Nitric Oxide Synthase Lies Downstream from Vascular Endothelial Growth Factor–induced but Not Basic Fibroblast Growth Factor–induced Angiogenesis

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Abstract

Systemic administration of the nitric oxide (NO) synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) to rabbits bearing a corneal implant blocked vascular endothelial growth factor (VEGF), but not basic fibroblast growth factor (bFGF)-induced angiogenesis. L-NAME completely blocked angiogenesis induced by VEGF-transfected MCF-7 breast carcinoma cells and the cells remained dormant in the cornea. Postcapillary endothelial cell migration and growth induced by VEGF were blocked by both the NO synthase inhibitor N^{ω}-mono-methyl-L-arginine and by the guanylate cyclase inhibitor LY 83583. We conclude that NO is a downstream imperative of VEGF-, but not bFGF-induced angiogenesis, and propose that the NO synthase/guanylate cyclase pathway is a potential target for controlling tumor angiogenesis in response to VEGF. Our studies support recent evidence that VEGF and bFGF induce angiogenesis by different mechanistic pathways using the $\alpha_v\beta_5$ and $\alpha_v\beta_3$ integrins, respectively. (*J. Clin. Invest.* 1997. 99:2625–2634.)

Key words: neovascularization • endothelium • nitric oxide • vascular endothelial growth factor

Introduction

Vascular endothelial cell growth factor (VEGF)[1] is a secreted endothelial-specific growth factor that is strongly angiogenic in vivo (1, 2). Human VEGF exists as a result of differential splicing in at least four isoforms of 121, 165, 187, and 207 amino acids (3). The observation that VEGF expression is induced by hypoxia (4), together with elevated expression of VEGF in solid tumors, points to a key role for VEGF in tumor angiogenesis (5–8). This is supported by transfection of human $VEGF_{121}$ into MCF-7 human breast carcinoma cells or mouse $VEGF_{164}$ into SK-MEL-2 human melanoma cells where it was shown that VEGF expression enhanced tumor growth and vascular density (9, 10). Several studies have shown that administration of VEGF antibodies is effective in blocking tumor growth (11, 12), and viral delivery of a dominant negative VEGF receptor to vascular endothelium has been shown to retard xenografted glioblastoma growth in nude mice (13). Similarly, there exists much evidence for a role for basic fibroblast growth factor (bFGF) in tumor angiogenesis (14, 15).

The postreceptor signaling pathways underlying VEGF actions on endothelial cells are still unclear. VEGF has been shown to elevate intracellular inositol 1,4,5-trisphosphate and calcium levels, and to stimulate tyrosine phosphorylation and vWf release in cultured human umbilical vein endothelial cells (16, 17). Moreover, VEGF effects on permeability and vascular tone are coupled to nitric oxide (NO) production (18–20).

The observation that in vivo angiogenesis is accompanied by vasodilation, and that many angiogenic factors are vasodilators, prompted us to search for evidence of an involvement of NO in angiogenesis. Endothelium-dependent relaxation is known to arise from endothelium-derived NO inducing cyclic guanosine monophosphate (GMP) in the vascular smooth muscle cell (21). The synthesis of NO by endothelial cells can be blocked by L-arginine analogs such as N^{ω}-mono-methyl-L-arginine (L-NMMA) and L^{ω}-nitro-L-arginine methyl ester (L-NAME), while D-isomers are ineffective. In previous studies, we have shown that chemical mediators, which activate the constitutive NO synthase, as well as NO donors, such as sodium nitroprusside, promote endothelial cell proliferation and migration in vivo and in vitro, while inhibitors of NO synthase suppress these responses (22, 23). In addition, human monocyte-induced angiogenesis requires an L-arginine/NO synthase–dependent mechanism (24). In this study, we have investigated whether the NO synthase pathway is involved in the angiogenic activity of VEGF and bFGF. The role of NO in angiogenesis was assessed by NO synthase inhibition and an involvement of cyclic GMP in endothelial cell activation in vitro.

Methods

Cell line and culture. Coronary venular endothelial cells (CVEC) were obtained, as previously described, by a bead-perfusion technique through the coronary sinus (25). Cells were maintained in culture in DME supplemented with 10% bovine calf serum (CS) and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) on gelatin-coated dishes. Cells between passage 15 and 25 were used in these experiments.

Low-passage human breast carcinoma cells MCF-7 (wild-type, WT) and MCF-7 cells overexpressing recombinant $VEGF_{121}$ (V12 cells) were cultured as described (9).

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1. *Abbreviations used in this paper:* bFGF, basic fibroblast growth factor; CS, calf serum; CVEC, coronary venular endothelial cells; GMP, guanosine monophosphate; L-NAME, N^{ω}-nitro-L-arginine methyl ester; L-NMMA, N^{ω}-mono-methyl-L-arginine; NO, nitric oxide; VEGF, vascular endothelial growth factor; WT, wild-type.

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Determination of NO synthase activity. CVEC were seeded in 100-mm culture dishes and allowed to grow to 90% confluence. Cells were treated for 24 h with test substances in DME plus 0.1% CS. At the end of incubation, cells were washed and scraped in Dulbecco's PBS. Samples were kept at −80°C until use. Cells were homogenized in buffer containing 0.32 M sucrose, 20 mM Hepes buffer, 1 mM EDTA, and 1 mM dithiothreitol. The calcium-dependent and calcium-independent isoforms of NO synthase were assayed as previously reported (23).

Measurement of cyclic nucleotide levels. Cyclic AMP and cyclic GMP levels were measured in cell extracts from confluent monolayers by RIA using iodinated tracers as previously reported (20, 22). Cell monolayers were treated with 1 mM 3-isobutyl-5-methyl-xanthine and 10 μM indomethacin for 15 min before stimulation. After stimulation, cells were rinsed with PBS and removed by scraping in ice-cold 10% trichloroacetic acid. After centrifugation, cyclic nucleotide levels were assayed in the supernatant, while protein concentration was measured in the pellet by Bradford assay (26). The amount of cyclic nucleotide was quantified as femtomoles per milligram of protein.

Proliferation assays. Cell proliferation was quantified by total cell number and by DNA synthesis (20). 10^3 cells resuspended in 1% CS were seeded in each well of 96-multiwell plates. After adherence (3–4 h) the medium was replaced with 1% CS DME containing test substances and incubated for 48 h. DNA synthesis was measured after 24 h by 5-bromo-2′-deoxyuridine (BrdU) uptake and immunocytochemical processing (cell proliferation kit; Amersham Corp., Arlington Heights, IL). The number of cells and labeled nuclei was counted in five random fields of each well at a magnification of 100 with the aid of a 21-mm^2 ocular grid. To assess the effect of NO synthase and guanylate cyclase inhibition, L-NMMA (200 μM) and LY 83583 (1 μM) were added to the cells 1 h before the test substances.

Cytotoxic effect of L-NMMA and LY 83583 was measured by trypan blue exclusion in cell suspension after 2 h of incubation.

Chemotaxis assay. The Neuro Probe 48-well microchemotaxis chamber was used to assess cell migration as described (23). The growth factors, prepared in 1% CS medium, were placed in the lower wells. 10^4 cell suspension was added to each upper well. The chamber was incubated at 37°C for 4 h. To assess the effect of NO synthase and guanylate cyclase inhibition, L-NMMA (200 μM) and LY 83583 (1 μM) were given to the cells for 30 min at room temperature before seeding. After fixation, nonmigrating cells on the upper surface of the filter were removed and migrated cells were stained and counted (at a magnification of 400) in 10 random fields per well. Each experiment was performed in triplicate and migration was expressed as the number of total cells counted per well.

Cell adhesion assay. Cell adhesion was measured following a modification of the method reported by Taraboletti et al., in polystyrene microtiter 96-well plates coated with fibronectin (10 μg/ml) (27). Before seeding, 5×10^5/ml in 0.1% CS medium cells were treated with L-NMMA (200 μM) and LY 83583 (1 μM) for 60 min at room temperature. The plate was incubated for 90 min at 37°C. The wells were gently washed two times with PBS and the adherent cells were fixed with methanol and stained with Diff-Quik. The number of adherent cells was counted by microscopic examination in seven random fields at a magnification of 200. Data are reported as total cells counted per square millimeter.

Sprouting assay. Endothelial cell sprouting was measured as the migration and growth of quiescent adherent endothelial cells during a 72-h exposure to angiogenic factors (28). 5×10^4 cells were seeded into a rectangular silicon gasket inside a six-well plate in 10% CS medium. Once at confluence, cells were serum-starved overnight and the silicon gasket was removed. The start for cell movement was defined by the four sides of the monolayer, marked with a scalpel, on the outside of the tissue culture dish. LY 83583 and SQ 22536 (both 1 μM) were added to the cells 3 h before the growth factors. Assays were performed in 1% CS medium. Total cell counting and the distance migrated were determined microscopically with a squared ocular grid (225 μm × 225 μm) after cell staining with Diff-Quik. 80 cell counts were performed in each experiment and experiments were carried out in quadruplicate.

Angiogenesis in vivo: rabbit cornea assay. Corneal assays were performed in female New Zealand albino rabbits (Charles River, Calco, Como, Italy) as described (29, 30). The test substance, either as a cell suspension or slow-release pellet incorporating recombinant growth factor, was implanted into the micropocket. Recombinant growth factors ($VEGF_{165}$ and bFGF, both from R&D Systems Europe Ltd., Abingdon, United Kingdom) were prepared as slow-release pellets by incorporating the protein under test into an ethynyl-vinyl copolymer (Elvax-40). In the experiments with cell suspensions, rabbits received 2.5×10^5 wild-type MCF-7 cells in the left cornea, and an equal number of V12 cells in the right cornea. Subsequent daily observation of the implants was made with a slit lamp stereomicroscope without anesthesia. An angiogenic response was scored positive when budding of vessels from the limbal plexus occurred after 3–4 d and capillaries progressed to reach the implanted pellet according to the scheme previously reported. The number of positive implants over the total implants performed was scored during each observation. The potency of angiogenic activity was evaluated on the basis of the number and growth rate of newly formed capillaries, and an angiogenic score was calculated (vessel density × distance from limbus) (23). A density value of 1 corresponded to 0–25 vessels per cornea, 2 from 25–50, 3 from 50–75, 4 from 75–100 and 5 for > 100 vessels. The distance from the limbus was graded with the aid of an ocular grid. Corneas were removed at the end of the experiment as well as at defined intervals after surgery and/or treatment, and fixed in formalin for histological examination. Endothelial cells of intracorneal microvessels were immunolabeled with anti–CD-31 monoclonal antibody (clone JC/70; Dako, A/S Glostrup, Denmark) at 1:300 dilution for 12 h at room temperature (31).

To evaluate the effect of NO synthase inhibition on the response to the angiogenic effectors, freshly prepared L-NAME (0.5 g/liter) was given in the drinking water ad libitum. The efficacy of L-NAME administration was evaluated at the end of the treatment as acetylcholine-induced relaxation in rabbit aortic rings preconstricted with phenylephrine as previously described (23).

Statistical analysis. Results are expressed as means±SEM for (n) experiments. Multiple comparisons were performed by one-way ANOVA and individual differences were tested by Fisher's test after the demonstration of significant intergroup differences by ANOVA.

Table I. Cyclic AMP and Cyclic GMP Levels in Postcapillary Endothelial Cells Exposed to VEGF and bFGF

		Cyclic GMP	
	Cyclic AMP	− L-NMMA	+ L-NMMA
Basal	4836±678	36.5±5	42±5
VEGF 10 ng/ml	4459±509	96.5±15*	51.4±8
bFGF 10 ng/ml	6563±316	50.4±5	48±10
NaNP 100 μM	4772±900	68±13*	55.2±6
PGE_1 10 μM	18919±1225*	37±3	ND

Cyclic AMP and cyclic GMP levels were evaluated by RIA in CVEC extracts. Cells, grown to 90% confluence in 100-mm petri dishes, were stimulated with the growth factors in the presence of 60 U/ml superoxide dismutase for 10 min. Cells were treated with 200 μM of the NO synthase inhibitor L-NMMA 1 h before the addition of stimuli. Data are reported as femtomoles per milligram of proteins. Numbers represent means±SEM of six determinations. *ND*, not done. *$P < 0.001$ versus basal condition.

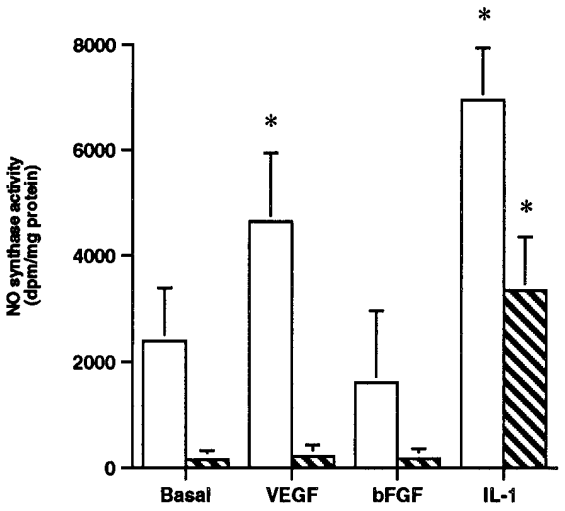

Figure 1. NO synthase activity in CVEC exposed to VEGF and bFGF. The calcium-dependent (*empty columns*) and calcium- and calmodulin-independent (*hatched columns*) NO synthase activities were measured in the supernatant of CVEC exposed to VEGF and bFGF (10 ng/ml each) and to IL-1 (50 U/ml) for 24 h. The ratio between labeled citrulline (dpm) and mg protein assayed in cell homogenate was taken as NO synthase activity. Data are the means±SEM of five determinations. $*P < 0.05$, significantly different from basal condition.

Results

NO synthase and soluble guanylate cyclase mediate the activity of VEGF, but not bFGF, on microvascular endothelial cells. To determine whether VEGF could stimulate the NO synthase pathway, enzyme activity and the levels of cyclic GMP were measured in postcapillary CVEC. NO signal transduction in target cells involves the activation of soluble guanylate cyclase and elevation of intracellular cyclic GMP. Subconfluent CVEC cells were exposed to 10 ng/ml of each growth factor for 10 min. The levels of cyclic GMP in the cell were doubled by VEGF but had no effect on cyclic AMP (Table I). In contrast, bFGF had no effect on cyclic GMP accumulation but slightly increased cyclic AMP production. Sodium nitroprusside (NaNP) (100 μM) and prostaglandin E_1 (PGE_1) (10 μM) were used as positive stimulators of cyclic GMP and cyclic AMP, respectively. The effect of VEGF on cyclic GMP elevation was blocked in the presence of the NO synthase inhibitor L-NMMA, thus demonstrating that VEGF was linked to the NO synthase pathway. Consistent with these observations, VEGF increased the calcium-dependent NO synthase isoform about twofold and the calcium-independent isoform by 36%; conversely bFGF did not affect either isoform of the enzyme (Fig. 1).

As VEGF elevated both NO synthase activity and cyclic GMP levels, the involvement of the NO synthase pathway was

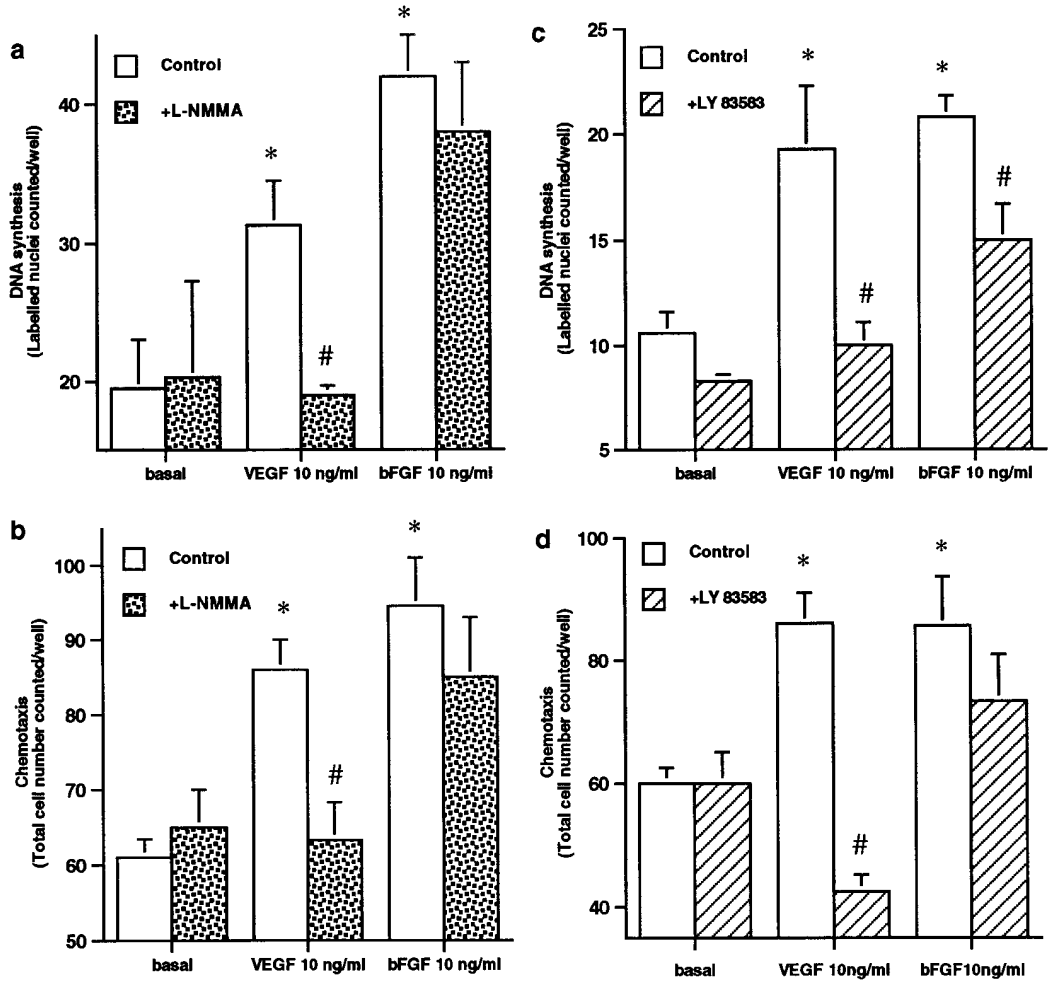

Figure 2. Effect of NO synthase and guanylate cyclase inhibition on VEGF- and bFGF-induced DNA synthesis and chemotaxis in CVEC. Cells were treated with 200 μM L-NMMA (*a* and *b*) or 1 μM LY 83583 (*c* and *d*). DNA synthesis (*a* and *c*) was determined by incorporation of BrdU in cells exposed to the growth factors for 48 h. Labeled nuclei were counted with the aid of an ocular grid at ×100. Data are presented as the total labeled nuclei counted per well. Migration (*b* and *d*) was assayed in a 48-well microchemotaxis chamber as described in Methods. The chamber was incubated at 37°C for 4 h. Data are means±SEM from at least three experiments. $*P < 0.05$, significantly different from basal control; $^{\#}P < 0.05$, significantly different from growth factors alone.

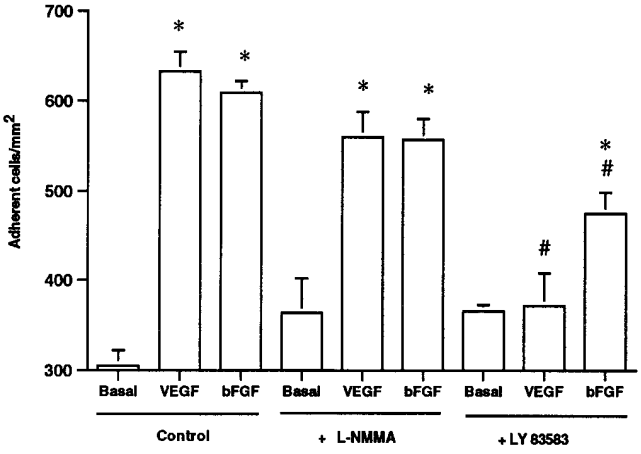

Figure 3. Effect of NO synthase and guanylate cyclase inhibition on cell adhesion induced by VEGF and bFGF. Cell adhesion in response to the growth factors (10 ng/ml each) was evaluated in fibronectin-coated polystyrene microtiter plates. Cells were pretreated with L-NMMA (200 μM) and LY 83583 (1 μM) for 60 min before seeding. After 90 min of incubation at 37°C, adherent cells were counted microscopically in seven random fields at ×200. Data are reported as total cells counted per square millimeter. $^{*}P < 0.05$, significantly different from basal controls; $^{\#}P < 0.05$, significantly different from growth factors alone.

investigated on the biological activities of VEGF. VEGF-induced endothelial cell proliferation and migration were assessed during NO synthase and soluble guanylate cyclase inhibition. Preincubation with 200 μM of L-NMMA suppressed the activity of VEGF (10 ng/ml) on endothelial cell DNA synthesis and chemotaxis, but had no effect on that induced by bFGF (Fig. 2, *a* and *b*). The treatment with the soluble guanylate cyclase inhibitor LY 83583 (1 μM) (32) strongly inhibited VEGF-induced mitogenic and migratory activity on cultured endothelium (Fig. 2, *c* and *d*) and reduced bFGF mitogenic activity. Neither inhibitor impaired cell vitality as measured by trypan blue exclusion. Since adhesion is required for migration and mitosis, endothelial cell adhesion was studied during NO/cyclic GMP inhibition. A 90-min exposure to either VEGF or bFGF doubled the number of adherent endothelial cells. L-NMMA or LY 83583 treatment did not impair CVEC adhesion in control conditions. However, while the NO synthase inhibitor did not substantially modify the adhesion promoted by either growth factor, the guanylate cyclase inhibitor completely abolished the VEGF effect and reduced bFGF-induced adhesion (Fig. 3).

Effect of soluble guanylate cyclase inhibition on the long-term replication and sprouting of CVEC in response to VEGF and bFGF. An involvement of the NO/cyclic GMP signaling pathway in mediating the longterm effects of VEGF on cultured endothelium was assessed by treating CVEC cells primed to sprout with the soluble guanylate cyclase inhibitor LY 83583. CVEC cells were exposed to VEGF and bFGF for 72 h, and cell growth and migration were assessed simultaneously as sprouting of cells from a confluent and growth-arrested monolayer. The effect was compared to that in the presence of the inhibitor of adenylate cyclase SQ 22536 (33). Fig. 4 *a* and Fig. 5 show the effect of the guanylate cyclase inhibitor LY 83583 on basal, VEGF-stimulated, and bFGF-stimulated endothelial cell growth and progression after 72 h of culture. Pretreatment with 1 μM LY 83583 for 3 h had no effect on basal growth and migration. However, when cells were stimulated with 10 ng/ml of VEGF, a marked reduction in both cell growth (73%) and migration (58%) was observed (Fig. 4 *a* and Fig. 5, *b* and *e*). In contrast, when bFGF (10 ng/ml) was added to the cells, neither cell replication nor cell movement was sensitive to the presence of LY 83583.

The adenylate cyclase inhibitor SQ 22536 increased both the number of replicating cells under both basal conditions and in response to VEGF (Fig. 4 *b*). SQ 22536 had no effect on the activity of bFGF.

Role of nitric oxide in VEGF- and bFGF-induced angiogenesis in vivo. To investigate a potential role of NO in angiogen-

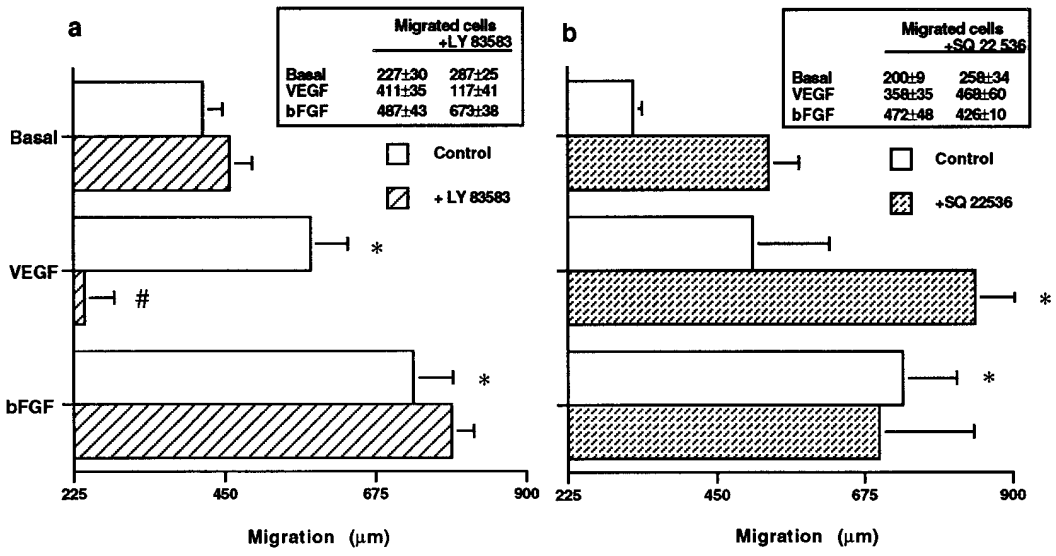

Figure 4. Effect of cyclic GMP and cyclic AMP inhibition on endothelial cell sprouting induced by VEGF and bFGF. Endothelial cell sprouting was assayed, as reported in Methods, on monolayers of adherent and synchronized cells. LY 83583 and SQ 22536 (1 μM each) were used to inhibit cyclic GMP and cyclic AMP production, respectively. 10 ng/ml of the growth factors were added for 72 h. Data are the distance traveled in μm by VEGF- and bFGF-stimulated cells. The insets show the total number of cells counted. Cell counting and distance migrated were determined microscopically with a squared ocular grid (225 μm × 225 μm). Data are means±SEM from three experiments. $^{*}P < 0.05$, significantly different from basal control; $^{\#}P < 0.05$, significantly different from VEGF alone.

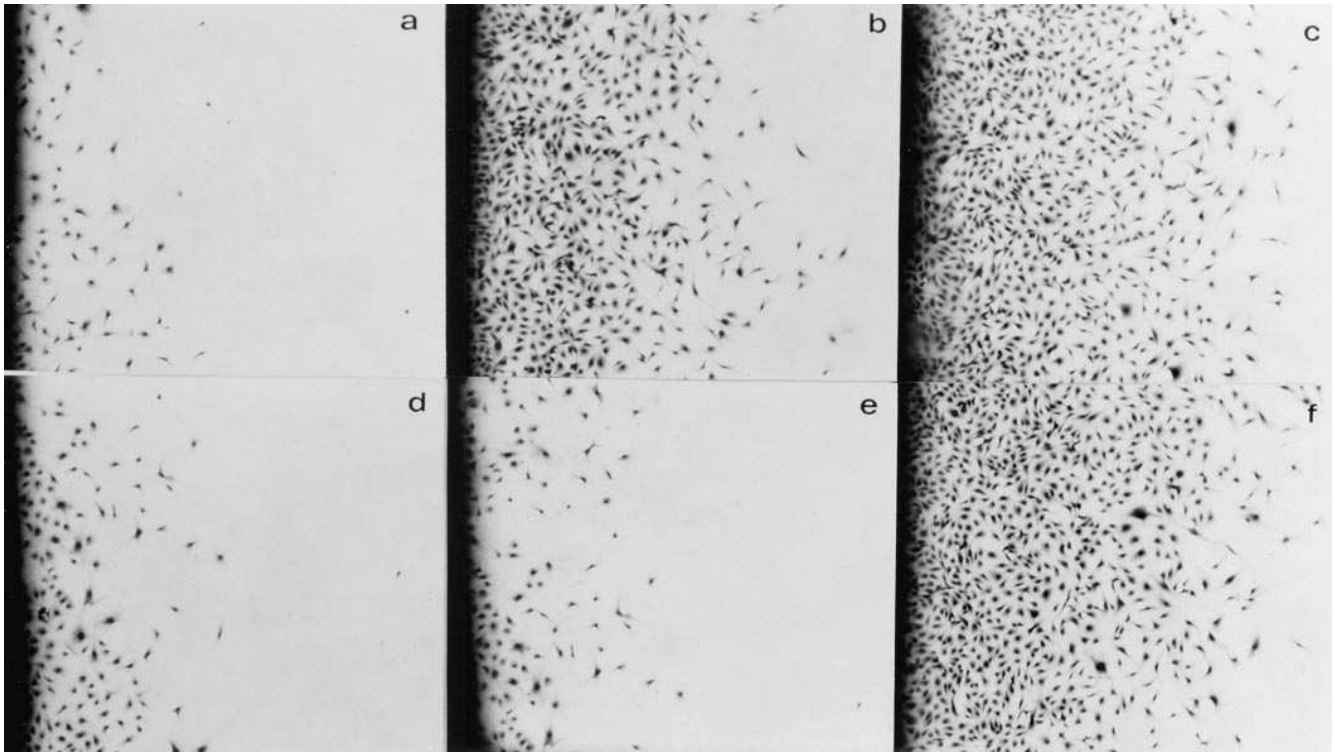

Figure 5. Representative pictures of CVEC sprouting. *a*, *b*, and *c* show basal, VEGF-, and bFGF-induced CVEC sprouting. *d*, *e*, and *f* show the effect of 1 μM LY 83583 on basal, VEGF, and bFGF growth and migration. Original magnification, ×4.

esis in vivo, we examined the effect of systemic NO synthase inhibition by L-NAME on VEGF- and bFGF-induced angiogenesis. L-NAME was administered to rabbits via drinking water 7 d before corneal implant and for 10 d further after implant. Rabbit corneas were implanted with slow-release pellets containing $VEGF_{165}$ or bFGF (both at 100 ng/pellet). VEGF and bFGF induced a strong angiogenic response with 100 and 90% of implants being positive, respectively. The angiogenic response quantitated by both the number of new vessels and their rate of growth was greater for bFGF than $VEGF_{165}$ (Fig. 6). Systemic administration of L-NAME (0.5 g/liter) for 1 wk before surgery greatly reduced VEGF-induced angiogenesis (Fig. 6 *a*), but had no significant effect on angiogenesis elicited by bFGF (Fig. 6 *b*).

L-NAME blocks angiogenesis in vivo induced by MCF-7 VEGF transfectants. To extend the above studies to tumor angiogenesis, we looked for a role of NO in angiogenesis induced by $VEGF_{121}$ overexpression by MCF-7 breast carcinoma cells (9). We examined the effect of L-NAME on the growth of WT and V12 cells implanted into the rabbit cornea. The implant of V12 cells gave a strong angiogenic response within 1 wk (Fig. 7 *a* and 8 *b*). The dense vascular network elicited by V12 transfectants was efficiently blocked by L-NAME treatment for as long as 20 d after surgery (Fig. 7 *a* and 8 *c*). Conversely, WT cells induced the growth of a few slowly elongating capillaries from the preexisting limbal vessels. This angiogenesis was insensitive to treatment with L-NAME (Fig. 7 *b*). Histological sections of representative corneas are shown in Fig. 9. As expected, with a V12 cell implant, we observed erythrocyte containing vessels (Fig. 9, *b* and *e*). No vessels were detected in V12 cell implant from a rabbit receiving L-NAME (Fig. 9, *c* and *f*) or in control empty corneas (Fig. 9 *d*). A measure of specificity of the treatment was seen in the results obtained with D-NAME, the inactive isomer of L-NAME, which gave only weak inhibition of V12-induced angiogenesis (Fig. 10).

A direct cytotoxic effect of L- or D-NAME on the MCF-7 transfectants, an effect that could have impaired angiogenesis, was ruled out by in vitro experiments showing that neither compound at 200 μM reduced cell growth (Table II). Cell replication was supported by the addition of either L- or D-isomer, suggesting that these effects are not related to the NO pathway.

Discussion

Recent reports have shown that VEGF-stimulated angiogenesis proceeds by an integrin-mediated angiogenic pathway dis-

Table II. Effect of L-NAME and D-NAME on Growth of WT and V12 MCF-7 Cells In Vitro

Treatment	WT	V12
	Total cell number counted per well × 10^{-4}	
None	12.9±1.3	14.2±0.5
L-NAME (200 μM)	21.1±1.7*	20.7±1.8*
D-NAME (200 μM)	22.6±1.4*	26.0±0.9*

9,000 cells/well were seeded in 24-well plates in 10% FCS medium and left for 48 h. Cells were then treated with 200 μM of L-/D-NAME in 5% FCS medium for 48 h. Cells were fixed with methanol and stained with Diff-Quik. Values (mean±SEM) are expressed as total cell number per well. *$P < 0.05$, statistically different from no treatment condition.

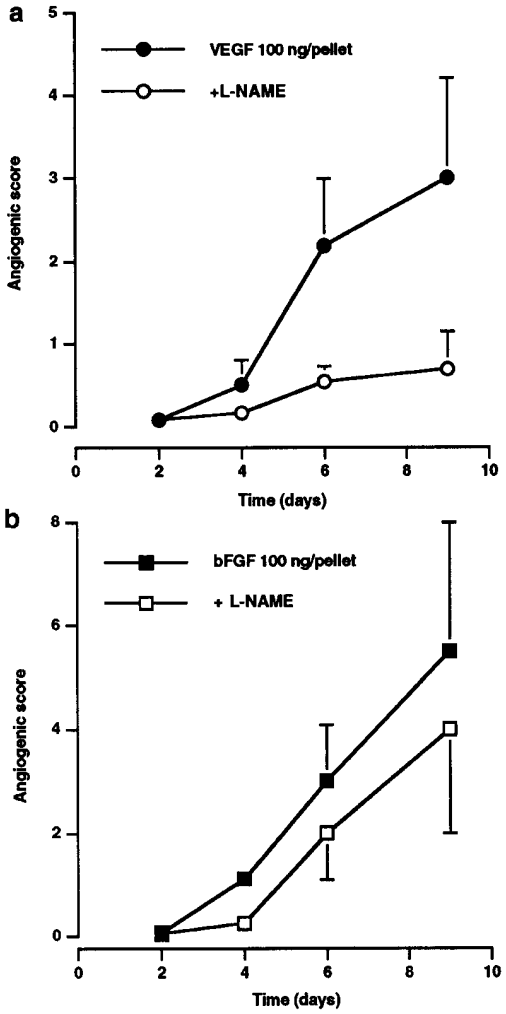

Figure 6. Effect of NO synthase inhibition on angiogenesis induced in response to recombinant $VEGF_{165}$ (*a*) and by bFGF (*b*). The growth factor (100 ng)–containing pellet was placed into the left cornea and a control pellet in the right cornea of albino rabbits. Each factor was tested in two distinct experiments with similar results. To evaluate the effect of NO synthase inhibition on the response to the angiogenic effectors, L-NAME was given in the drinking water ad libitum (0.5 g/liter). Data (means±SD) are expressed as angiogenic score and are the results from four corneal implants from one representative experiment.

tinct from that stimulated by bFGF (34). The aim of this study was to assess whether NO was involved in VEGF- or bFGF-induced angiogenesis. The data reported indicate that NO is a downstream imperative of VEGF- but not bFGF-induced angiogenesis. Postcapillary endothelial cell mobilization and growth induced by VEGF were blocked by the NO synthase inhibitor L-NMMA and by the guanylate cyclase inhibitor LY 83583; however, these inhibitors did not persistently modify bFGF effects. In vivo inhibition of NO synthase blocked angiogenesis induced by VEGF and by a highly angiogenic carcinoma population. In contrast, the angiogenic activity of bFGF was not affected.

Previous reports have indirectly implicated a role for NO synthase in angiogenesis (23, 24). For example, it was shown that angiogenic activity was only released from bacterial endotoxin–treated macrophages in the presence of L-arginine (24). Although the source of angiogenic activity was not identified in the above study, it was blocked nonetheless by the NO synthase inhibitors. Similarly, L-arginine was shown to favor healing and angiogenesis in gastric ulcerations while NO synthase inhibitors delayed it (35). Other observations have indicated a cytotoxic/cytostatic effect of NO on the vascular development of the chorioallantoic membrane suggesting a diversity of effects in embryonal versus adult tissue (36, 37). In previous studies, we have shown that vasodilating peptides such as substance P promote angiogenesis via the NO/cyclic GMP pathway (23, 38). Here we demonstrate that NO synthase lies downstream of a specific extracellular angiogenic signal, i.e., VEGF.

In breast cancer, the expression and levels of VEGF correlate with high microvessel density, and both features are associated with poor prognosis (8). Transfection of $VEGF_{121}$ into human breast carcinoma cell line (V12 cells) has been shown previously to enhance tumor growth and vascular density in

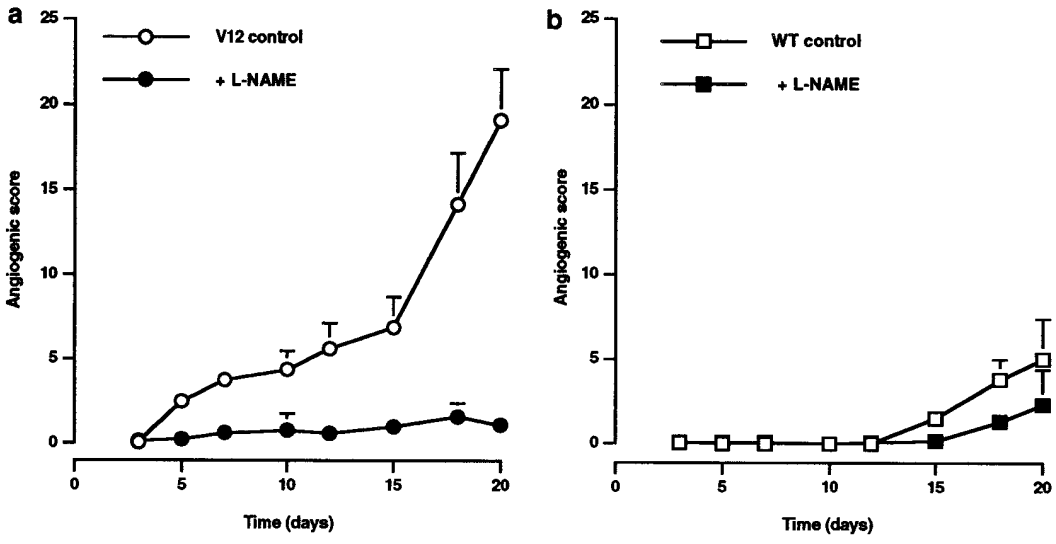

Figure 7. Effect of NO synthase inhibition on angiogenesis induced by VEGF-transfected carcinoma cells. MCF-7 cells overexpressing $VEGF_{121}$ (V12 clone) (*a*) or WT cells (*b*) were implanted into the right and left cornea of albino rabbits, respectively. Data are expressed as in Fig. 4 and were from five corneal implants.

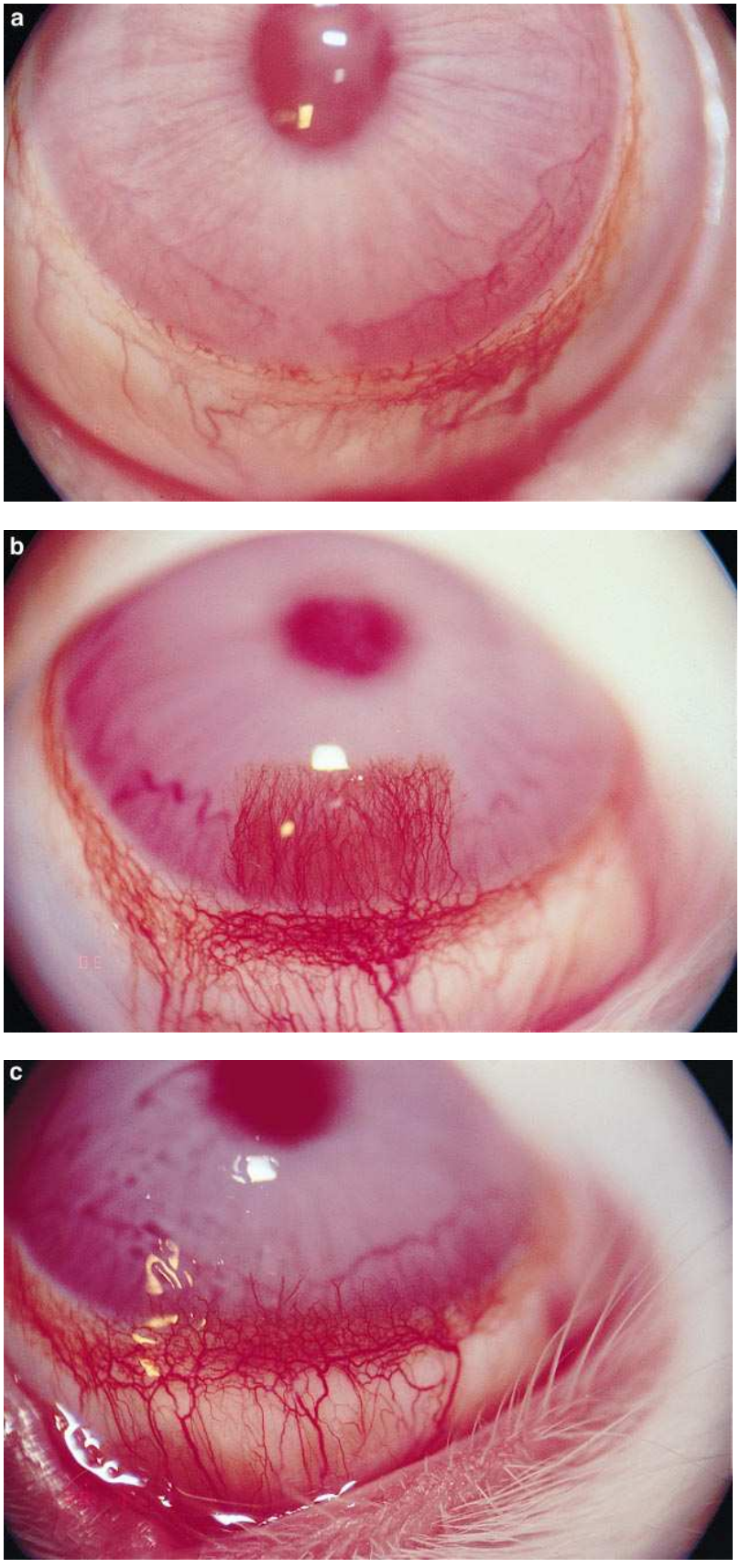

Figure 8. The effect of NO synthase inhibition on angiogenesis in vivo in response to WT and VEGF-transfected MCF-7 cells. Corneal angiogenesis induced by WT MCF-7 (*a*) and by $VEGF_{121}$ transfectants (V12 clone) (*b*). (*c*) The angiogenic response elicited by V12 cells after L-NAME treatment. WT MCF-7 cells induced the growth of several slowly progressing capillaries from the preexisting limbal vessels. V12 cells induced a strong angiogenic response which was blocked by NO synthase inhibition. All pictures were taken at day 18 after surgical implant through a slit stereomicroscope (Nikon, Tokyo, Japan). ×18.

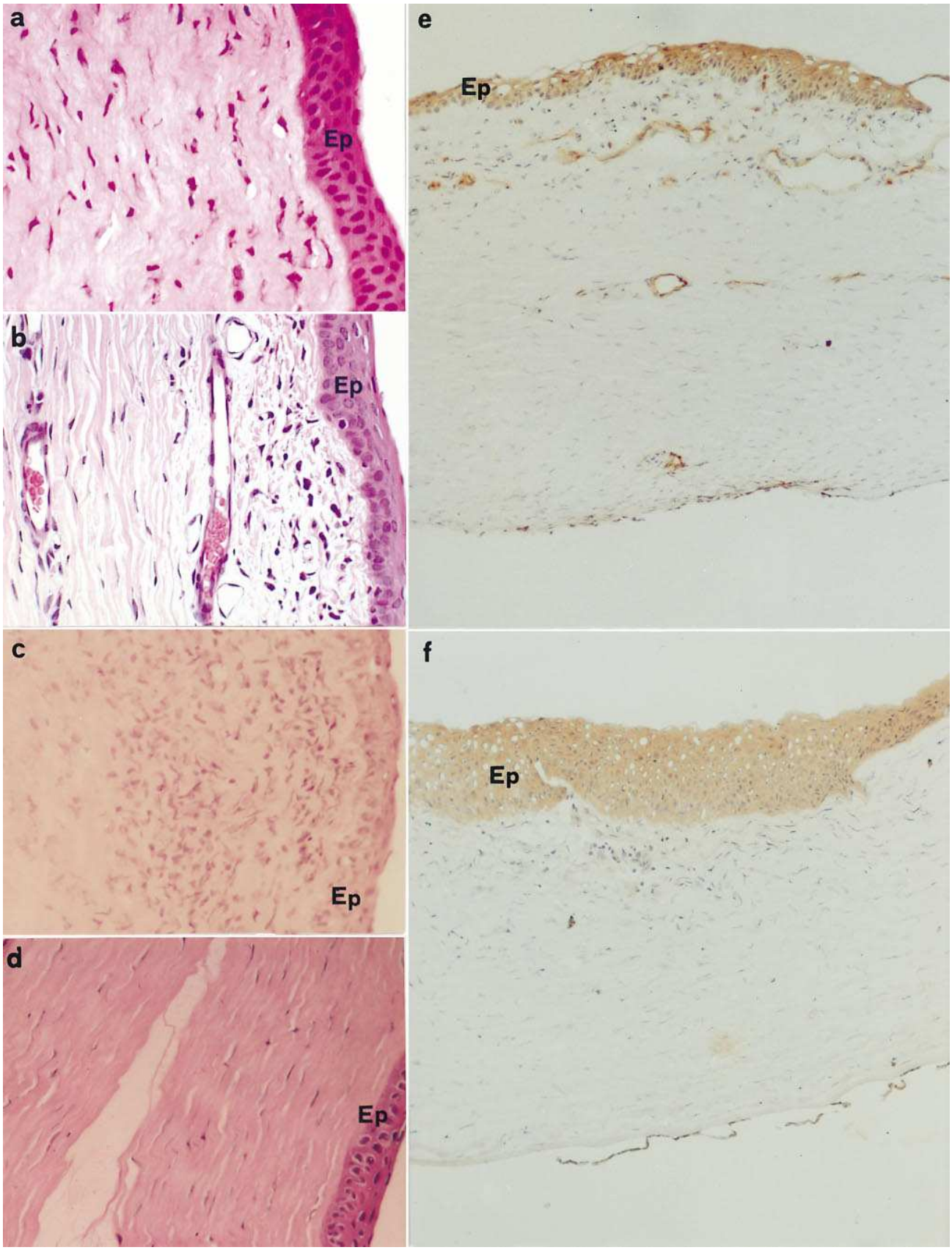

Figure 9. Histological sections of corneas 20 d after implant. (*a*) WT MCF-7 cell implant; (*b*) V12 cell implant showing erythrocyte containing vessels; (*c*) V12 cell implant from a rabbit receiving L-NAME; (*d*) control empty cornea; (*e*) anti–CD-31 antibody immunostaining of endothelial cells in V12-induced corneal angiogenesis in controls; and (*f*) after L-NAME treatment. *Ep*, corneal epithelium.

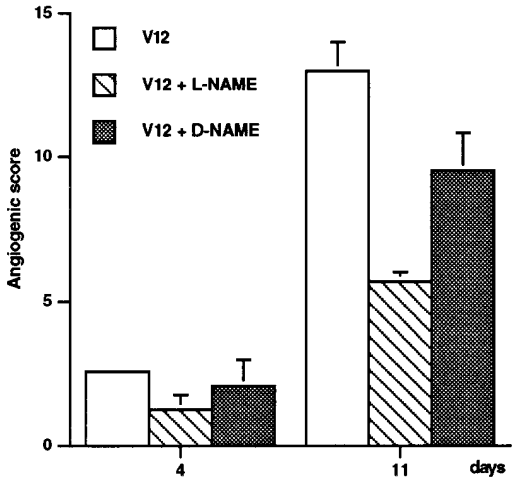

Figure 10. Specificity of NO synthase inhibition on VEGF-induced tumor angiogenesis. To assess the specificity of NO synthase inhibition on angiogenesis induced by VEGF transfectants (V12 clone), the effect of the systemic administration of L-NAME was compared to that of the inactive enantiomer D-NAME. L-NAME (*dashed bars*) inhibits VEGF-induced angiogenesis, while the inactive stereoisomer D-NAME (*filled bars*) has little effect.

vivo and promotes a strong angiogenic response (9). Here, we show that V12 cells induced a strong angiogenic response, which was efficiently blocked by NO synthase inhibition. Conversely, angiogenesis induced by mock-transfected MCF-7 cells was slight, appeared late, and was insensitive to L-NAME treatment. Thus, the VEGF effect appears to be selectively linked to the NO pathway. In fact, L-NAME, but not D-NAME, completely blocked neovascularization induced by the VEGF transfectants, while the cells remained dormant in the cornea.

Increased NO levels have been found in human tumors (39, 40). Transfection of the inducible NO synthase into a colon adenocarcinoma line gave a cell line that, despite growing more slowly in vitro, promoted tumors that grew more rapidly and were more vascularized than WT cells (41). Other observations that agree that NO is a specific signal for tumor vascularization show that blocking NO synthase activity retards the growth of xenografted tumors (42, 43), and excessive production of NO sustains tumor growth (44). Consistently, dexamethasone exerts an antiangiogenic effect leading to reduced tumor growth (45). Thus, several data exist in support of NO as a signal transducer in tumor angiogenesis. Based on these considerations, we hypothesize that NO released by capillaries in proximity of a tumor under the control of a local growth factor may favor tumor angiogenesis.

The data presented here clearly demonstrate that NO production significantly contributes to the growth-promoting effect of VEGF, but not for the growth-promoting effect of bFGF. Inhibition of the soluble guanylate cyclase impairs the adhesion induced by either VEGF or bFGF and this effect is possibly involved in the reduction of DNA synthesis and migration. However, in the long run, while bFGF can overcome this impairment and proceed to complete its morphogenetic program for angiogenesis, VEGF does not. The specificity of the NO synthase inhibitors on VEGF-induced angiogenesis, and not bFGF-induced angiogenesis, provides new evidence for the existence of the two angiogenic pathways defined by the $\alpha_v\beta_5$ and $\alpha_v\beta_3$ integrins (34). Thus, although the NO pathway integrates several chemical and physical modulators of the angiogenic process, not all angiogenic factors depend on this signaling cascade. Indeed, L-NAME does not block the intrinsic angiogenic activity of the WT cells used in this study. This highlights the problem that arises as a result of tumors secreting multiple angiogenic factors, namely, that the blocking of any single angiogenic factor is unlikely to be an effective antitumor strategy. L-NAME joins thalidomide and linomide as the only orally active antiangiogenic compounds. Our data indicate that the calcium-dependent isoform is preferentially involved in the VEGF-induced effect, but more work with specific inhibitors of the NO synthase isoforms is needed to fully elucidate the role of NO in angiogenesis. Nevertheless, the nitric oxide pathway remains a promising target for consideration in pro- and antiangiogenic therapeutic strategies.

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