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Endostatin Inhibits Microvessel Formation in the *ex Vivo* Rat Aortic Ring Angiogenesis Assay

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Endostatin has demonstrated potent antiangiogenic and antitumor activity in mouse models. We have investigated the *ex vivo* rat aortic ring assay and a human vein model to assess the biological activity of murine and human endostatin. Rat aortic rings were exposed to recombinant murine endostatin (*Spodoptera frugipera;* Calbiochem, San Diego, CA) or recombinant human endostatin (*Pichia pastoris;* EntreMed, Rockville, MD). After 5 days, murine endostatin (500 μg/ml) demonstrated inhibition of microvessel outgrowth with dose-dependent effects (down to 16 μg/ml). No significant inhibition was observed with human endostatin in the rat assay. Human endostatin at 250 and 500 μg/ml inhibited outgrowths from human saphenous vein rings after a 14-day incubation. Electron microscopy assessed the formation of basal lamina, confirming that the microvessels were progenitors of patent vessels. Immunostaining for Factor VIII or CD34 demonstrated that the microvessel cells were endothelial. BrdU incorporation assays supported the presence of proliferating endothelial cells, correlating with neovascularization from the aortic wall. We conclude that the rat aortic ring assay confirms the antiangiogenic activity of murine but not human endostatin, suggesting that the model may have species specificity. However, the human form shows biological activity against human vascular tissue. © 2000 Academic Press

Angiogenesis is a critical process allowing the growth, invasion, and metastasis of solid tumors. Without the development of a new capillary network, a tumor will not grow beyond a few cubic millimeters (1). The angiogenic signaling cascade is hypothesized to respond to a net balance of positive and negative endogenous regulators (2). It is speculated that the microenvironment of quiescent vascular tissue favors inhibitors of angiogenesis while abnormal angiogenic foci, such as the microenvironment of solid tumors, favor stimulators. Based on the evidence that progressive tumor growth is angiogenesis-dependent, antiangiogenic compounds are being developed and evaluated in the clinic, with initial findings reporting therapeutic benefit (3–5).

Endostatin, a 20-kDa C-terminal fragment of collagen XVIII, is currently in preclinical development as a novel antiangiogenic agent. Mouse endostatin was initially isolated from the conditioned media of a murine hemangioendothelioma cell line (EOMA) and identified as 184 amino acids at the noncollagenous carboxy terminus (domain NC1) of collagen XVIII (6–8). Mouse endostatin, a potent inhibitor of angiogenesis *in vitro* and *in vivo,* has also demonstrated potent antitumor activity *in vivo* without the development of resistance (6, 9). Circulating and tissue forms of human endostatin have been identified, showing the human form lacks 12 N-terminal amino acids and a C-terminal lysine, as compared to the mouse form (10, 11). These differences have been speculated to be the result of carboxypeptidases (C-terminal lysine deletion) and distinct cleavage or releasing mechanisms from the parent molecule collagen XVIII by as yet unknown proteases (12 N-terminal aa deletion). Nevertheless, the two forms show 86% identity and >90% similarity, suggesting high structural and possibly functional correlation (10).

The antiangiogenic mechanism of endostatin is not clearly described. X-ray crystallography of the endostatin molecule predicted a prominent heparan sulfate binding site, suggesting it may inhibit binding of angiogenic factors such as basic fibroblast growth factor

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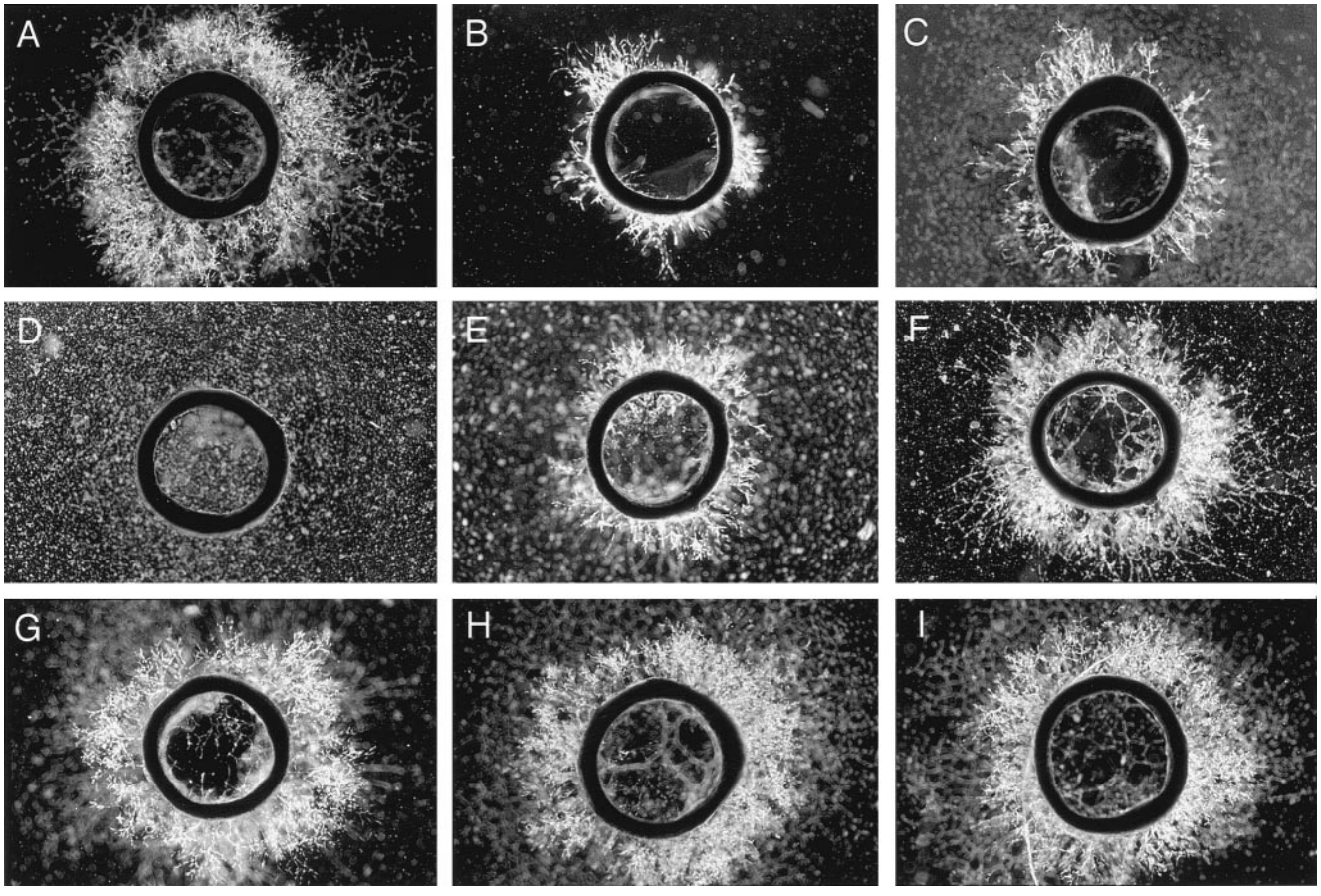

FIG. 1. Dose-dependent antiangiogenic activity of murine endostatin on rat aortic rings. (A) Control medium. (B) CAI at 12 μg/ml. (C) Suramin at 50 μg/ml. (D) 500 μg/ml endostatin. (E) 162 μg/ml endostatin. (F) 100 μg/ml endostatin. (G) 64 μg/ml endostatin. (H) 40 μg/ml endostatin. (I) 24 μg/ml endostatin. Photographs are representative rings from duplicate assays.

(bFGF) or vascular endothelial growth factor (VEGF) to heparan sulfate proteoglycans (12). However, *in situ* binding studies in intact human tissues demonstrate endostatin binding is not mediated by heparan sulfate proteoglycans and does not compete with bFGF-binding sites, providing evidence for a different mechanism of action (13). Recently, *in vitro* studies on endothelial cells with the recombinant human and murine form of endostatin in a *Pichia pastoris* system suggest slightly distinct biological activities (14, 15). Mouse endostatin treatment on cow pulmonary artery endothelial (C-PAE) and bovine aorta endothelial (BAE) cells caused apoptosis, the human form failed to show antiproliferative activity or apoptotic activity. However, when human umbilical vein (HUVE) and human microvascular endothelial cell-lung (HMVE-L) cells were tested, the human form inhibited proliferation and caused apoptosis and G_1 cell cycle arrest (14). The distinction may be due to subtle differences in the endostatin-receptor mechanisms across species.

Based on the sequence differences of human and murine endostatin and the distinct *in vitro* activities of the two forms, we investigated the more complex *ex vivo* rat aortic ring assay model to compare the biological activity of human and murine endostatin. We report the rat aortic ring assay supports the antiangiogenic activity of murine endostatin at high concentrations, but not the human form. However, modifications to this model with human tissue has provided evidence for the biological activity of human endostatin, suggesting species-specificity of the human form in the rat *ex vivo* model. Taken together, our data and previous *in vitro* data suggest the human and murine form of endostatin may have subtly different effector properties on different tissue types, which may impact the relative potency of the antiangiogenic effect. Discovery of a putative endostatin receptor will provide a better understanding of the emerging subtle distinctions in biological activity of murine and human endostatin.

METHODS

Rat aortic ring cultures. Twelve-well tissue culture grade plates were covered with 250 μl of Matrigel (Becton–Dickinson, Bedford, MA) and allowed to gel for 30–45 min at 37°C, 5% CO_2. Thoracic aortas were excised from eight to ten week-old male Sprague–Dawley rats (range 292–307 g) and the fibroadipose tissue was

removed. The aortas were sectioned into 1 mm long cross sections, rinsed eight times with EGM-2 (Clonetics Corp.), placed on the Matrigel coated wells, covered with an additional 250 μl Matrigel, and allowed to gel for 30–45 min at 37°C, 5% CO_2. The rings were cultured for 24 h in 2 ml of EGM-2. After the 24-h incubation, the medium was removed and replaced with 1 ml EBM (Clonetics Corp.), supplemented with 2% FBS and 10 μg/ml gentamicin. Recombinant murine endostatin (Calbiochem, San Diego, CA) was reconstituted in EBM and added as a single treatment on Day 1. Carboxyamidotriazole (CAI) and suramin were tested as positive controls in DMSO (v/v $<$ 0.5%) (NCI, Bethesda, MD) (17, 18). Aortic rings were photographed on Day 5.

Human venous ring cultures. A specimen of human saphenous vein was harvested during the course of surgery on an IRB approved protocol. Veins were cut into 2-mm-long cross sections, rinsed in EGM-2, and cultured under the same conditions as above. Human veins were treated on Day 1 with recombinant human endostatin (EntreMed, Rockville, MD) and photographed on Day 14.

Quantitation of ring microvessel outgrowth. Microvessel outgrowth area was quantified using the NIH Image 1.62 Software (NIH, Bethesda, MD). Briefly, ring cultures were photographed and a digital image was generated. Images were analyzed by manually encircling the outgrowth area and computing square pixels. Mean pixel area was computed from duplicate images. Statistical analysis was performed using the Kruskal–Wallis, Spearman rank, or Wilcoxon rank-sum test as noted.

Factor VIII staining/BrdU incorporation assay. Rat aortic segments cultured in control media were rinsed thoroughly with phosphate-buffered saline (PBS), pH 7.4, and frozen at −70°C in OCT compound (Sakura Finetek, Torrance, CA). Rings were cut into 8-μm sections and DNA synthesis was assessed according to the instructions of the Boehringer Mannheim 5-bromo-2′-deoxy-uridine Labeling & Detection Kit II (Indianapolis, IN). Additionally, the presence of endothelial cells in new microvessel growths was confirmed by staining the aortic sections for Factor VIII-related antigen as previously described (19). Frozen section preparation and staining for Factor VIII-related antigen and CD34 were performed by Molecular Histology, Inc. (Gaithersburg, MD).

Electron microscopy. Electron microscopy was performed on the aortic sections to assess the formation of basement membranes of the vessel walls. Areas exhibiting cellular outgrowths from the aortic rings and embedded in Matrigel were marked on the tissue culture flask with a pen using an inverted microscope. The cells were fixed *in situ* in the tissue culture flask with 2.5% glutaraldehyde in phosphate buffer saline (pH 7.4), postfixed in 1% osmium tetroxide, and dehydrated through graded alcohols and propylene oxide. Subsequently, the marked areas were removed from the tissue culture flask en-bloc in Matrigel, cross-sectioned and embedded flat in Maraglass 655 (Ladd Research Industries, Burlington, VT). Ultrathin sections were stained with uranyl acetate-lead citrate and examined with a CM10 Philips electron microscope (Mahwah, NJ).

RESULTS

Murine Endostatin

Recombinant murine endostatin was reconstituted in endothelial cell basal media (EBM) and incubated on rat aortic rings at varying concentrations for 5 days. The concentrations tested ranged from 2 μg/ml to 500 μg/ml. Carboxyamidotriazole and suramin have both been used as positive controls in this model (16, 17) and inhibited outgrowths at 12 μg/ml and 50 μg/ml, respectively (Figs. 1B and 1C). Complete inhibition was observed at the highest concentration of endostatin tested, 500 μg/ml, with loss of inhibition starting below 100 μg/ml (Figs. 1D–1I). Quantitative image analysis confirmed the dose-dependent activity of murine endostatin against rat microvessel outgrowth. There was no statistical difference of outgrowth area for control wells across plates by the exact Kruskal–Wallis test. The Spearman rank correlation coefficient between concentration and the outgrowth area was −0.90 ($p <$ 0.0001 for the null hypothesis, or no correlation). Statistically significant inhibition was achieved at concentrations ranging from 24 500 μg/ml (Fig. 2).

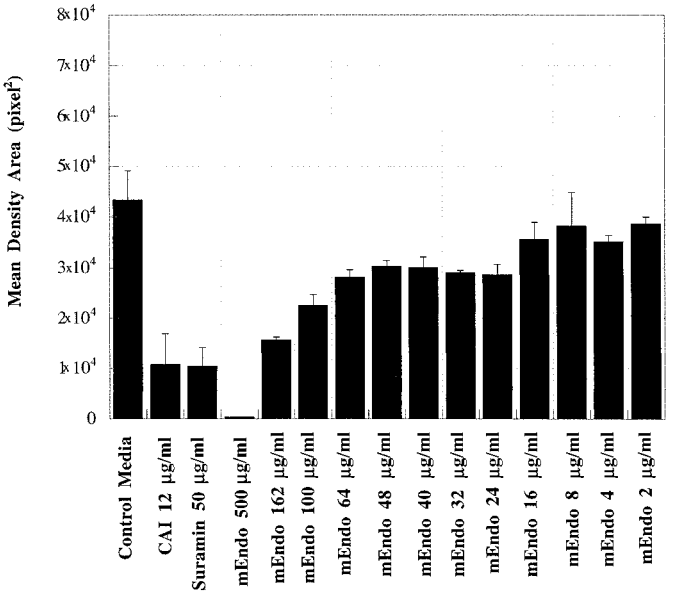

FIG. 2. Quantification of microvessel outgrowth with murine endostatin treatment on rat aortic rings. Image analysis of rat aortic rings demonstrate dose-dependence of antiangiogenic activity of murine endostatin ranging from 2 to 500 μg/ml. Error bars represent standard deviations of duplicate assays. The Spearman rank correlation coefficient between concentration and outgrowth area = −0.90.

Factor VIII and BrdU Incorporation

To confirm the outgrowths from the aortic rings were endothelial cells, treated and control rings were removed from the Matrigel and frozen for section analysis. BrdU incorporation assays on sectioned rings confirmed the presence of proliferating cells (Fig. 3A). Furthermore, factor VIII-related antigen staining on the outgrowths also supported the presence of endothelial cells (Fig. 3B). The histochemical data suggest the outgrowths were proliferating endothelial cells.

Basal Lamina and Lumen Formation

Electron micrographs were prepared to analyze the morphology of the aortic outgrowths. The micrographs

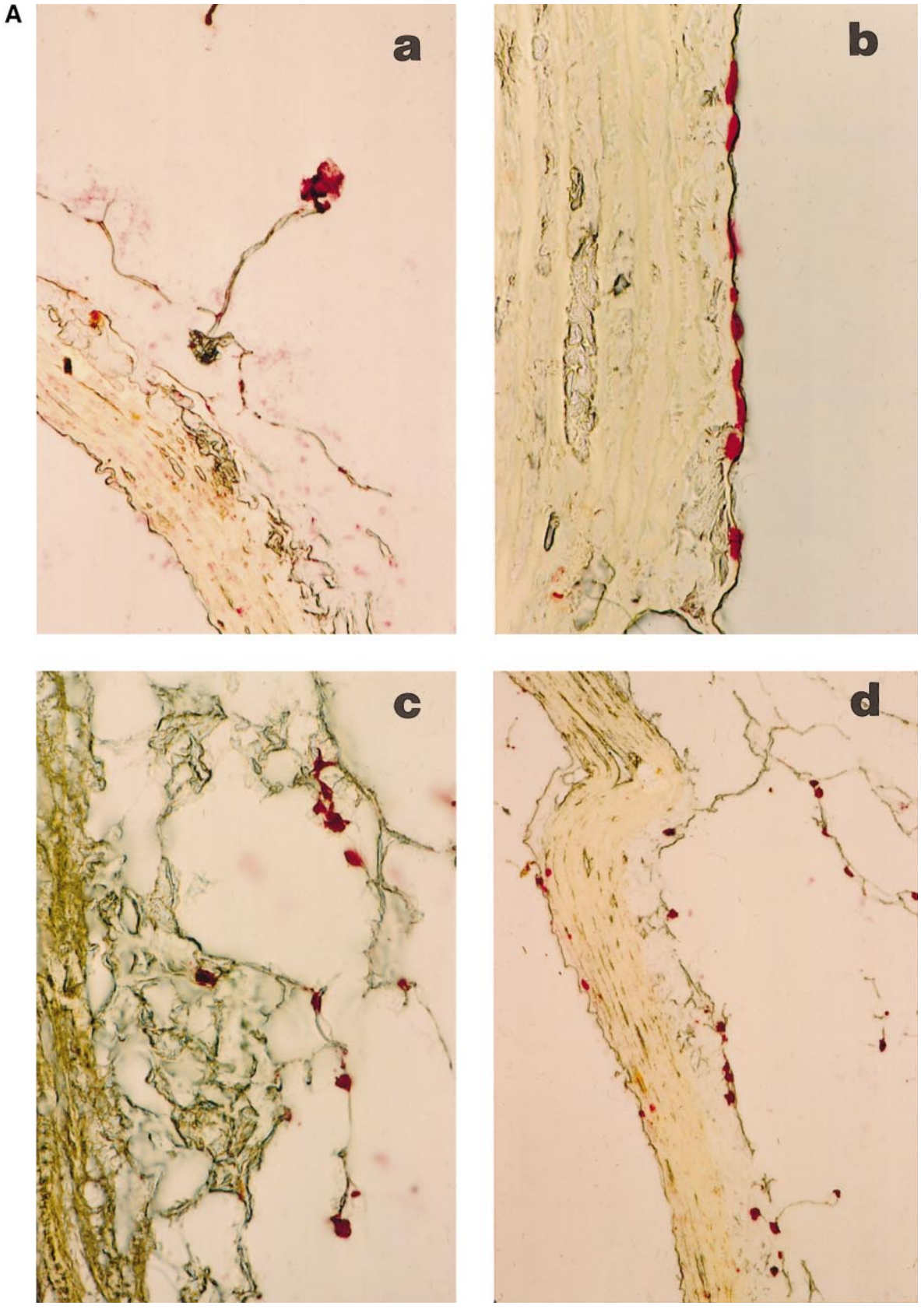

FIG. 3. Histochemical staining for 5-bromo-2′-deoxyuridine (BrdU) and Factor VIII on rat aortic endothelium. (A) BrdU uptake in vascular endothelial cells demonstrating proliferation activity. Note the linear spread exterior to the aortic wall in (b) and extraaortic proliferation in (c). (d) Shows both linear and externalization of cells. Original magnification ×100. (B) Immunohistochemical Factor VIII labeling of adventitial endothelial cells. Chromogen is diaminobenzidine. Original magnification ×200.

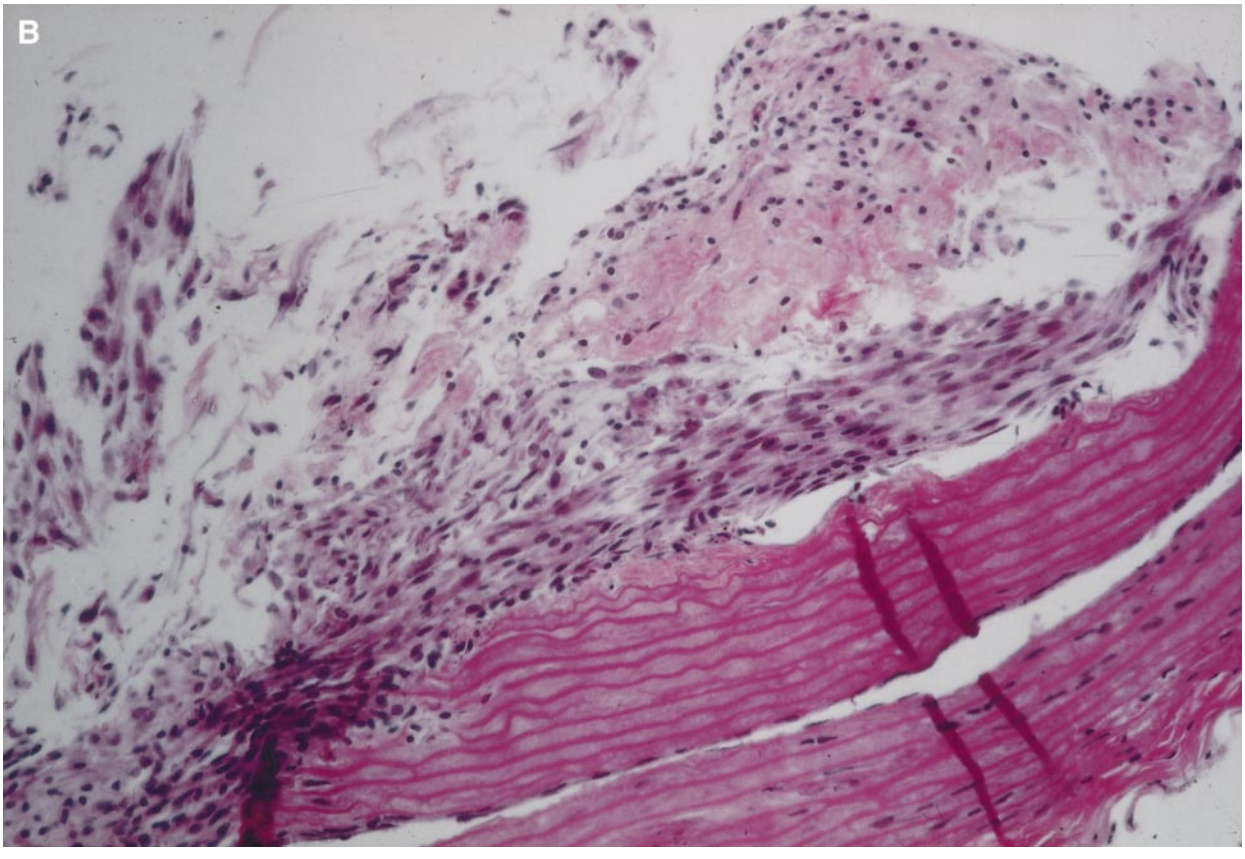

FIG. 3—*Continued*

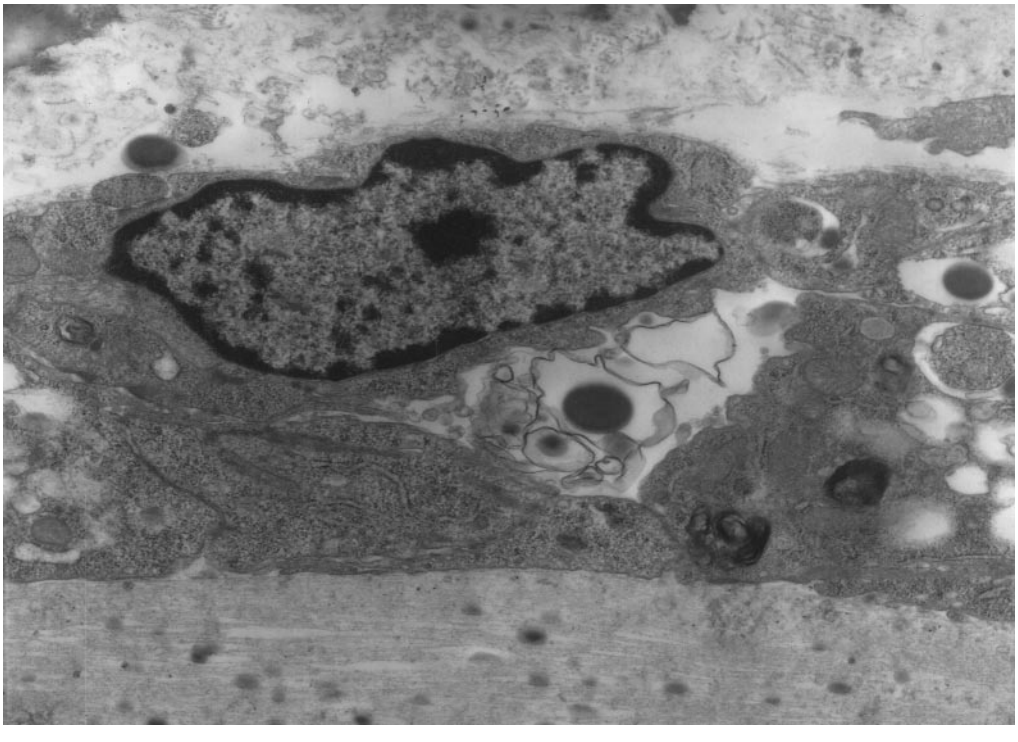

FIG. 4. Electron micrograph of endothelial cells (EC) basal lamina and lumen formation in rat aortic neovessels. Electron microscopy confirmed the formation of a basal lamina by outgrowing endothelial cells. The micrograph also suggests three dimensional organization of ECs into a lumen-like structure. Original magnification ×25.

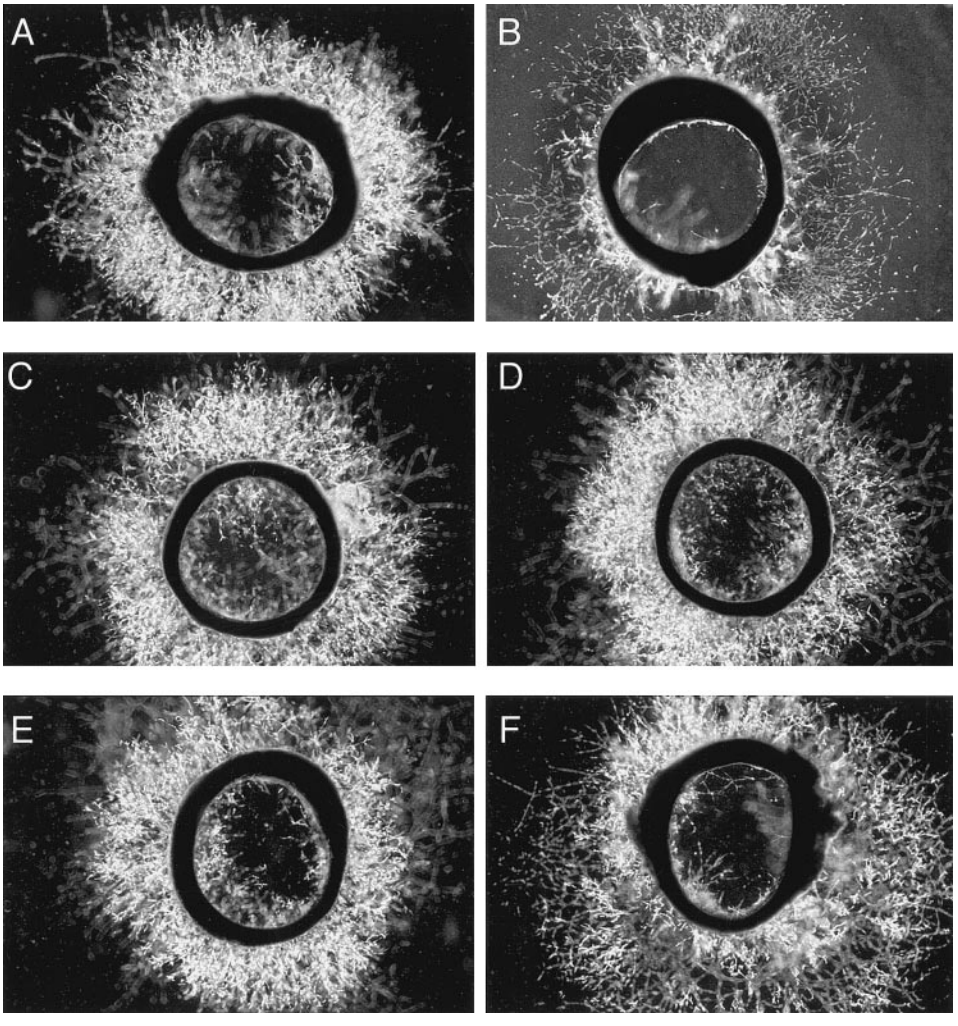

FIG. 5. Lack of antiangiogenic activity of human endostatin on rat aortic rings. (A) Control medium. (B) Suramin at 50 μg/ml. (C) 500 μg/ml endostatin. (D) 250 μg/ml endostatin. (E) 125 μg/ml endostatin. (F) 63 μg/ml endostatin. Photographs are representative rings from duplicate assays.

confirmed the formation of basal lamina in proliferating cells and the formation of a lumen-like structure (Fig. 4). These studies confirmed the presence of proliferating endothelial cells (BrdU, Factor VIII) in the aortic outgrowths along with formation of basement membranes, suggesting neovascularization from the aortic wall and the microvessels observed were progenitors of patent vessels.

Human Endostatin

Recombinant human endostatin (EntreMed, Inc.) was tested on the rat aortic ring assay. Even at comparable concentrations to the mouse range of activity, i.e., 500 and 250 μg/ml, human endostatin did not inhibit microvessel formation compared to controls (Fig. 5). CAI was used a positive control. To investigate whether the activity of the human form was sensitive to the length of incubation, daily doses of fresh human endostatin were tested on rat aortic rings, but failed to significantly inhibit microvessel formation (data not shown).

Next, we investigated a human model of the aortic ring assay to compare against the rat system. Control experiments with sections of human adrenal veins indicated the optimal incubation time would be approximately 10–14 days (data not shown). Human endostatin inhibited the outgrowth from human vein tissue at 250 or 500 μg/ml (Figs. 6 and 7). Statistical analysis showed a trend towards significant inhibition between human endostatin treated veins and controls ($p = 0.13$) by the exact version of the Wilcoxon rank-sum test.

Human Vein Sections Were Positive for CD34

Sections of the human saphenous vein rings were frozen and analyzed for CD34 immunoreactivity. The human outgrowths were positive for CD34, supporting

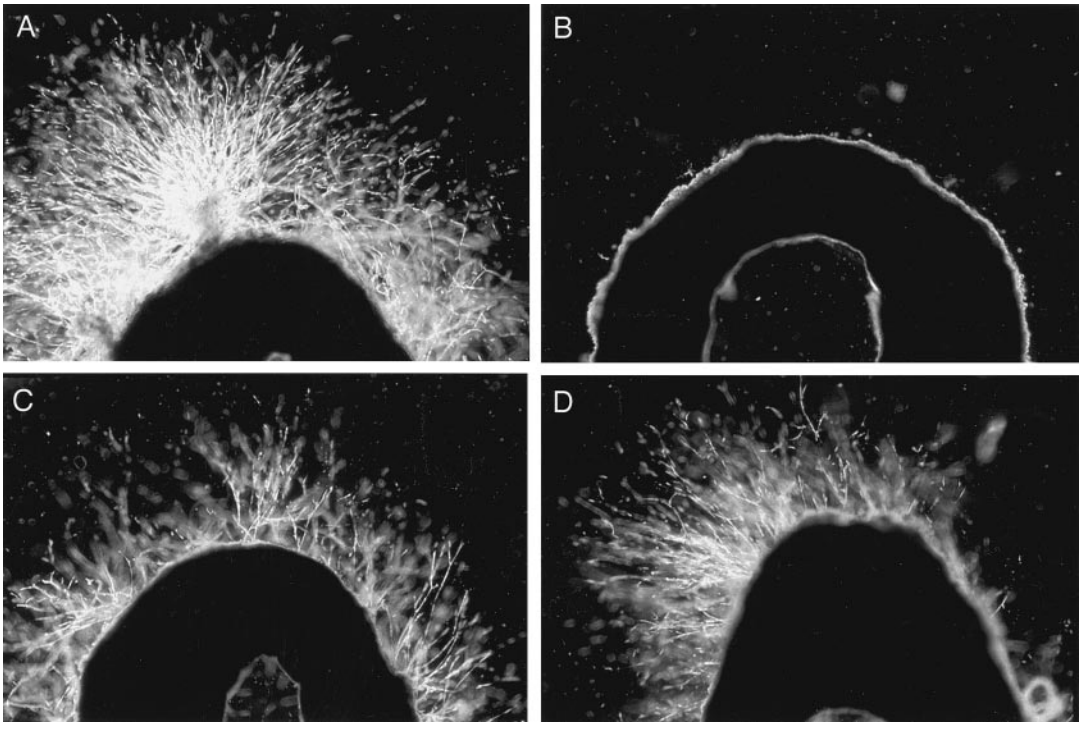

FIG. 6. Antiangiogenic activity of human endostatin on human vein outgrowths. (A) Control medium. (B) CAI at 12 μg/ml. (C) 500 μg/ml endostatin. (D) 250 μg/ml endostatin. Photographs are representative rings from duplicate assays.

the presence of endothelial cells in the microvessel outgrowth (Fig. 8).

DISCUSSION

The present study indicates the antiangiogenic effect of endostatin in the rat aortic ring angiogenesis assay is species-specific. Mouse endostatin inhibited vessel outgrowth at high concentrations (100 to 500 μg/ml range) and was dose-dependent. Human endostatin failed to significantly inhibit vessel outgrowth in this model. To confirm the outgrowths were proliferating endothelial cells and progenitors of patent vessels, aortic ring sections were analyzed for BrdU incorporation by *in situ* staining, immunoreactivity with endothelial-specific antibodies by frozen section staining, and lumen formation morphology by electron microscopy. The histological data from these studies confirmed the presence of proliferating endothelial cells with basal lamina and three dimensional structure that resemble lumen formation. In summary, the rat aortic outgrowths correlate with microvascular events of *in vivo* neovascularization, and while mouse endostatin was an active antagonist against these events, the human form was not. Interestingly, the μg/ml concentration range showing activity for the murine for is much higher than previously reported ng/ml concentrations of endostatin needed for an endothelial cell antiproliferative effect *in vitro* (6, 14, 15).

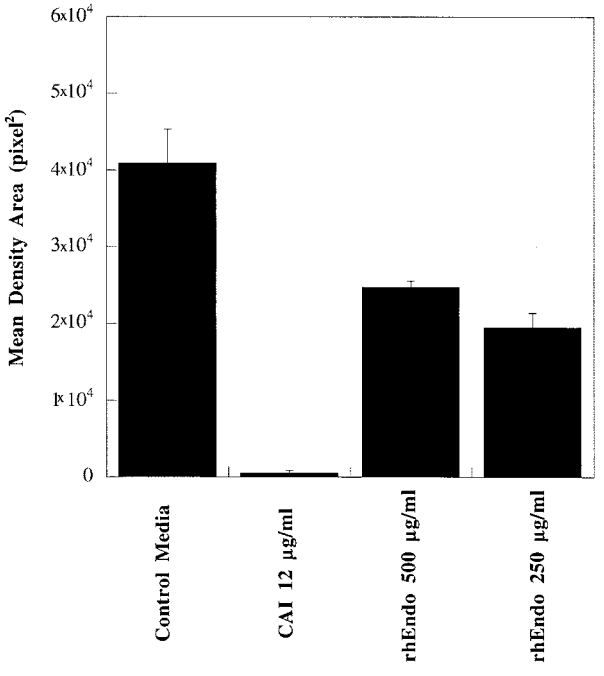

FIG. 7. Quantification of microvessel outgrowth with human endostatin treatment on human vein rings. Image analysis of outgrowth area demonstrated inhibition by human endostatin at 250 μg/ml and 500 μg/ml. Error bars represent standard deviations of duplicate assays. The Wilcoxon rank sum test for treated vs control veins determined, $p = 0.13$.

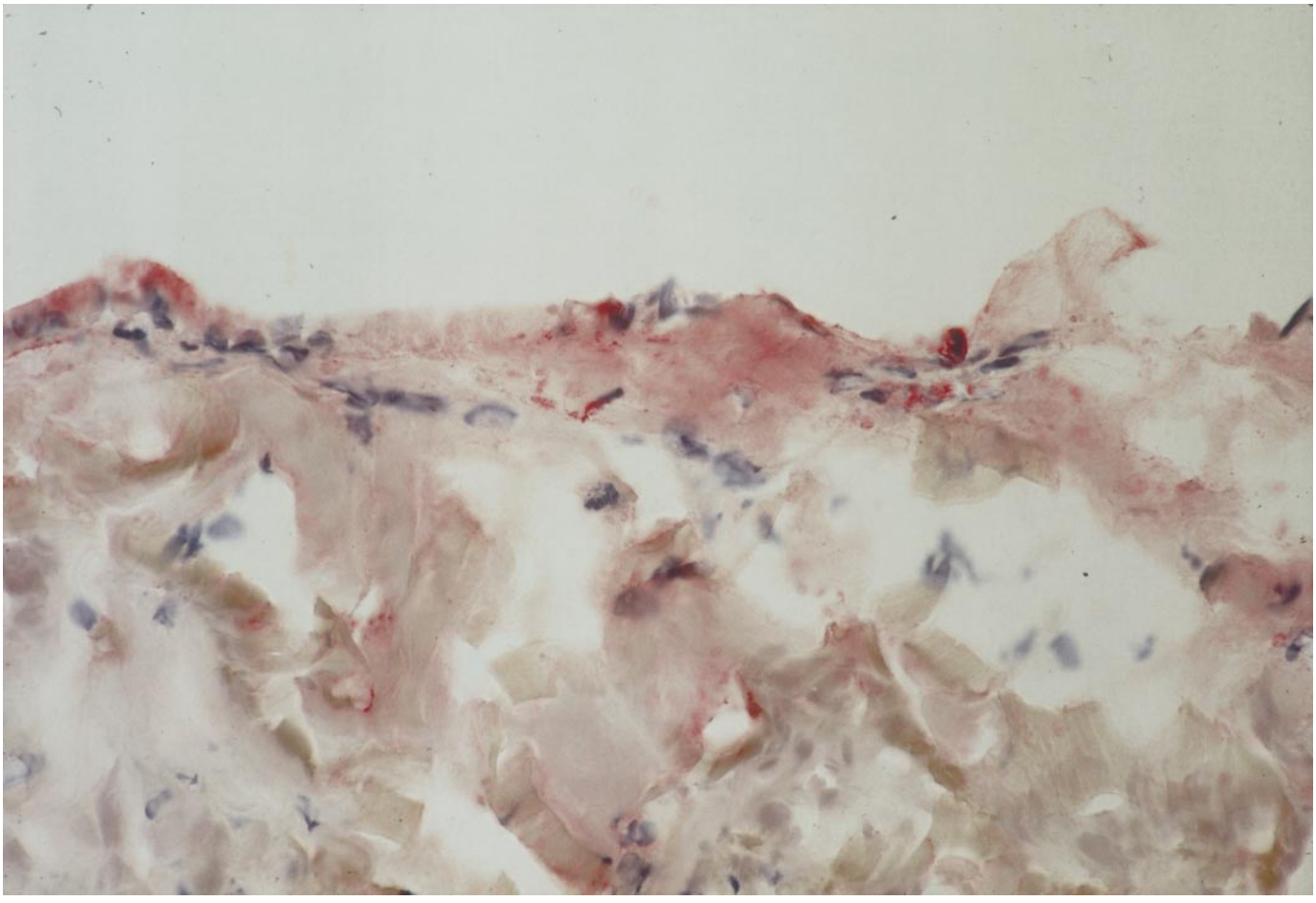

FIG. 8. High magnification of immunohistochemical labeling for CD34 expression on human endothelial cells still attached to the venous wall, ×400.

The lack of activity of human endostatin in this model prompted an investigation of a human system. A specimen of human saphenous vein was sectioned and treated with human endostatin. After fourteen days, human endostatin demonstrated inhibitory activity of vessel outgrowth at 250 and 500 μg/ml. To compare the immunoreactivity of the human tissue to the rat, sections of the human vein were prepared and analyzed for CD34. The CD34-positive results suggest, like the Factor XVIII positive rat sections, that the human outgrowths were endothelial cells. To our knowledge, this is the first report of the antiangiogenic activity of human endostatin on human tissue other than *in vitro* antiproliferative effects.

There are several possible explanations for the distinct biological activity profiles of the human and mouse endostatin in the rat aortic ring assay. One is species-specificity of the as yet unknown endostatin receptor. The murine endostatin may cross react with rodent vascular tissue receptors while the human form may not. This species-specificity may be influenced by the differences in the amino acid sequence between the two forms. While the 12 N-terminal amino acids and C-terminal lysine of the recombinant human form were present in the protein used in this study, differences in internal amino acid sequence between the human and the mouse form may be significant enough to alter the protein folding and tertiary structure of endostatin.

Another interesting possibility is the effect of the expression system on the activity of recombinant endostatin products. It has recently been noted that the presence of the C-terminal lysine of both forms is sensitive to the recombinant expression system used. Boehm *et al.* have observed the C-terminal lysine of murine endostatin to be missing in a *Pichia pastoris* expression system (16). Full-length murine and human endostatin in this study were cloned by disrupting the *P. pastoris* KEX1 gene, encoding a carboxypeptidase, which in *Saccharomyces cerevisiae* has been known to cleave lysine and arginine residues from the C-terminus of peptides and proteins (16). It is unknown what effect the C-terminal lysine will have on the antiangiogenic activity of both endostatin forms (16). It is noted that even the same expression system (*P. pastoris*) yields slightly different recombinant human forms of endostatin.

Our data from the rat aortic ring assay provide a distinct system in which mouse endostatin exhibits potent antiangiogenic activity. While the human form is not active against rat outgrowths, preliminary data from our human system suggest it is active against human vascular tissue. Our data supports the published *in vitro* activity of human endostatin, which has been specific and limited to human derived cell lines (14, 15). Currently, we are exploring other sources of human tissue to validate the human ring assay. Further investigation into endostatin's mechanism of action will provide more insight into the subtle but distinct biological activity profiles of human and mouse endostatin.

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