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Neutrophil-Activating Protein-2- and Interleukin-8-Mediated Angiogenesis

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Abstract In this study, we investigated the anti-angiogenic potential of nitric oxide (NO) donors and anti-integrin compounds against neutrophil-activating protein-2 (NAP-2), interleukin-8 (IL-8), and basic fibroblast growth factor (FGF-2)-induced angiogenesis. In vivo, recombinant human NAP-2 and FGF-2 induce a potent and comparable angiogenic response in the chick embryo chorioallantoic membrane (CAM). We demonstrate that NO donors and anti-integrin agents are capable of abrogating either NAP-2- or FGF-2-induced angiogenesis in the CAM model. The NO donor, S-nitroso N-acetyl penicillamine (SNAP), blocked either NAP-2- or FGF-2-mediated angiogenesis in the CAM. Similarly, angiogenesis stimulated with NAP-2 or FGF-2 was blocked by antagonist of the αvβ3 integrin in the CAM model. However, the inhibition of NAP-2 and IL-8 by the anti-integrin compound is significantly less than the inhibition observed with FGF-2 as the angiogenic stimulus. Similarly, the ability of these mechanisms to also inhibit endothelial cell differentiation was demonstrated. Taken together, these data illustrate the involvement of multiple pathways in the mechanisms of action for the α-chemokine- and cytokine-mediated angiogenesis. These approaches may be a useful tool for the inhibition of angiogenesis associated with human tumor growth or with neovascular, ocular, and inflammatory diseases where chemokines and cytokines are involved. J. Cell. Biochem. 102: 412–420, 2007.

Key words: angiogenesis; cytokines; chemokines; NAP-2; IL-8; FGF-2; endothelial cells; nitric oxide; anti-integrin inhibitors

Angiogenesis is the development of new blood vessels from preexisting blood vessels. Physiologically, angiogenesis prepares the womb for egg implantation, ensures proper development of mature organisms, and plays a key role in wound healing. By contrast, it supports the pathological conditions associated with a number of disease states such as cancer, inflammation, and ocular diseases. Angiogenesis or neovascularization is a multistep process controlled by the balance of pro- and anti-angiogenic factors. The latter stages of this process involve proliferation and the organization of endothelial cells (ECs) into tube-like structures. Mitogenic cytokines such as basic fibroblast growth factor (FGF-2) and vascular endothelial growth factor (VEGF) are thought to be key players in promoting EC growth and differentiation.

The EC produces a number of factors that regulate vascular tone and growth. Nitric oxide (NO), the free radical gas, is one of the most important regulators and is formed as a byproduct of the conversion of L-arginine to L-citrulline [Ignarro et al., 1987; Palmer et al., 1987, 1988]. NO is synthesized by one of three enzyme isoforms of NO synthase (NOS). Endothelial NOS (eNOS) and neuronal NOS (nNOS) are constitutively expressed, but inducible NOS (iNOS) is expressed in response to various cellular stimuli. Hence, the iNOS isoform is thought to be the key regulator of NO formation in response to cytokine stimulation. In addition to the well-characterized vasodilatory and anti-platelet effects, NO also regulates the proliferation and migration of vascular smooth muscle cells (VSMCs) and fibroblasts

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[Garg and Hassid, 1989; Cui et al., 1994; Dubey et al., 1995]. Recently, it has been demonstrated that increased expression of iNOS and nitrite production induced by lipopolysaccharide (LPS) are correlated with the progression of angiogenesis [Pipili-Synetos et al., 2000].

The EC is the pivotal cellular component of the angiogenic process and responds to many cytokines through its cell-surface receptors and intracellular signaling mechanisms. ECs in culture are capable of forming tube-like structures with a lumen. Therefore, ECs are not only a prerequisite for neovascularization but are also the basal structural requirement. With the potent effects of NO as both a vasodilator and a regulator of endothelium permeability, the idea that NO plays a pivotal role in angiogenesis is understandable. However, the data generated thus far are conflicting and demonstrate both proangiogenic and anti-angiogenic effects [Powell et al., 2000; Jones et al., 2004; Isenberg et al., 2005; Milkiewicz et al., 2006; Zheng et al., 2006]. The arginine analog L-NAME enhances FGF-2-mediated angiogenesis but not VEGF-mediated angiogenesis in an in vivo model of angiogenesis [Norrby, 1998]. EC differentiation (tube formation) is altered in a dose-dependent manner by NO-modulating compounds [Ray-Chaudhury et al., 1996; Babaei et al., 1998].

The purpose of this study was to determine if alteration of NO levels plays an important role in the inhibition of α-chemokine- and cytokine-induced angiogenesis.

MATERIALS AND METHODS

Reagents

Human recombinant VEGF was obtained from Invitrogen (Carlsbad, CA). Human FGF-2 obtained from Invitrogen was used in the chorioallantoic membrane (CAM), and FGF-2 obtained from R&D Systems (Minneapolis, MN) was used in the mouse Matrigel plug model. XT199 was synthesized at DuPont Pharmaceuticals Inc. (Wilmington, DE). Chemokines, cytokines, NO donors, and other commonly used reagents were obtained from Sigma Co. (St. Louis, MO).

Nitric Oxide Measurements

Concentrations of NO metabolites were measured in the medium of HUVE cell after exposure to S-nitroso N-acetyl penicillamine (SNAP) at various concentrations for 72 h. Medium samples were processed and subjected to a fluorometric assay, as previously described [Powell et al., 2000].

Neovascularization on the Chick Chorioallantoic Membrane

In vivo neovascularization was examined by the method previously described by Auerbach et al. [1974]. Ten-day-old embryos were purchased from Spafas, Inc. (Preston, CT) and were incubated at 37°C with 55% relative humidity. A small hole was punctured with a hypodermic needle in the shell concealing the air sac. A second hole was punctured in the shell on the broad side of the egg, directly over an avascular portion of the embryonic membrane, as observed during candling. A false air sac was created beneath the second hole by the application of negative pressure to the first hole, which caused the CAM to separate from the shell. A window, approximately 1.0 cm^2, was cut in the shell over the dropped CAM with the use of a small crafts grinding wheel (Dremel, Division of Emerson Electric Company; Racine, WI), which allowed direct access to the underlying CAM. Filter disks of No. 1 filter paper (Whatman International; United Kingdom) were soaked in 3 mg/ml cortisone acetate (Sigma) in a solution of 95% ethanol and water and subsequently air-dried under sterile conditions. FGF-2 (Invitrogen) was used to grow vessels on the CAMs of 10-day-old chick embryos. Sterile filter disks adsorbed with FGF-2 dissolved in PBS at 1 μg/ml were placed on growing CAMs. At 24 h, test compounds or control vehicle were added directly to CAMs topically.

Microscopic Analysis of CAM Sections

CAM tissue directly beneath FGF-2-saturated filter disk was resected from embryos treated 48 h previously with compound or control. Tissues were washed three times with PBS. Sections were placed in a 35 mm Petri dish (Nalge Nunc; Rochester, NY) and were examined under a SV6 stereomicroscope (Karl Zeiss; Thornwood, NY) at 50× magnification. Digital images of CAM sections adjacent to filters were collected using a 3-CCD color video camera system (Toshiba America; New York, NY) and analyzed with the Image-Pro Plus software (Media Cybernetics; Silver Spring, MD). The number of vessel branch points contained in a circular region equal to the area of a filter disk was counted for each section.

Percent inhibition data are expressed as the quotient of the experimental value minus the negative control value divided by the difference between the positive control value and the negative control value.

Endothelial Cell Differentiation (Tube Formation)

Differentiation by ECs was examined using a method developed by Grant et al. [1991]. Matrigel growth factor reduced (GFR) (Becton Dickinson) was thawed overnight at 4°C. Using cold pipette tips, 250 ml of Matrigel GFR was placed in a cold 24-well multiwell plate (Corning). Matrigel GFR was allowed to polymerize during incubation at 37°C for 30 min. Cells were trypsinized, centrifuged, and subsequently washed twice in PBS. After counting, HUVE cells were plated at 40,000 cells/well in EBM containing FGF-2 50 ng/ml at an initial volume of 125 ml in a 24-well plate. After 1–2 h of incubation at 37°C with 5% CO_2 and 95% humidity to allow cell attachment, 125 ml samples containing varied concentrations of SNAP dissolved in EBM medium were added. Plates were incubated overnight at 37°C with 5% CO_2 and 95% humidity. Subsequently, the medium was decanted, and cells were fixed and stained using a modified Hema 3 Stain kit (Fisher; Swedesboro, NJ).

Microscopic Analysis of Endothelial Cell Differentiation

Digital images of microtiter well sections were collected using a DKC5000 3-CCD color video camera system (Sony Corporation, Inc.; Japan) and analyzed with the Image-Pro Plus software (Media Cybernetics). The area and major axis length of stained cells having a tubular morphology on the Matrigel surface were counted from five images/well. Area data are expressed in units of mm^2, and length data are expressed as length/area in units of mm/mm^2. Percent inhibition data are expressed as the quotient of the experimental value minus the negative control value (EBM medium) divided by the difference between the positive control value and the negative control value.

Mouse Matrigel Angiogenesis Model

Male C57B1/6 mice, 6–8 weeks old (Charles River Laboratories; Wilmington, MA) were housed 10 per cage in a room maintained at 20 ± 2°C with a humidity of 50 ± 10% and a 12 h light-dark cycle. The animals were fed a standard pelleted mouse chow (Agway Inc.; Waverly, NY). The murine Matrigel model was conducted according to previously described methods conducted in our laboratories [Powell et al., 2000]. Matrigel was thawed overnight at 4°C and placed on ice. Aliquots of Matrigel were placed into cold polypropylene tubes, and FGF-2 (R&D Systems) or NO donors were administered at 5 mg/kg in Alza minipump implanted subcutaneously within 1 h of Matrigel injection. Tubes were mixed by rotating end over end at 4°C. Matrigel with FGF-2, NO donor, or control Matrigel was subcutaneously injected into the ventral midline of the mice. At day 14, animals were sacrificed and the solidified gels were removed. Gels were then analyzed for presence of new vessels by the hemoglobin assay and microscopic staining [Powell et al., 2000].

Hemoglobin Assay

The removed gels were analyzed for neovascularization by a method previously described [Okada et al., 1995]. Control and experimental gel implants were placed in a micro centrifuge tube containing 0.5 ml of cell lysis solution (Sigma) and crushed with a pestle. Subsequently, the tubes were incubated overnight at 4°C and centrifuged at 1,500*g* for 15 min on the following day. A 200 μl aliquot of cell lysate was added to 1.3 ml of Drabkin's reagent solution (Sigma) for each sample. The solution was then analyzed on a spectrophotometer at a 540 nm wavelength. The absorption of light is proportional to amount of hemoglobin contained in the sample or to the degree of neovascularization.

RESULTS

Effects of SNAP on Endothelial Cell Nitric Oxide

Determination of NO concentrations by Greiss reaction revealed that serum-induced cultures contain NO metabolites at an almost 1:1 ratio (Table I). The concentration of nitrate and nitrite in proliferating cultures was slightly lower but not statistically significant than basal cultures. However, more nitrates were detectable in both conditions. The addition of the NO donor, SNAP, at 10 mM raised the concentration of the NO metabolites nitrite and nitrate by greater than 10- and 20-fold, respectively (Table I). But perhaps more significantly, the metabolite ratio, nitrate to nitrite, changed from nearly equimolar to greater than 3:1 (Table I).

TABLE I. Nitric Oxide Metabolite Concentrations in Cell Medium

	Nitrate + nitrite (μM)	Nitrite (μM)	Nitrate (μM)
EGM	29.8 ± 3.4	13.8 ± 3.6	16.0 ± 2.9
EGM + SNAP (1 mM)	677.1 ± 30.2	172.3 ± 2.7	504.9 ± 18.5
EBM	35.6 ± 4.2	14.2 ± 2.7	21.3 ± 3.4
EBM + SNAP (1 mM)	596.1 ± 29.3	156.5 ± 3.1	439.6 ± 22.5

Values of the combined nitrate + nitrite concentration and the nitrite concentration represent the mean ± SEM, N = 3. The nitrate value is derived from the difference of the means of the two measured values.

Inhibition by an NO Donor in an In Vitro Model of Angiogenesis

ECs undergo important morphological changes in the later phases of neovascularization that give rise to tubular structures with lumens. We further investigated the angiogenesis phenomena in an in vitro setting by examining EC differentiation on Matrigel matrices. Cultures of HUVE cells were plated at a subconfluent level on solidified Matrigel in 24-well plates and incubated in the presence of FGF-2 (50 ng/ml) with and without SNAP at various concentrations, ranging from 1 μM to 1 mM. HUVE cells cultured in this assay form tube-like structure on the surface of the matrix. By contrast, HUVE cells treated with 1 μM SNAP and higher failed to develop the level of tubes associated with control cultures. Quantitative measurements of tube formation revealed that SNAP inhibited the cytokine-stimulated tube formation by 116% (Fig. 1). The results demonstrate that the inhibitory effects of NO donors may lie in their ability to prevent this critical step in the angiogenic process.

Dose-Dependent Inhibition of Cytokine- and Chemokine-Induced Angiogenesis in the CAM Model

Our previous experiment demonstrated that donors of NO are capable of inhibiting angiogenesis [Powell et al., 2000]. To determine whether this effect of NO on FGF-2-induced angiogenesis is applicable to chemokines, we treated 10-day-old fertilized chick embryos with filters saturated with PBS, filters saturated with neutrophil-activating protein-2 (NAP-2) or FGF-2 (1 μg/ml), or filters saturated with cytokines (1 μg/ml) + SNAP (0.5, 5.0, and 50 μg). In the presence of NAP-2 and FGF-2 alone, adjacent CAM membranes undergo morphological changes and become vascularized. The angiogenic index of neovascularization in FGF-2 treated is approximately twofold greater than control CAMs. A dose-dependent relationship existed between the amount of SNAP and the level of inhibition observed (Fig. 2A,B), and the level of inhibition of FGF-2-induced angiogenesis observed (87%) at the highest concentration is significant (ANOVA; $P < 0.05$). SNAP proved to be an effective inhibitor of chemokine-induced angiogenesis stimulated with NAP-2 at the highest concentration as well. The relative extent of inhibition against FGF-2 by a 50 μg dose of SNAP was almost 20% higher than its effectiveness against NAP-2-mediated angiogenesis (68%). The concentration of NO metabolites in the CAM fluid was assayed by the Greiss reaction. The addition of 5 and 50 μg of SNAP to the fertilized chicken embryos was capable of raising the concentration of NO metabolites in the CAM fluid. This aqueous fluid, which is adjacent to the CAM membrane, had a nitrate concentration range of 270–370 nM and a nitrite concentration range of 72–101 nM (data not shown).

Inhibition of Angiogenesis Through Dosing of Donors of NO in the Mouse Matrigel Plug Model

We examined the level of cytokine-induced angiogenesis that developed in a Matrigel plug containing FGF-2 when exposed to donors of NO. As shown in Table II, FGF-2 containing Matrigel induces about a threefold increase in the amount of hemoglobin found in the excised Matrigel. Both NO donors, SNAP and SNAG, abrogated the neovascularization of the Matrigel significantly (Student's t-test, $P < 0.05$; n = 5). Treatment of the mice with 5 mg/kg of SNAP or SNAG following surgical implantation of a subcutaneous mini-pump resulted in an 85% decrease in the angiogenesis index (Table II).

Inhibition of Angiogenesis by Anti-integrin Inhibitors

We examined the level of cytokine- and chemokine-induced angiogenesis that devel-

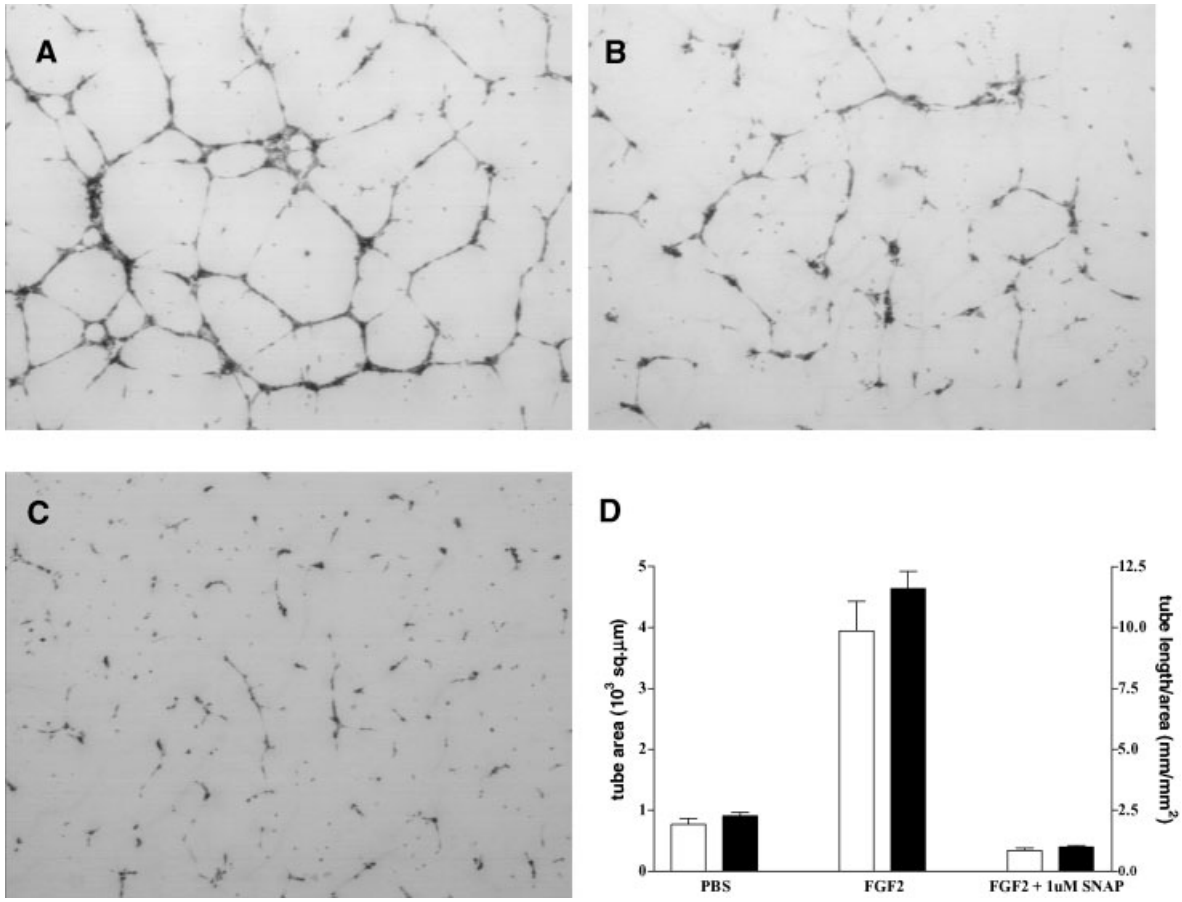

Fig. 1. NO donors inhibit differentiation of tube formation in HUVE cells induced by FGF-2 in vitro. Cells were plated at subconfluent levels on Matrigel matrices and incubated at 37°C in 5% CO_2 and 95% humidified atmosphere. After 16 h, cells were stained and photographed, and measurements were made by image analysis. Representative photographs of endothelial cells taken at 16 h after (**A**) FGF-2, (**B**) PBS, and (**C**) FGF-2 + 1 uM SNAP administration. **D**: Tube area and tube length/area of HUVE cells in different media. Each bar represents mean ± SEM, n = 4.

oped in the CAM model when exposed to anti-integrin inhibitors. To assess the effects of αvβ3 antagonist on chemokine-induced neovascularization, NAP-2, FGF-2, or interleukin-8 (IL-8) were applied to CAMs of 10-day-old chick embryos. After 24 h, embryos were treated topically with the αvβ3 antagonist XT199, and blood vessel growth was allowed to proceed for an additional 48 h. As shown in Figure 3, CAMs treated with PBS became vascularized in the area adjacent to the cytokine containing filter disc. In contrast, CAMs treated with 120 ng XT199 reduced the FGF-2-induced level of vascularization by 90%. However, XT199 was less effective in reducing chemokine-induced angiogenesis. NAP-2- and IL-8-mediated neovascularization was inhibited by 43% and 65%, respectively (Fig. 3). In both instances, the percent inhibition of chemokine-induced vascularization was statistically significant from that seen with FGF-2 ($P < 0.05$, Student's t-test; N = 5). When a combination of NAP-2 and IL-8 was applied to CAMs, no inhibition of blood vessel growth was observed after the addition of the αvβ3 antagonist XT199 at the same dose that inhibited either NAP-2 or IL-8 alone (data not shown). This perhaps might be due to an additional mechanism(s) that may be involved in α-chemokine–mediated angiogenesis, or higher doses of XT199 might be required to down-regulate the greater level of angiogenesis stimulation with the combination of NAP-2 and IL-8.

DISCUSSION

Previous studies have investigated the effect of NO-modulating compounds on angiogenesis

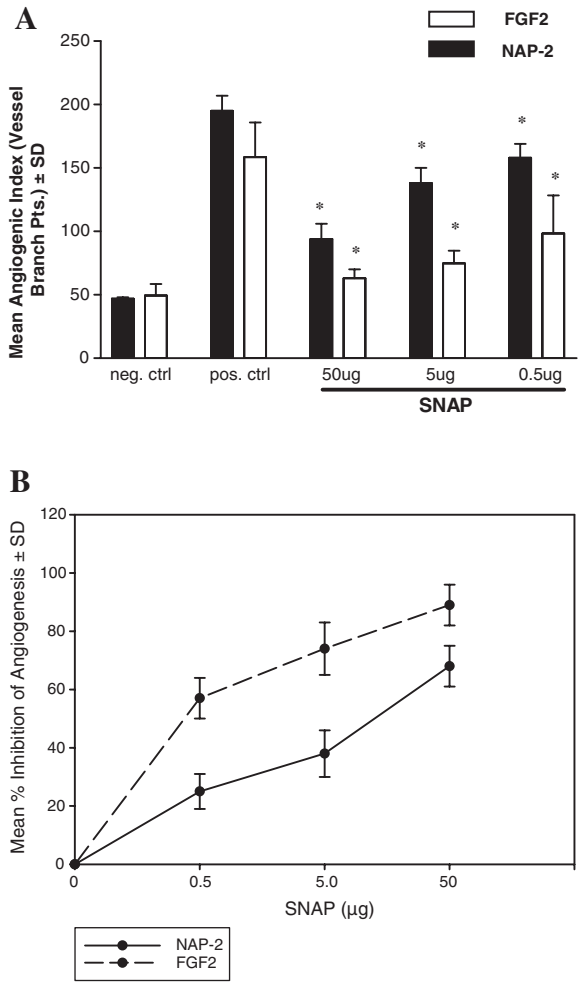

Fig. 2. **A**: NO donors dose-dependently inhibit neovascularization induced by NAP-2 and FGF-2 in vivo. CAMs of 10-day-old chicken embryos were treated with cytokines FGF-2 or NAP-2 or PBS-saturated filters. After 24 h, various amounts of SNAP were applied to FGF-2-treated CAMs. After 48 h, CAMs were resected and photographed, and measurements were made by image analysis. FGF-2 (*open bars*); NAP-2 (*closed bars*). $^*P < 0.01$ as compared to respective positive control for FGF-2 or NAP-2. **B**: NO donors dose-dependently inhibit neovascularization induced by FGF-2 or NAP-2 in vivo. CAMs of 10-day-old chicken embryos were treated with FGF-2 or PBS-saturated filters. After 24 h, various amounts of SNAP were applied to FGF-2-treated CAMs. The percent inhibition of neovascularization was observed at each dose of SNAP. Each line represents mean ± SEM, n = 3. $^*P < 0.01$, ANOVA for difference between cytokine-treated and cytokine with SNAP.

using inhibitors and donors of NO. The in vivo and in vitro systems used for studying the modulation of angiogenesis by NO have demonstrated some interesting and often contradictory results [RayChaudhury et al., 1996; Babaei et al., 1998; Norrby, 1998; Powell et al., 2000]. Because of the difficulty in measuring local NO metabolite concentrations, it is hard to say with complete certainty that overproduction of NO metabolites is the responsible mechanism, but data examining the subprocesses of angiogenesis would support that concept. Our results are consistent with a report by Norrby [1998], which identified NO as the suppressor of FGF-2-mediated angiogenesis in a different in vivo model. Furthermore, our data support the notion put forth by Pipili-Synetos et al. [2000]: by utilizing a direct pharmacological method for increasing NO, we have demonstrated the anti-angiogenic effects of NO in vivo and in vitro.

Proliferation and differentiation of ECs is a key role in the angiogenic process in the pathophysiological state, because this particular cell population undergoes phenotypic and morphological changes, as evidenced by increased growth and tube formation. This study has identified NO as both an inhibitor and a stimulator of EC proliferation. Our results are consistent with RayChaudhury et al. [1996] and Lopez-Farre et al. [1997]. It has been shown that treatment of proliferating EC cultures with high concentrations (>1 mM) of the NO donor, SNAP, inhibits serum-induced growth. The levels of nitrite and nitrate are not significantly higher in basal cultures than in serum-induced cultures. Under these conditions, both NO metabolites are found at nearly equimolar concentrations. Malinski et al. [1993] showed that following transient cerebral arterial occlusion, rat brain tissue was reported to have levels up to 4 μM. Thus, in situations of transient oxygen deprivation, anti-proliferative concentrations of NO may develop in the microenvironment prior to entering apoptosis. Additionally, we and others have demonstrated the anti-proliferative and anti-angiogenic effects of integrin blockade [Friedlander et al., 1995; Yeh et al., 1998; Yeh et al., 2001]. XT199 abrogates cell proliferation in a dose-dependent manner. The ability of anti-integrin compounds to inhibit growth is accomplished through interfering with the ECM binding with αvβ3. This interaction thwarts cell survival signaling and allows cells to enter apoptosis.

The morphological changes of EC that occur during angiogenesis are not resistant to the effects of SNAP. We have shown that a low dose of SNAP, relative to that required to have an effect on cell survival, has negative effects upon these angiogenesis-associated changes. SNAP

TABLE II. Inhibition of Neovascularization in the Murine Matrix Implant Model

Group	Dose[a] (mg/kg)	Hemoglobin[b] (g/dl) ± SEM	Mean % inhibition ± SEM
Negative control		0.45 ± 0.10	
Positive control (FGF-2)		1.45 ± 0.16	
FGF-2 + SNAP	5	0.63 ± 0.07[c]	85 ± 10
FGF-2 + SNAG	5	0.63 ± 0.14[c]	85 ± 16

[a]Subcutaneous mini-pump.
[b]Mean, n = 5.
[c]$P < 0.05$ versus positive control.

(1 μM) significantly interrupts the formation of tube-like structures on Matrigel matrices upon stimulation by FGF-2. The importance of the ECM in modulating the response of ECs to external cues is further underscored by our studies indicating decreased survival and increased NO levels in the absence of the matrix [Powell et al., 2000]. Neutrophil co-culture with ECs has been shown to induce shedding of VCAM, ICAM, and some selectins [Boehme et al., 2000]. In a pathophysiological model, neutrophils co-incubated with ECs in the presence of sera from patients with systemic lupus erythematosus have also demonstrated EC destruction. Perhaps in the absence of the ECM-dependent signaling, the cell–cell interaction between neutrophils and ECs produces NO that leads to oxidative stress and the

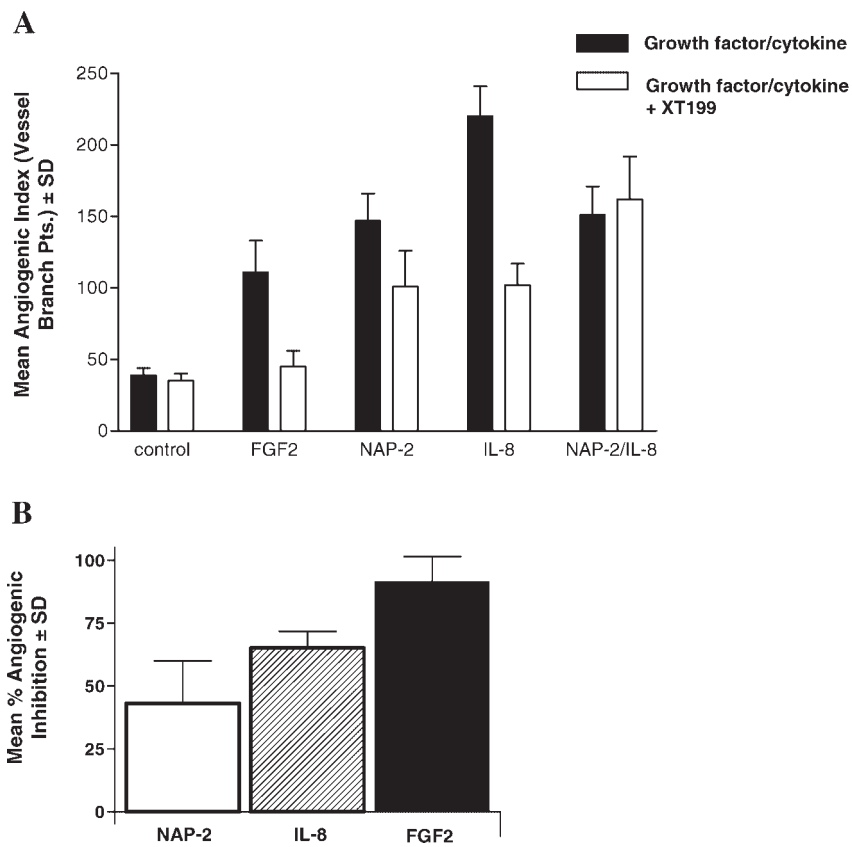

Fig. 3. The αvβ3 antgonist, XT199, inhibits neovascularization induced by NAP-2, IL-8, and FGF-2 in vivo. **A**: CAMs of 10-day-old chicken embryos were treated with XT199 (*open*) or PBS (*closed*). CAMs were resected and photographed, and measurements were made by image analysis. Each bar represents mean ± SEM, n = 5. **B**: The percent inhibition of neovascularization observed with 120 ng of XT199, FGF-2 (*open bar*), IL-8 (*diagonal lines*), and NAP-2 (*closed bar*). Each bar represents mean ± SEM, n = 5. *$P < 0.05$, Student's *t*-test for difference between FGF-2-treated and all others.

formation of free radicals, which results in decreased survival. When the ECM is present, the integrin avidity is modulated such that the same signal that produced decreased survival now induces a cellular differentiation cascade. Both observations would be consistent with neutrophil function during the classical inflammatory response because the endothelium will be perturbed at the site of tissue injury and the oxidative stress will provide a mechanism for disposing of damaged endothelium. In addition, the other function of neutrophils is to initiate tissue remodeling at the injury site, and this is likely to be with matrix degradation and extravasation of ECs.

In this study, which uses FGF-2 as an angiogenic stimulus, we have identified the NO donor SNAP as a dose-dependent inhibitor of the angiogenic process in vivo. The angiogenic index provided a direct measure of the extent of vascularization that is contained in the area of the CAM. Significant inhibition of the angiogenic response in this model was seen only at the higher amounts of SNAP. This may suggest that there is some threshold level of NO required to elicit an NO-dependent anti-angiogenic response that is not being achieved by lower amounts of SNAP. Efforts to block cytokine-induced neovascularization with factors other than FGF-2 and NAP-2 (e.g., HGF) with SNAP proved to be effective as well (data not shown). From measurements of NO metabolite concentrations in the adjacent CAM fluid, we did not find significant differences between treated and control embryos. We believe that the volume of the embryo reduces the magnitude of the concentration change observed in the microenvironment. In experiments by RayChaudhury et al. [1996], they found that a single exposure to NO inhibits EC proliferation for up to 72 h with recovery thereafter. Our vascular measurements in this paradigm are taken at 48 h post-NO exposure.

Using an anti-integrin compound, we have identified the αvβ3 antagonist XT199 as an inhibitor of the cytokine- and chemokine-induced angiogenic process in vivo. The angiogenic index for FGF-2, NAP, and IL-8 at 1 μg/ml is comparable and not statistically different. Significant inhibition of the angiogenic response in this model was observed when 120 ng of XT199 administered in CAMs, in comparison to controls. The effectiveness of this anti-αvβ3 compound was greater against FGF-2 than either NAP-2 or IL-8. This may suggest that there is an additional mechanism involved in a chemokine-mediated angiogenesis that is not being addressed by XT199. Attempts to block a combination of NAP-2 and IL-8 chemokine-induced neovascularization with XT199 proved to be ineffective as well.

In considering an explanation of the observations presented herein, one should consider a few points. The combination of NAP-2 and IL-8 does not provide synergy or additivity because they have highest affinity for a common receptor, the CXCR2. Second, the receptors become refractory and undergo desensitization from prolonged exposure to NAP-2 and IL-8. The primary target cells of NAP-2 and IL-8 are neutrophils, which undergo haptotaxis and activation in response to these α-chemokines. Neutrophils store VEGF in their granules and express VEGF in response to IL-8. As demonstrated a few years ago, VEGF and FGF-2 mediate angiogenesis by utilizing distinct integrin pathways [Friedlander et al., 1995]. Hence, FGF-2 but not VEGF is responsive to anti-αvβ3 integrin modulation. This provides a reasonable explanation for the difference in inhibition observed between FGF-2 and the α-chemokines presented in this study.

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