and that its administration may represent an important therapeutic approach for the treatment of human malignancies.

Materials and Methods

Chemicals, Reagents, and Antibodies

Purified ECM proteins including collagen (pepsin solubilized) types-I and -IV lacking 7S and NC1 domains, fibronectin, and vitronectin were obtained from Sigma (St. Louis, MO). Bovine serum albumin (BSA), ethanol, methanol, and acetone were all obtained from Sigma. OTC embedding compound was obtained from VWR International (Bridgeport, NJ). Monoclonal antibodies (mAbs) LM609 (anti- $\alpha \nu\beta 3$), P4C10 (anti- $\beta 1$), and P1F6 (anti- $\alpha \nu\beta 5$) were kindly provided by Dr. David Cheresh, Scripps Research Institute (La Jolla, CA). mAbs directed to $\alpha 1$, $\alpha 2$, and $\alpha 3$ integrin subunits were obtained from Chemicon (Temecula, CA). Fluorescein isothiocyanate- and rhodamine-labeled goat anti-mouse secondary antibodies were obtained from BioGenex Inc. (San Ramon, CA). Polyclonal antibody AB769 directed to CD-31 was obtained from BD Pharmingen (San Diego, CA). X-Gal was obtained from Promega (Madison, WI). ApoptTag apoptosis detection kit was obtained from Roche (Indianapolis, IN). Recombinant $\alpha 2(IV)$ NC1 domain was kindly provided by BioStratum Inc. (Durham, NC). Recombinant $\alpha 5(IV)$ NC1 domain was kindly provided by Dr. Billy Hudson (Vanderbilt, TN). The control peptide used was a commercially synthesized 25-mer based on the sequence from human $\alpha 2(IV)$ chain of collagen type-IV. The specific amino acid sequence of the control peptide is NH₂-CKGIDMPGTPGLKGDRGSPGMDGFC-COOH.

Cells and Cell Culture

Human M21 melanoma cell line was kindly provided by Dr. David Cheresh (Scripps Research Institute). Human SKOV-3 ovarian carcinoma cells were obtained from American Type Culture Collection (Rockville, MD). Cell lines were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 1.0% sodium pyruvate, glutamate, and Pen-Strep (Life Technologies, Inc.). Cells were maintained as subconfluent cultures before use and harvested with trypsin-ethylenediaminetetraacetic acid (Life Technologies, Inc.).

Cell Adhesion Assay

Cell adhesion assays were performed essentially as described.²⁰ Briefly, nontissue culture 48-well plates were coated with either recombinant $\alpha 2(IV)$ NC1 domain (100 μ g/ml) or purified ECM proteins including collagen-I and -IV (10 μ g/ml) fibronectin (10 μ g/ml), or vitronectin (5 μ g/ml) for 18 hours at 4°C. Subconfluent cells (M21 and SKOV-3) were harvested, washed, and resuspended in adhesion buffer (RPMI 1640 medium containing 1.0 mmol/L MgCl₂, 0.2 mmol/L MnCl₂, and 0.5% BSA) in the presence or absence of mAbs (75 μ g/ml) or recombinant $\alpha 2(IV)$ NC1 and $\alpha 5(IV)$ NC1 domains (100 μ g/ml). Cells were added to the coated wells in a total volume of 200 μ l and allowed to attach for 30 to 60 minutes at 37°C.

Nonattached cells were removed by washing and attached cells were stained with crystal violet.²⁰ Wells were washed and cell-associated crystal violet was eluted with 100 μ l of 10% acetic acid. Cell adhesion was quantified by measuring the optical density of the eluted crystal violet at a wavelength of 600 nm.²⁰

Chick Embryo Tumor Growth Assay

The chick embryo tumor growth assays were performed essentially as described.²¹ Briefly, 10-day-old chick embryos were prepared by separating the chorioallantoic membrane (CAM) from the shell membrane. Single cell suspensions from subconfluent cultures of SKOV-3 cells (5.0×10^6 /embryo) were added to the CAM in a total volume of 40 μ l. Twenty-four hours later, the embryos were either untreated or injected intravenously with a single dose of recombinant α 2(IV)NC1 or α 5(IV)NC1 domains (30 μ g/embryo) in a total volume of 100 μ l per embryo. The embryos were allowed to grow for a total of 7 days at which time they were harvested and the tumors were resected, trimmed free of surrounding CAM tissue, washed, and wet weights determined. Experiments were performed twice with 5 to 10 embryos per condition.

Nude Mouse Tumor Growth Assay

Subconfluent M21 melanoma cells were harvested, washed, and resuspended in sterile phosphate-buffered saline (PBS). Tumor cells (2.0×10^6) were injected subcutaneously into nude mice.²² Three days after tumor cell injections, when small palpable tumors were detected, mice were either untreated or injected (intraperitoneally) with recombinant α 2(IV)NC1 domain or control normal mouse IgG at various concentrations. The mice were treated daily for a total of 21 days. To assess the effects of α 2(IV)NC1 domain on pre-existing tumors, tumors were allowed to grow to ~ 80 mm³ (day 6) before treatment was initiated. Tumor size was monitored with calipers and tumor volumes were estimated using the formula $V = L^2 \times W/2$, where V = volume, L = length, and W = width. Experiments were completed two to three times with similar results.

Human/Mouse Chimeric Model

The human/mouse chimeric tumor growth model was performed essentially as described with some modifications.²³ Briefly, fresh human neonatal foreskins (Cooperative Human Tissue Network, Cleveland, OH) were surgically transplanted on nude mice.²³ Human M21 melanoma cells were injected within the human skin graft.²³ Tumors were allowed to grow within the human skin for 3 days until small palpable tumors were detected. Three days after intradermal injection of tumor cells, mice were treated with daily intraperitoneal injections of α 2(IV)NC1 domain or control for a total of 26 days. Tumor size was monitored with calipers and tumor volumes were estimated using the formula $V = L^2 \times W/2$, where V =

volume, L = length, and W = width. Experiments were completed two times with similar results.

Immunohistochemical and Immunofluorescence Analysis

Tumors derived from control and α 2(IV)NC1 domain-treated mice were dissected, washed, embedded in OTC, snap-frozen, and 4.0- μ m sections were cut with a cryostat.²² To quantify tumor-associated angiogenesis, sections were incubated with 2.5% BSA in PBS to block any nonspecific binding. Endogenous peroxidase activity was quenched by incubation with hydrogen peroxide (0.03%). Tissues were next incubated with anti-CD-31 antibody at a concentration of 10 μ g/ml for 2 hours at 37°C, washed with PBS, and incubated with peroxidase-labeled goat anti-rabbit secondary antibody. Quantification of CD-31-positive blood vessels was performed essentially as described²⁴ with some modifications. The number of CD-31-positive blood vessels per microscopic field was counted in hot spots (fields containing at least five vessels). Ten fields were counted for each tumor section with three tumors per condition.

To evaluate the relative levels of cellular apoptosis, we used the ApopTag apoptosis detection kit. Briefly, tumors from control and α 2(IV)NC1 domain-treated mice were dissected, washed, embedded in OTC, snap-frozen, and 4.0- μ m sections were cut with a cryostat.²² To quantify cellular apoptosis, sections were incubated with 2.5% BSA in PBS to block any nonspecific binding. Tumor sections were stained with ApopTag apoptosis detection reagents according to the manufacturer's instructions. The relative levels of apoptosis were estimated by laser-scanning image analysis.²⁵ Briefly, stained tissue sections were scanned using a Kodak ID imaging system (Eastman-Kodak, Rochester, NY) and the pixel density of positive staining regions ($\times 200$ microscopic fields) $n = 3$ per tumor specimen was quantified using Kodak image analysis software as has been described previously.²⁵ In further experiments, co-staining was performed to determine whether CD-31-positive blood vessels were undergoing apoptosis. Briefly, tumors from control and α 2(IV)NC1 domain-treated mice were dissected, washed, embedded in OTC, snap-frozen, and 4.0- μ m sections were cut with a cryostat. The tissues were next co-incubated with both ApopTag reagents and anti-CD-31 antibody as we have previously described.²⁶ Sections were washed and incubated with rhodamine-conjugated goat anti-rabbit secondary as described above. Tumor sections were analyzed and photographed with a BX20 Olympus compound microscope fitted with epifluorescences (New Jersey Scientific) with an attached charge-coupled device digital camera.

Analysis of Cellular Senescence

To examine the levels of cellular senescence within tumors, we assessed the relative levels of senescence-associated β -galactosidase (SA- β -Gal) as previously described.^{27,28} Briefly, tumors from control and α 2(IV)NC1

domain-treated mice were dissected, washed, embedded in OTC, snap-frozen, and 4.0- μ m sections were cut with a cryostat. To quantitate cellular senescence, sections were first fixed in 3% formaldehyde for 2 to 3 minutes then washed with PBS four times for 5 minutes each. The tissue sections were stained with SA- β -Gal staining solution containing 1.0 mg/ml X-Gal in 20 mmol/L sodium phosphate, 20 mmol/L citric acid, 5 mmol/L potassium ferricyanide, 2 mmol/L magnesium hexacyano, and 150 mmol/L sodium chloride at pH 6.0.^{27,28} The tissues were incubated for 24 to 48 hours at 37°C, washed three times in H₂O, and mounted with Permount (Fisher, Pittsburgh, PA). Tissue sections were analyzed and photographed with a BX20 Olympus compound microscope fitted with a charge-coupled device digital camera. Quantification of the relative levels of senescence was performed by scoring (+, ++, +++) the tissue sections for cellular expression of SA- β -Gal. In particular, five $\times 200$ microscopic fields were evaluated for each of three distinct tumors per condition. Tissues with an average of less than two positive cells per field were given a score of +, two to five positive cells per field ++, and greater than five positive cells per field +++.

To assess the relative levels of senescence in cells grown *in vitro*, a similar staining procedure was applied. Briefly, 48-well nontissue culture plates were coated with either collagen type-IV or other ECM components at a concentration of 10 μ g/ml. Subconfluent cells were harvested, washed, and resuspended in 1% serum containing medium and plated in the presence or absence of $\alpha 2(\text{IV})\text{NC1}$ domain or controls. The cells were allowed to incubate for 12 to 24 hours at 37°C. The cells were next carefully washed two times with PBS and fixed with 2.0% formaldehyde and 0.2% glutaraldehyde for 2 minutes at room temperature. The cells were washed three times with PBS and stained with SA- β -Gal staining solution as described above for 12 to 24 hours.^{27,28} Tumor cells were analyzed and photographed with a BX20 Olympus compound microscope fitted with a charge-coupled device digital camera. *In vitro* tumor cell senescence was quantified by counting the number of enlarged granular cells staining positive for SA- β -Gal per $\times 200$ microscopic field. Three fields were evaluated for each well with three wells per condition.

Statistical Analysis

Statistical analysis was performed using the InStat statistical program for Macintosh computers. Data were analyzed for statistical significance using Student's *t*-test. *P* values <0.05 were considered significant.

Results

Immobilized Recombinant $\alpha 2(\text{IV})\text{NC1}$ Domain Supports Tumor Cell Adhesion *In Vitro*

ECM components such as collagen type-IV are known to support integrin-dependent tumor cell interactions. Our previous studies, as well as studies from other laborato-

Figure 1. Immobilized $\alpha 2(\text{IV})\text{NC1}$ domain supports tumor cell adhesion. Nontissue culture-treated 48-well plates were coated with either recombinant $\alpha 2(\text{IV})\text{NC1}$ domain or BSA (100 μ g/ml). Subconfluent tumor cells (M21 and SKOV-3) were harvested, washed, and resuspended in adhesion buffer and allowed to attach to the coated wells. Cell adhesion was quantified by measuring the optical density of eluted dye from adherent cells at a wavelength of 600 nm. Data bars represent the mean optical density $\pm$ SD from triplicate wells. Experiments were completed three times with similar results.

ries have demonstrated that genetically distinct NC domains can interact with endothelial cells and support cell adhesion *in vitro*.^{14,29} To expand these studies, we assessed the capacity of recombinant $\alpha 2(\text{IV})\text{NC1}$ domain to directly support tumor cell adhesion. Nontissue culture-treated plates were coated with either recombinant $\alpha 2(\text{IV})\text{NC1}$ domain or BSA as a control. Tumor cells (human M21 melanoma or SKOV-3 ovarian carcinoma) were allowed to adhere to the coated substrates. As shown in Figure 1, both human M21 melanoma and SKOV-3 ovarian carcinoma cells readily attached to immobilized $\alpha 2(\text{IV})\text{NC1}$ domain while exhibiting little interactions with BSA. These findings are in agreement with our previous studies with endothelial cells and suggest that recombinant $\alpha 2(\text{IV})\text{NC1}$ domain of collagen type-IV can specifically interact with these human melanoma and carcinoma cells.

Tumor Cell Interactions with Recombinant $\alpha 2(\text{IV})\text{NC1}$ Domain Depend on Integrin Receptors

Integrin receptors are considered the major family of cell surface receptors mediating cell-ECM interactions.¹³ Previous studies have suggested that integrin receptors expressed on endothelial cells can interact with distinct NC1 domains of collagen type-IV.^{14–19} In fact, our studies indicated that endothelial cells interact with immobilized $\alpha 2(\text{IV})\text{NC1}$ domain by multiple integrins, including $\alpha \nu \beta 3$, $\alpha \nu \beta 5$, and $\alpha 3 \beta 1$.¹⁴ To assess whether melanoma cells interact with $\alpha 2(\text{IV})\text{NC1}$ domain using similar sets of integrins, *in vitro* cell adhesion assays were performed in the presence or absence of function blocking anti-integrin antibodies. As shown in Figure 2A, a monoclonal antibody (mAb) specifically directed to the $\beta 1$ integrin

Figure 2. Tumor cell adhesion to immobilized $\alpha 2$ (IV)NC1 domain is integrin-dependent. Nontissue culture-treated 48-well plates were coated with recombinant $\alpha 2$ (IV)NC1 domain (**A** and **B**) or various purified ECM proteins (**C–G**). Subconfluent M21 melanoma and SKOV-3 carcinoma cells were resuspended in adhesion buffer in the presence or absence of specific antagonists and allowed to attach to the coated wells. Cell adhesion was quantified by measuring the optical density of eluted dye from adherent cells at a wavelength of 600 nm. Data bars represent the mean optical density $\pm$ SD from triplicate wells. **A** and **B**: Effects of anti-integrin mAbs (75 μ g/ml) on M21 melanoma cell attachment to immobilized $\alpha 2$ (IV)NC1 domain. **C–G**: Effects of soluble recombinant $\alpha 2$ (IV)NC1 domain (100 μ g/ml) on M21 (**C–F**) and SKOV-3 cell (**G**) attachment to immobilized ECM proteins. NT, no treatment; anti- $\beta 1$, mAb P4C10; anti- $\alpha \nu \beta 5$, mAb P1F6; anti- $\alpha \nu \beta 3$, mAb LM609; anti- $\alpha 1$, mAb 1973Z; anti- $\alpha 2$, mAb 1950Z; anti- $\alpha 3$, mAb 1952Z; $\alpha 5$ (IV)NC1, recombinant NC1 domain from the $\alpha 5$ chain of human collagen type-IV. Experiments were completed two to three times with similar results.

subunit inhibited M21 cell adhesion to $\alpha 2$ (IV)NC1 domain by $\sim 60\%$, whereas a function-blocking antibody directed to $\alpha \nu \beta 5$ had little if any effect. Interestingly, a mAb directed to integrin $\alpha \nu \beta 3$ blocked M21 cell adhesion to $\alpha 2$ (IV)NC1 domain by $\sim 40\%$, suggesting a role for both $\alpha \nu \beta 3$ and at least one $\beta 1$ -containing integrin. Importantly, although our previous studies suggested that endothelial cells can interact with $\alpha 2$ (IV)NC1 domain using $\alpha \nu \beta 3$, $\alpha \nu \beta 5$, and $\alpha 3\beta 1$, M21 melanoma cells do not use $\alpha \nu \beta 5$,

but rather $\alpha \nu \beta 3$ and at least one other $\beta 1$ -containing integrin.

To determine which $\beta 1$ integrin may contribute to M21 melanoma cell interactions with $\alpha 2$ (IV)NC1 domain, similar cell adhesion assays were performed in the presence or absence of function-blocking antibodies directed to distinct collagen-binding integrin α subunits. As shown in Figure 2B, a function-blocking antibody directed to the $\alpha 1$ subunit inhibited cell adhesion by $\sim 50\%$, whereas antibodies directed to the $\alpha 2$ and $\alpha 3$ integrin subunits exhibited little if any effect. Moreover, a combination of anti- $\alpha 1$ and anti- $\alpha \nu \beta 3$ mAbs inhibited M21 cell adhesion by $\sim 85\%$ as compared to controls (data not shown). These data suggest that M21 melanoma cells predominately use $\alpha 1\beta 1$ and $\alpha \nu \beta 3$ integrins to facilitate tumor cell adhesion to $\alpha 2$ (IV)NC1 domain. Given these results, it is possible that addition of soluble recombinant $\alpha 2$ (IV)NC1 domain may inhibit tumor cell adhesion to specific ECM components. To test this possibility, we allowed M21 melanoma cells to attach to purified ECM proteins in the presence or absence of soluble $\alpha 2$ (IV)NC1 domain. As shown in Figure 2C, soluble $\alpha 2$ (IV)NC1 domain (100 μ g/ml) inhibited M21 cell adhesion to collagen type-IV by $\sim 50\%$ as compared to control $\alpha 5$ (IV)NC1 domain. In contrast, $\alpha 2$ (IV)NC1 domain failed to inhibit M21 cell adhesion to other ECM proteins including collagen type-I, fibronectin, and vitronectin (Figure 2; D to F). Importantly, soluble $\alpha 2$ (IV)NC1 domain did not detach cells that had already bound to these ECM ligands (data not shown). To confirm the anti-adhesive activity of recombinant $\alpha 2$ (IV)NC1 domain in a second independent tumor cell type, we examined human SKOV-3 ovarian carcinoma cells. As shown in Figure 2G, $\alpha 2$ (IV)NC1 domain inhibited SKOV-3 cell adhesion to collagen type-IV by $\sim 50\%$ as compared to controls. Consistent with our studies on M21 melanoma cells, SKOV-3 carcinoma cells also primarily use $\alpha 1\beta 1$ and $\alpha \nu \beta 3$ to interact with immobilized $\alpha 2$ (IV)NC1 domain (data not shown). These results indicate that the inhibitory activity of $\alpha 2$ (IV)NC1 domain may be associated with an ECM- and/or integrin-specific mechanism.

Recombinant $\alpha 2$ (IV)NC1 Domain Inhibits SKOV-3 Tumor Growth in Vivo

Tumor cell interactions with the ECM have been shown to play a critical role in regulating tumor growth and progression.^{30–34} Therefore we examined the effects of recombinant $\alpha 2$ (IV)NC1 domain on SKOV-3 tumor growth *in vivo* using the chick embryo tumor growth model. SKOV-3 carcinoma cells were implanted on the CAMs of 10-day-old chick embryos. Twenty-four hours later the embryos were treated with a single intravenous injection of $\alpha 2$ (IV)NC1 domain or control $\alpha 5$ (IV)NC1 domain. At the end of a 7-day incubation period, the resulting tumors were removed and wet weights determined. As shown in Figure 3, a single injection of recombinant $\alpha 2$ (IV)NC1 domain significantly ($P = 0.035$) inhibited SKOV-3 ovarian tumor growth by $\sim 50\%$ as compared to either no treatment or control. Taken together, these findings suggest that the anti-adhesive and anti-tumor effects of

Figure 3. Recombinant $\alpha 2(IV)NC1$ domain inhibits SKOV-3 carcinoma tumor growth *in vivo*. Subconfluent SKOV-3 carcinoma cells were implanted on the CAMs of 10-day-old chick embryos. Twenty-four hours later the embryos were treated with a single intravenous injection of $\alpha 2(IV)NC1$ or $\alpha 5(IV)NC1$ domains (30 μg /embryo). At the end of a 7-day incubation period the resulting tumors were removed and wet weights determined. Data bars represent the mean tumor weights $\pm$ SE from 8 to 10 embryos per condition. Experiments were completed two times with similar results.

$\alpha 2(IV)NC1$ domain are not restricted to M21 melanoma and may impact a variety of tumors from distinct histological origins.

Recombinant $\alpha 2(IV)NC1$ Domain Inhibits Tumor Growth in Murine Models

Our studies as well as work from other laboratories have provided evidence that specific NC1 domains from collagen type-IV can inhibit growth factor- and tumor-induced angiogenesis *in vivo*.^{14–19} Moreover, studies have indicated that the growth of malignant tumors depends on angiogenesis. In this regard, we expanded our previous studies to examine the effects of recombinant $\alpha 2(IV)NC1$ domain on tumor growth in murine models. To determine whether recombinant $\alpha 2(IV)NC1$ domain exhibited a dose-dependent inhibition of tumor growth, human M21 melanoma cells were injected subcutaneously in nude mice. After development of palpable tumors (3 days), mice were treated daily (intraperitoneally) with $\alpha 2(IV)NC1$ domain at various concentrations. As shown in Figure 4A, administration of recombinant $\alpha 2(IV)NC1$ domain resulted in a dose-dependent inhibition of tumor growth with maximal inhibition of $\sim 70\%$ ($P < 0.05$) observed at a dose of 2.5 mg/kg. Administration of control protein at doses up to 5.0 mg/kg had little if any effect. In similar experiments, M21 tumors were allowed to grow until they reached a mean tumor size of ~ 80 mm³ (6 days) at which time, mice were treated daily with $\alpha 2(IV)NC1$ domain at a concentration of 2.5 mg/kg. As shown in Figure 4B, $\alpha 2(IV)NC1$ domain significantly ($P < 0.05$) inhibited the growth of established M21 melanoma tumors by $\sim 60\%$ as compared to controls.

Figure 4. Recombinant $\alpha 2(IV)NC1$ domain inhibits tumor growth *in vivo*. The effects of recombinant $\alpha 2(IV)NC1$ domain on the growth of human M21 melanomas were examined in murine xenograft models. **A:** Tumor-bearing mice were treated (intraperitoneally) daily with increasing concentrations of $\alpha 2(IV)NC1$ domain or control (normal mouse IgG). **B:** Tumors (M21) were allowed to grow to a mean size of ~ 80 mm³ before daily treatments (intraperitoneally) with $\alpha 2(IV)NC1$ domain or control (2.5 mg/kg). Data bars represent the mean tumor volumes $\pm$ SE from five to six mice per condition. **C:** Human M21 melanoma cells were injected into full-thickness human skin grafts transplanted on the backs of nude mice. After establishment of tumors (3 days), mice were treated (intraperitoneally) daily with $\alpha 2(IV)NC1$ domain (2.5 mg/kg) or vehicle control (PBS). Data bars represent the mean tumor volumes $\pm$ SE from five to six mice per condition.

A common limitation of many murine xenograft models involves the growth of malignant tumors in tissue microenvironments not typically observed in the human disease. To confirm the effects of $\alpha 2(IV)NC1$ domain on melanoma tumor growth, we used the human/mouse chimeric model in which human M21 melanoma cells were allowed to grow within full thickness human skin transplanted on nude mice. In agreement with our previous findings, daily administration of $\alpha 2(IV)NC1$ domain in mice harboring M21 tumors growing within full thickness human skin resulted in significant ($P < 0.05$) inhibition by $\sim 75\%$ (Figure 4C). These findings suggest that recombinant $\alpha 2(IV)NC1$ domain not only inhibits human M21 tumor growth within the subcutaneous compartment, but also blocks tumor growth within full thickness human skin where human cutaneous melanomas typically arise.

Recombinant $\alpha 2(IV)NC1$ Domain Inhibits Tumor-Associated Angiogenesis *in Vivo*

Previously published reports have indicated that $\alpha 2(IV)NC1$ domain can inhibit angiogenesis stimulated by purified growth factors and certain tumors.^{14,35–37} To assess the effects of recombinant $\alpha 2(IV)NC1$ domain on tumor-associated angiogenesis in our models, we examined M21 tumors from either control or $\alpha 2(IV)NC1$ domain-treated mice. Frozen sections from these tumors were stained for the presence of CD-31, a known marker

Figure 5. Recombinant $\alpha 2$ (IV)NC1 domain inhibits tumor-associated angiogenesis *in vivo*. Tumors (M21) from control and $\alpha 2$ (IV)NC1-treated mice were snap-frozen and 4.0- μ m tissue sections prepared. Tumor blood vessels were examined by immunohistochemistry using anti-CD-31 antibody. CD-31-positive vessel counts were performed on 10 microscopic fields from three tumors for each experimental condition. Data bars represent the mean CD-31 vessel counts $\pm$ SE from the three tumors per experimental condition. Original magnifications, $\times 200$.

of blood vessels. Tumors from mice treated with $\alpha 2$ (IV)NC1 domain exhibited a marked reduction in tumor-associated blood vessels from areas near the margin of the tumors. To quantify the apparent change in microvascular density, tumor-associated angiogenesis was estimated by counting the number of blood vessels from 10 microscopic fields from each of three distinct tumors per experimental condition.²⁴ As shown in Figure 5, tumor blood vessels from mice treated with $\alpha 2$ (IV)NC1 domain were significantly ($P = 0.001$) reduced by $\sim 45\%$ as compared to controls, confirming the anti-angiogenic activity of $\alpha 2$ (IV)NC1 domain. These findings suggest that the inhibition of tumor growth observed may be associated at least in part with inhibition of tumor angiogenesis.

Elevated Levels of Apoptosis in M21 Tumors from Mice Treated with Recombinant $\alpha 2$ (IV)NC1 Domain

Previous studies have suggested that $\alpha 2$ (IV)NC1 domain can inhibit endothelial cell proliferation and induce endothelial but not tumor cell apoptosis *in vitro*.^{35–37} To examine potential mechanisms by which $\alpha 2$ (IV)NC1 domain may inhibit tumor growth, we examined M21 tumors from control and $\alpha 2$ (IV)NC1 domain-treated mice for the relative levels of apoptosis by terminal dUTP nick-end labeling (TUNEL) staining. Frozen sections of tumors from control and $\alpha 2$ (IV)NC1 domain-treated mice were analyzed using the ApopTag apoptosis detection reagent. In control-treated tumors, random apoptotic staining was detected in numerous scattered cells staining TUNEL-positive (Figure 6A). In contrast, elevated levels of apoptosis were detected localized in discrete foci (Figure 6A, middle and right) throughout tumors treated with

$\alpha 2$ (IV)NC1 domain (Figure 6A). In fact, tumors from mice treated with $\alpha 2$ (IV)NC1 domain (2.5 mg/kg) were associated with a significant ($P = 0.043$) increase in apoptosis by approximately fourfold as compared to controls (Figure 6B). Importantly, $\alpha 2$ (IV)NC1 domain failed to induce apoptosis in tumor cells *in vitro* (data not shown) suggesting that the apoptosis observed within the tumors may be because of indirect mechanisms. Given previous reports that $\alpha 2$ (IV)NC1 domain can induce apoptosis in endothelial cells but not in tumor cells, we examined whether angiogenic blood vessels within the tumor may be undergoing apoptosis. To this end, we co-stained tumor sections from control and $\alpha 2$ (IV)NC1 domain-treated mice with an antibody directed to CD-31 to mark blood vessels and with ApopTag reagent to detect apoptotic cells. As shown in Figure 6C (top left) CD-31-positive (red) tumor blood vessels within control-treated tumors could be readily detected. Moreover, little if any apoptosis (green) was observed in association with tumor blood vessels. In contrast, apoptosis (green) could be detected in association with (yellow co-localization) blood vessels from $\alpha 2$ (IV)NC1 domain-treated tumors (Figure 6C, top right). It is important to point out that although examples of apoptotic blood vessels could be detected (yellow), not all vessels were apoptotic. Interestingly, tumors from mice treated with $\alpha 2$ (IV)NC1 domain were also associated with elevated levels of apoptosis in cells other than CD-31-positive endothelial cells (Figure 6C, bottom). Taken together, these results suggest that $\alpha 2$ (IV)NC1 domain treatment may cause enhanced apoptosis within both nonendothelial cells and tumor-associated blood vessels *in vivo*.

Elevated Levels of Cellular Senescence in M21 Tumors from Mice Treated with Recombinant $\alpha 2$ (IV)NC1 Domain

Integrin-mediated cellular interactions with the ECM are known to regulate a variety of processes involved in tumor growth, including cell adhesion, migration, apoptosis, proliferation, and cell cycle control. Interestingly, studies have suggested that chemotherapeutic agents and radiation therapy may modulate cellular senescence.^{38–41} In fact, cellular senescence has been observed in murine tumors and biopsies from human patients after chemotherapy.^{38–41} In this regard, we assessed whether $\alpha 2$ (IV)NC1 domain could alter the levels of cellular senescence in tumors *in vivo*. To examine the relative levels of cellular senescence, we assessed the levels of SA- β -Gal, a well-known marker for cellular senescence.^{27,28} Frozen sections of tumors from control and $\alpha 2$ (IV)NC1 domain-treated mice were stained for SA- β -Gal using X-Gal as previously described.²⁷ As shown in Figure 7A, top, little if any cellular senescence was detected in tumors from control-treated mice. In contrast, tumors from mice treated with increasing doses of $\alpha 2$ (IV)NC1 domain exhibited elevated levels of SA- β -Gal-positive staining senescent cells scattered throughout the tumors (Figure 7A, middle and bottom). Importantly, tumors ($n = 3$) from mice treated with $\alpha 2$ (IV)NC1

Figure 6. Tumors from mice treated with recombinant $\alpha 2(\text{IV})\text{NC1}$ domain exhibit enhanced apoptosis. Tumors (M21) from control and $\alpha 2(\text{IV})\text{NC1}$ domain-treated mice were snap-frozen and 4.0- μm tissue sections prepared. Tumor-associated apoptosis was detected by TUNEL staining and tumor blood vessels were examined by immunohistochemistry using anti-CD-31 antibody. **A:** Representative examples of tumors exhibiting apoptosis within control and $\alpha 2(\text{IV})\text{NC1}$ domain-treated M21 melanoma tumors after TUNEL staining (brown). **B:** Quantification of the relative levels of apoptosis within tumors from control and $\alpha 2(\text{IV})\text{NC1}$ domain-treated mice. Data bars represent the mean apoptotic staining $\pm$ SE from three distinct tumor specimens. NT, no treatment; $\alpha 2(\text{IV})\text{NC1}$, recombinant $\alpha 2(\text{IV})\text{NC1}$ domain treated (2.5 mg/kg); control, control peptide treated (2.5 mg/kg). **C:** Co-localization (yellow) of apoptosis (green) and CD-31-positive (red) tumor blood vessels within control and $\alpha 2(\text{IV})\text{NC1}$ domain-treated M21 tumors. **Top:** Representative examples of CD-31-positive blood vessels. **Bottom:** Representative examples of apoptosis in non-CD-31-expressing cells. Original magnifications: $\times 200$ (**A**); $\times 630$ (**C**).

domain exhibited enhanced senescence staining (score +++) as compared to either tumors from untreated or control-treated mice (score +). Because inhibition of angiogenesis may result in reduction in growth factors, oxygen, and other nutrients to the tumor, it is possible that the induction of cellular senescence may be an indirect result of $\alpha 2(\text{IV})\text{NC1}$ domain-inhibiting angiogenesis. Alternatively, $\alpha 2(\text{IV})\text{NC1}$ domain may directly induce senescence in tumor cells.

To determine whether $\alpha 2(\text{IV})\text{NC1}$ domain can directly induce senescence in tumor cells, *in vitro* assays were performed with M21 melanoma cells cultured on a collagen type-IV substrate. M21 cells in the presence or absence of $\alpha 2(\text{IV})\text{NC1}$ domain or control peptide were seeded in microtiter wells coated with collagen type-IV. The cells were allowed to incubate for 24 hours and then stained for the presence of SA- β -Gal. As shown in Figure

7B (top), scattered SA- β -Gal-positive staining senescent cells were detected in untreated cultures growing on collagen type-IV. Interestingly, M21 cells treated with exogenous $\alpha 2(\text{IV})\text{NC1}$ domain exhibited an enhancement of SA- β -Gal staining senescent cells after a 24-hour incubation period (Figure 7B, middle). In contrast, cells cultured under identical conditions treated with a control collagen peptide showed only minimal senescence staining (Figure 7B, bottom). In fact, $\alpha 2(\text{IV})\text{NC1}$ domain caused a significant ($P = 0.038$) increase in tumor cell senescence by approximately threefold as compared to control (Figure 7C). Importantly, recombinant $\alpha 2(\text{IV})\text{NC1}$ domain failed to induce senescence in cells cultured on fibronectin (data not shown). These novel findings suggest for the first time, that recombinant $\alpha 2(\text{IV})\text{NC1}$ domain may induce M21 tumor cell senescence and that this induction of tumor cell senescence is substrate-dependent.

Discussion

An expanding body of evidence has been accumulating concerning the requirement of cellular interactions with ECM components such as collagen in the regulation of invasive cellular processes.^{2-5,7} In fact, modulation of collagen structure and integrity has been suggested to alter integrin-mediated cell cycle control.⁴²⁻⁴⁵ Moreover, genetic evidence from collagen- and integrin-deficient mice has implicated these molecules as important regulators in biological events including proliferation, invasion, tumor growth, and angiogenesis.^{13,44} Previous studies have demonstrated that not only is cellular interactions with triple helical collagen important, but integrin-mediated ligation of cryptic epitopes hidden within its triple helical structure may alter integrin signaling and play crucial roles in angiogenesis and tumor growth.^{20,22} Thus, it is critical that we obtain a more complete understanding of the role that the ECM plays in regulating both normal tissue homeostasis and pathological diseases.

Recent work has provided convincing evidence that $\alpha 2$ (IV)NC1 domain can inhibit endothelial cell migration and proliferation, and induce endothelial but not tumor cell apoptosis *in vitro*.^{14,35-37} Importantly, our studies confirm these findings because recombinant $\alpha 2$ (IV)NC1 domain failed to induce tumor cell apoptosis *in vitro* yet did induce apoptosis *in vivo*. Studies have also suggested that specific NC domains can down-regulate FLIP, inhibit the phosphorylation of Akt and Fak, and disrupt mTOR function, which may play a role in their capacity to induce apoptosis in endothelial cells.^{14-19,35-37} However, little is known concerning the potential effect of $\alpha 2$ (IV)NC1 domain on tumor cell behavior. Here, we demonstrate that recombinant $\alpha 2$ (IV)NC1 domain can mediate tumor cell adhesion in an integrin-dependent manner using $\alpha \nu \beta 3$ and $\alpha 1 \beta 1$.

To further expand our previous work, we examined the effects of recombinant $\alpha 2$ (IV)NC1 domain on the growth of melanoma tumors in murine tumor models. Recombinant $\alpha 2$ (IV)NC1 domain significantly inhibited human M21 melanoma tumor growth in a dose-dependent manner with a maximal inhibition (70%) observed at ~2.5 mg/kg. However, a common limitation associated with xenograft models is that they often do not mimic the tissue microenvironment in which the tumors are typically found in humans. In this regard, cutaneous human melanoma is thought to arise in part, from a series of genetic mutations within melanocytes present in the basal layers of the epidermis.⁴⁶ Our findings suggest that daily (intra-peritoneal) administration of recombinant $\alpha 2$ (IV)NC1 do-

Figure 7. Recombinant $\alpha 2$ (IV)NC1 domain enhances tumor cell senescence. Tumors (M21) from control and $\alpha 2$ (IV)NC1 domain-treated mice were snap-frozen and 4.0- μ m tissue sections prepared. Tumor senescence was assessed by monitoring the expression of SA- β -Gal. **A:** Representative examples of SA- β -Gal expression (blue) within control and $\alpha 2$ (IV)NC1 domain-treated M21 melanoma tumors. **B:** Representative examples of SA- β -Gal expression (blue) within control and $\alpha 2$ (IV)NC1 domain (250 μ g/ml)-treated M21 cells cultured on collagen type-IV. **C:** Quantification of the relative numbers of senescence cells within control and $\alpha 2$ (IV)NC1 domain-treated M21 melanoma cells cultured on collagen type-IV. Data bars represent the mean cell counts per $\times 200$ microscopic fields $\pm$ SE from triplicate wells. Experiments were completed three times with similar results. Original magnifications: $\times 200$ (A); $\times 150$ (B).

main potently inhibited human M21 melanoma tumor growth within full thickness human skin transplanted on nude mice. These findings confirm our previous results and suggest for the first time that $\alpha 2(\text{IV})\text{NC1}$ domain has the capacity to inhibit human melanoma tumor growth within a human tissue microenvironment.

Although data from our laboratory and previously published studies from other investigators, suggest that recombinant $\alpha 2(\text{IV})\text{NC1}$ domain can inhibit angiogenesis and tumor growth, the molecular mechanisms by which it functions is still not completely understood. Recent work from a variety of laboratories has provided compelling evidence that the inhibitory response in tumors to therapeutic intervention (chemotherapy and radiation) may be associated in part with the induction of cellular senescence.^{38–47,48} Senescence can be thought of as a physiological or cellular program of growth arrest. Cellular senescence can be triggered by a number of events including telomere shortening, DNA damage, and by other cellular stresses induced by chemotherapeutic reagents and radiation.^{38–40,46} Typical characteristics of senescent cells include an enlarged and flattened morphology with an increase in granularity. In addition, important biochemical changes that have been linked with cellular senescence include, increased expression of p21^{Waf1/CIP-1}, and p16^{ink4a}, an enhanced nuclear accumulation of actin and the expression of SA- β -Gal.^{38–47,48} In fact, expression of SA- β -Gal is used extensively as a reliable indicator of cellular senescence *in vitro* and *in vivo*.^{38–47,48} Importantly, although senescent cells do not grow, they remain metabolically active and have the potential to produce inhibitors and/or stimulators of angiogenesis and tumor growth.^{36–38,44} Thus, the net therapeutic benefit from the induction of cellular senescence may depend on the overall balance between expression of any inducers or inhibitors. Recent studies have suggested that certain tumors from murine models and patients treated with chemotherapy exhibit enhanced levels of cellular senescence.^{36,38,44} Consistent with these observations, our studies indicate a specific increase in the number of enlarged flattened tumor cells expressing SA- β -Gal after treatment with $\alpha 2(\text{IV})\text{NC1}$ domain *in vitro*. Importantly, this increase in relative levels of SA- β -Gal-expressing senescent cells was not observed when cells were cultured on fibronectin, consistent with an integrin- and/or ECM-specific response. In this regard, it is interesting to note that changes in integrin-mediated tumor cell interactions with collagen have been reported to alter cell cycle progression.^{41,42} Moreover, alteration in Ras/MAP kinase-signaling pathways has also been shown to modulate cellular senescence.⁴⁸ Thus, it would be interesting to speculate that the induction of tumor cell senescence observed after $\alpha 2(\text{IV})\text{NC1}$ domain treatment may be associated in part with the alterations in integrin-mediated tumor cell interactions with collagen. Given this possibility, it would also be interesting to speculate that specific tumors with distinct integrin profiles such as those that express $\alpha \nu \beta 3$ and $\alpha 1 \beta 1$ might be more responsive to recombinant $\alpha 2(\text{IV})\text{NC1}$ domain than those tumors lacking these integrins. Studies are currently underway to examine this intriguing possibility.

Taken together, our findings suggest that although $\alpha 2(\text{IV})\text{NC1}$ domain likely impacts tumor growth via an anti-angiogenic mechanisms involving induction of endothelial cell apoptosis, it may also directly affect tumor cell adhesion and senescence via an integrin- and/or ECM-specific mechanism. The dual effects of recombinant $\alpha 2(\text{IV})\text{NC1}$ domain on both angiogenesis and tumor cells have important clinical implications. For example, recent studies have provided evidence that anti-angiogenic drugs such as vascular endothelial growth factor antagonists, in conjunction with conventional chemotherapy can enhance clinical tumor response in human trials.⁴⁹ Moreover, other studies suggest that induction of cellular senescence in tumors may enhance tumor sensitivity to conventional chemotherapy as well as reverse drug resistance in certain tumors.^{38–40} Our findings that $\alpha 2(\text{IV})\text{NC1}$ domain can inhibit tumor cell adhesion and induce tumor cell senescence, in conjunction with its previously reported anti-angiogenic activity, provide support for the notion that the use of $\alpha 2(\text{IV})\text{NC1}$ domain alone or in combination with standard chemotherapy and/or radiotherapy may represent an important new approach for the treatment of malignant human tumors.

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