Anti-angiogenic activity of human endostatin is HIF-1-independent *in vitro* and sensitive to timing of treatment in a human saphenous vein assay

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Abstract

Endostatin is a 20-kDa endogenous angiogenesis inhibitor that has recently been shown to inhibit the expression of vascular endothelial growth factor (VEGF), an angiogenic growth factor that is up-regulated by hypoxia via the HIF-1 transcription factor complex. To determine if the anti-angiogenic activity of endostatin involves a modulation of the HIF-1/VEGF pathway in cancer cells, experiments were conducted to establish what effect endostatin has on HIF-1 activity, HIF-1α protein production, and cellular localization in prostate cancer cells and endothelial cells. Endothelial cell tube formation was inhibited by endostatin purchased from Calbiochem (San Diego, CA) but not endostatin obtained from EntreMed (Rockville, MD). Subsequent experiments using Calbiochem endostatin showed that it did not alter HIF-1α protein production or cellular localization in any of the cell lines tested, nor did it alter HIF-1 transactivational activity in hypoxia. Whether or not this is also true *in vivo* remains to be determined. Nevertheless, these data suggest that the anti-angiogenic activity of endostatin is independent of the HIF-1/VEGF pathway. Immunocytochemical staining results do not indicate a decreased production of VEGF in Calbiochem endostatin-treated LNCaP or human umbilical vein endothelial cells (HUVEC). Treatment of rat aortic cross sections with human endostatin from Calbiochem resulted in a dose-dependent inhibition of microvessel outgrowth. Importantly, inhibition of vessel outgrowth by Calbiochem endostatin in a human saphenous vein angiogenesis assay required early treatment. In view of this *in vitro* data, we suggest that clinical trials involving endostatin treatment of late-stage disease may not adequately represent the efficacy of this drug in early-stage cancer. (Mol Cancer Ther. 2003;2:845–854)

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Introduction

Endostatin is a cleavage product from the COOH-terminal, non-helical portion of the collagen XVIII NC1 domain (1). It was first identified in a murine hemangioendothelioma cell line and subsequently shown to be a powerful anti-angiogenic peptide and inhibitor of endothelial cell proliferation (1). Endostatin had dramatic anti-tumor activity in mice treated s.c. A nearly complete regression of Lewis lung, melanoma, fibrosarcoma, and hemangioendothelioma primary tumors was achieved with 20 mg/kg daily endostatin treatments, with no observed toxicity. The anti-tumor activity was determined to be the result of an inhibition of endothelial cell proliferation, which in turn led to a high degree of tumor cell apoptosis (1). The results of other *in vivo* studies demonstrated the inhibitory effects of endostatin on rat primary mammary tumors (2) and renal tumors in nude mice (3).

The molecular details of the anti-angiogenic and pro-apoptotic activity of endostatin are under intense investigation following demonstration of its dramatic anti-tumor effects *in vivo*. Insight into the mechanism of endostatin's biological function arose following the determination of its crystal structure (4). Structural analyses indicated a high affinity for heparin (5–7). Structural studies thus suggest a function for the endostatin domain of collagen XVIII in mediating interactions with heparin sulfate proteoglycans either in the cell membranes or extracellular matrix (5). The anti-tumor activity of endostatin has been suggested to involve binding of heparin sulfate proteoglycans and subsequent disruption of mitogenic growth factor signals (5). Interestingly, domain NC1 is a stronger ligand than endostatin for sulfatides and the basement membrane proteins laminin-1 and perlecan (8). Thus, endostatin and NC1 appear to have distinct functions. Cleavage of endostatin from the NC1 domain via matrix metalloproteinase (MMP), cathepsin, and elastase activity (9, 10) is suggested to change it from a matrix-associated form to a soluble form (8), likely for the purpose of regulating unchecked vessel outgrowth and branching (1, 7). These observations are consistent with those of Dr. Judah Folkman's group that endostatin renders distant metastases to a state of dormancy (1, 11).

The mechanism whereby endostatin inhibits angiogenesis is not fully understood, although recent data indicate that endostatin can bind integrins on the surface of endothelial cells (12) and can disrupt focal adhesions of plasminogen activator to disrupt the cytoskeleton (13). Endostatin also down-regulates many growth- and apoptosis-related genes in endothelial cells (14), increases cellular Ca^{2+} concentration (15), and inhibits cyclin D1 (16). It is interesting to speculate therefore, that the antiproliferative activity of endostatin involves its binding cell surface proteins and producing ''outside-in'' signals for promotion of apoptosis and down-regulation of genes involved in the production of factors that degrade the extracellular matrix.

Another plausible, yet unexplored target mechanism of endostatin is the cellular hypoxic response. Hypoxia is a major pro-angiogenic phenomenon in solid tumors (17) that promotes angiogenesis via the HIF-1 transcription factor complex. Focal areas of hypoxia result as solid tumors grow and internal sites become further removed from the vasculature that nourishes the periphery (18). A positive feedback growth factor signaling loop results as hypoxia stimulates the synthesis of pro-angiogenic signals, most importantly the growth factor VEGF (vascular endothelial growth factor). More vasculature is recruited, the tumor grows, and the cycle continues. The molecular determinants of the hypoxic response are under intense investigation because this system represents a potentially useful therapeutic target to prevent angiogenesis and tumor growth. Cells constitutively produce HIF-1α, a transcription factor that can bind to a partner protein HIF-1β and trans-activate genes involved in angiogenesis as well as aerobic glycolysis. In normoxia, a specific prolyl hydroxylase oxidizes the HIF-1α subunit, allowing for the VHL protein to target it for proteasomal degradation. In hypoxia, HIF-1α is not hydroxylated and thus not degraded by VHL. It translocates to the nucleus, dimerizes with HIF-1β, and activates genes that contain a specific hypoxia responsive element (HRE). Such genes include VEGF, erythropoietin, and glycolytic genes (18). The list of known HIF-1-regulated genes continues to grow, although in general, the functions of these genes seem to function in cancer progression or glycolysis.

Published studies show that endostatin inhibits migration and proliferation of endothelial cells but not cancer cells (1, 19). However, the existence of a molecular effect on cancer cells has not been ruled out. We hypothesized that endostatin could interfere with angiogenesis indirectly by blocking HIF-1-mediated induction of VEGF expression in prostate cancer cells. The effect of endostatin on the HIF-1/VEGF pathway, to the best of our knowledge, has not been explored. Interestingly, endostatin purchased from Calbiochem (San Diego, CA) but not that obtained from EntreMed (Rockville, MD) could inhibit endothelial cell tube formation, prompting us to use Calbiochem endostatin in all subsequent bioassays. We also tested the effect of Calbiochem endostatin on the trans-activational activity of HIF-1 in a glioblastoma cell line.

Materials and Methods

The recombinant human endostatin used in our experiments is produced in a *Pichia pastoris* expression system by EntreMed and supplied to Calbiochem as 1 mg/ml preparations in 66 mM sodium phosphate dibasic, 17 mM citric acid, 59 mM NaCl, pH 6.2. We received endostatin (7.9 mg/ml) directly as a generous gift from EntreMed. We also separately purchased Calbiochem's endostatin. The separate preparations of endostatin used in our experiments are thus referred to as EntreMed and Calbiochem endostatin. Endostatin concentrations used in the tube formation assay reflect those previously shown to be effective in similar assays (20). Concentrations used in all other experiments reflect the range required to elicit a dose-dependent inhibition of angiogenesis in our rat aortic ring assays (21).

Endothelial Cell Tube Formation Assay

Twenty-four-well plates were coated with 100 μl of Matrigel (Becton Dickinson, Bedford, MA). Human umbilical vein endothelial cells (HUVEC) of less than passage 7 were then plated in duplicate at a concentration of 3×10^4 cells/well in 500 μl of EGM-2 (endothelial cell growth media) medium (Clonetics Corp., San Diego, CA) supplemented with 2% fetal bovine serum in the presence or absence of various concentrations of EntreMed endostatin (197.5, 395, 790, 1580, and 3160 ng/ml) or Calbiochem endostatin (62.5, 125, 250, 500, and 1000 ng/ml), and incubated at 37°C for 16 h. Subsequently, each well was washed in PBS, fixed in 100% methanol for 10 s, and stained with 50% DiffQuick solution II (Dade Behring Inc., Newark, DE). To analyze tube formation, four random fields of each well were digitally photographed. Tube area was quantified as the total number of pixels in thresholded images using MetaMorph software (Universal Imaging Corp., West Chester, PA). All experiments were repeated at least twice. Results were presented as mean ± SD. Comparisons were made with ANOVA followed by Dunnett's test with $P < 0.05$ as the criterion for statistical significance.

Mass Spectrometry

Mass spectra were acquired to compare endostatin samples obtained from both EntreMed and Calbiochem stocks. Protein solutions were made to 8 M urea and 10 mM DTT in 25 mM NH_4HCO_3, pH 8.2, and incubated at 56°C for 1 h. Solutions were subsequently made to 55 mM iodoacetamide and incubated for 45 min at ambient temperature in the dark. Solutions were diluted to give a final urea concentration less than 1 M, porcine trypsin (Promega, Madison, WI) was added to give a trypsin-to-protein ratio of 1:50 (w/w), and the sample incubated for 16 h at 37°C. Samples were desalted with C18 ZipTips (Millipore, Bedford, MA) as per manufacturer's protocols and stored at −20°C until mass spectral analysis. A sample (0.25 μl) was co-crystallized with 0.25 μl of α-cyano-4-hydroxycinnamic acid (20% w/v) in 50% ACN, 1% trifluoroacetic acid and spotted directly on a stainless-steel matrix-assisted laser desorption ionization (MALDI) plate. Mass spectra were acquired using an Applied Biosystems 4700 MALDI-TOF/TOF (time of flight) mass spectrometer (Applied Biosystems, Foster City, CA). For all mass spectra, the laser frequency

was 200 Hz, and for collision-induced dissociation (CID), the collision energy was 1 keV, using air as the collision gas. Spectra were visually inspected, and a number of peptides were chosen for collision-induced dissociation. MALDI spectra were internally calibrated (< 20 ppm) using trypsin autolysis products. Post-acquisition baseline correction and smoothing was carried out using software provided with the TOF/TOF instrument. Spectra were submitted to ProteinProspector (http://prospector.ucsf.edu/) for MS-Fit (peptide fingerprinting) or MS-Tag (tandem MS sequencing) database searching.

High Performance Liquid Chromatography Analysis of EntreMed and Calbiochem Endostatin

Endostatin (300 μl each of EntreMed and Calbiochem stock) was centrifuged in Centricon-3 spin columns (Millipore) for 1 h at 7500 × *g*. Fifty microliters of retentate were injected into a Waters Nova-Pak C18 high-performance liquid chromatography (HPLC) column (3.9 ft × 300 mm × 4 mm) and separated at room temperature at a flow rate of 1.0 ml/min. UV detection levels were 220, 248, and 215 nm (Hewlett Packard 1100 Series). The mobile phase consisted of distilled water and acetonitrile in a 30-min run consisting of a linear gradient of 5–50% acetonitrile (v/v).

Rat Aortic Ring Angiogenesis Assays

Twelve-well tissue culture grade plates were coated with 250 μl Matrigel (Becton Dickinson) and allowed to solidify for 30 min at 37°C, 5% CO_2. Thoracic aortas were excised from 7- to 10-week-old Sprague-Dawley rats and the fibroadipose tissue removed. The aortas were cut into 1-mm-long cross sections, rinsed eight times with EGM-2 media (Clonetics), placed on the Matrigel-coated wells, covered with an additional 250 μl Matrigel, and incubated for 30 min at 37°C, 5% CO_2. The rings were cultured for 24 h in 1 ml EGM-2. Following 24 h incubation, the medium was replaced with 1 ml of EBM (Clonetics) supplemented with fetal bovine serum (2%), ascorbic acid, hydrocortisone, heparin, and amphotericin. Calbiochem endostatin was prepared in EBM at the desired concentrations and added to the rings as single bolus treatments on day 1. Rings treated with the known anti-angiogenic compound Carboxyamidotriazole (12 μg/ml) were included as positive controls. All rings were photographed on day 5 and the vascular sprouts quantified as the mean pixel density obtained from image analysis of duplicate rings using Adobe Photoshop (Adobe Systems Inc., San Jose, CA).

Human Saphenous Vein Angiogenesis Assay

Specimens of human saphenous vein were obtained during the course of surgery at the National Cancer Institute, Bethesda, MD on an IRB approved protocol. Veins were cut into 2-mm-thick cross sections, rinsed in EGM-2, and cultured in the same manner as the rat aortic rings with the exception that 300 μl Matrigel were required to coat the plate and cover the ring. Human veins were treated on day 1 with the indicated concentrations of Calbiochem endostatin and photographed on day 10. Vascular sprouts were quantified as the mean pixel density obtained from image analysis of duplicate rings using Adobe Photoshop.

Endostatin ELISAs

HUVEC were seeded at approximately 70% confluence in 96-well culture plates in EGM-2 media (Clonetics) and treated with the indicated concentrations of Calbiochem endostatin after 24 h. The culture media were collected at 1, 24, and 48 h post-treatment time points and stored at −80°C. ELISAs were performed on combined media from duplicate cultures to detect levels of total endostatin. The ELISA detection method employed a polyclonal anti-endostatin antibody that detects total endostatin (Cytimmune, College Park, MD).

Immunoblotting

Prostate cancer cells (PC3) were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) in six-well plates and incubated for 16 h in a hypoxic chamber (Billups-Rothenberg, Inc., Del Mar, CA) at 37°C, 94.5% N_2; 5% CO_2; 0.5% O_2 or in normoxia with or without Calbiochem endostatin at various concentrations. Adherent cells were scraped from the plate and lysed in M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL) supplemented with protease inhibitor cocktail (Pierce) and 5 μM lactacystin (BioTrend, Köln, Germany) at 4°C for 1 h on a rotary shaker. The lysate was freeze-thawed and the insoluble debris removed by centrifugation at 15,000 × *g*. Protein quantification was via bicinchoninic acid (BCA) protein assay (Pierce). Thirty-microgram aliquots of each protein sample were electrophoresed through 4–12% polyacrylamide gels for 1 h at 180 V and the protein transferred to polyvinylidene difluoride membrane via a modified version of the semidry protein transfer method (22). Blotted membranes were blocked with PBS-Tween supplemented with 5% horse serum for 30 min and incubated with mouse monoclonal antibodies to human HIF-1α (Transduction Laboratories, Lexington, KY) at 1:500 dilution or β-actin (Abcam, Cambridge, United Kingdom) at 1:10,000 dilution. For signal detection, blots were incubated with a horseradish peroxidase-conjugated donkey anti-mouse IgG (Jackson Immunoresearch, West Grove, PA) at 1:10,000 followed by Supersignal West Pico Chemiluminescent Substrate (Pierce).

Immunocytochemistry

LNCaP and PC3 prostate cancer cell lines and a HUVEC endothelial cell line were each cultured to approximately 80% confluence on glass coverslips in 24-well plates and incubated with or without indicated concentrations of Calbiochem endostatin for 16 h in a hypoxic chamber (Billups-Rothenberg, Inc.) purged with 94.5% N_2; 5% CO_2; 0.5% O_2 or in normoxia at 37°C. The cells were fixed in 4% paraformaldehyde for 10 min and rinsed three times with PBS. The cells were rendered permeable by incubation for 2 min in 1% Triton X-100, 0.02% BSA in PBS and subsequently treated with blocking buffer (20% donkey serum in 2% BSA/PBS) for 30 min at 37°C. The cells were then incubated for 1 h at room temperature with an antibody mixture containing a mouse monoclonal antibody to human HIF-1α (Transduction Laboratories) at a 1:500 dilution and rabbit polyclonal antibody to human VEGF (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:2000 dilution in 2% donkey serum, 2% BSA/PBS.

Following three PBS rinses at room temperature, the cells were incubated for 2 h with a secondary antibody mixture containing a Texas Red-conjugated donkey anti-mouse secondary antibody (Jackson Immunoresearch) at a 1:2000 dilution and an AlexaFluor-conjugated donkey anti-rabbit secondary antibody (Molecular Probes, Eugene, OR) at a 1:2000 dilution in 2% donkey serum, 2% BSA/PBS. The cells were then washed three times in PBS and the nuclear DNA stained for 15 min with DAPI stain at a 1:10,000 dilution in 2% donkey serum, 2% BSA/PBS. The cells were washed with additional three rinses in PBS and mounted by inversion onto glass slides dotted with Gel/Mount (Biomeda, Foster City, CA). Cells were imaged on a DeltaVision imaging set (Applied Precision, Issaquah, WA). This system consists of an inverted microscope IX70 (Olympus America, Inc., Melville, NY) with a 1.40 NA 60× objective and FITC, rhodamine, and Cy5 filter sets; a Photometrix CH350 12-bit camera (Photometrics, Huntington Beach, CA) with a KAF 1400 chip; and a UNIX-based Silicon Graphics O2 workstation with SoftWoRx software installed. Pixels were not binned, the pixel size was 0.11 mm in x and y. Image size was 512 × 512. Images were scaled to 8 bit manually using the same range of scaling for all the images. Scaling was performed using Metamorph software (Universal Imaging, Downingtown, PA) and further adjustments to scaled images were performed using Adobe Photoshop.

Luciferase Reporter Gene Assay

Luciferase reporter experiments were performed using U251-HRE glioblastoma cells (23). These cells contain a plasmid construct (pGL3-HRE) in which tandem copies of the HRE recognition sequence for HIF-1 is inserted upstream of a luciferase gene. These cells thus express luciferase in a HIF-1-dependent fashion. U251-pGL3 control cells that express luciferase in a constitutive fashion were also tested. These cells were incubated with doses of Calbiochem endostatin up to 400 μg/ml to assess its effect on HIF-1 transactivational activity. Luciferase activity was recorded by luminometry (Packard TopCount NXT Microplate Scintillation & Luminescence Counter) and plotted as relative luciferase units (RLU), fold luciferase activity relative to media only controls in normoxia, or RLU/fold increase determined by dividing all RLU values by the normoxia media only control value.

Results

Effects of Endostatin on HUVEC Tube Formation

Calbiochem's endostatin significantly inhibited endothelial cell tube formation at 250, 500, and 1000 ng/ml. Lower concentrations did not have any effect on tube formation. Tube formation was not suppressed by EntreMed's endostatin at any of the concentrations tested (Fig. 1). This result prompted us to use endostatin purchased from Calbiochem for all subsequent bioassays described in this paper.

Mass Spectral Analysis of Calbiochem and EntreMed Endostatin

To determine if the endostatin obtained from EntreMed underwent degradation or other changes in chemical profile, tryptic digests of Calbiochem and EntreMed endostatin samples were performed. The full mass spectra of the resulting peptides from each digest are shown (Fig. 2, A and B). No major differences were observed between the two ensembles of peptides on examination of the full mass spectra. One candidate explanation for the inefficacy of the EntreMed stock would be that it contained the D104N

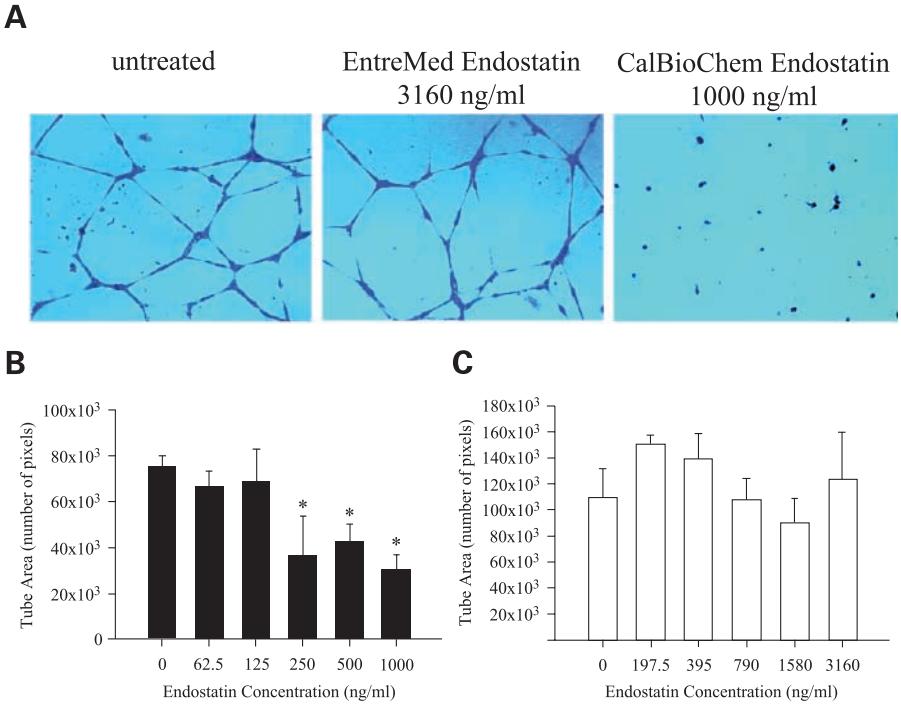

Figure 1. Comparative efficacy of Calbiochem and Entremed endostatin in a human umbilical vein tube formation assay. **A,** graphical depiction of tube formation. Quantitative analysis of tube formation for Calbiochem endostatin (**B**) and EntreMed endostatin (**C**).

mutation present in endostatin from some cancer patients' serum (24). Therefore, expansions of the area of the spectra containing the peak corresponding to Asp_{104} for both samples were inspected (Fig. 2C). The sequences of the tryptic peptides around Asp_{104} (IFSFDGK) both contained Asp at the 104 position as seen by the exact masses observed for both peptides (Fig. 2C). Thus, both Calbiochem's and EntreMed's endostatin samples have the wild-type amino acid sequence.

HPLC Analysis of EntreMed and Calbiochem Endostatin

HPLC analysis of both Calbiochem's and EntreMed's endostatin samples did not reveal differences with regard to retention time or UV profile (Fig. 3). The only observable difference between the two profiles was that more protein was present in the EntreMed sample, consistent with the higher initial concentration of that sample (Fig. 3).

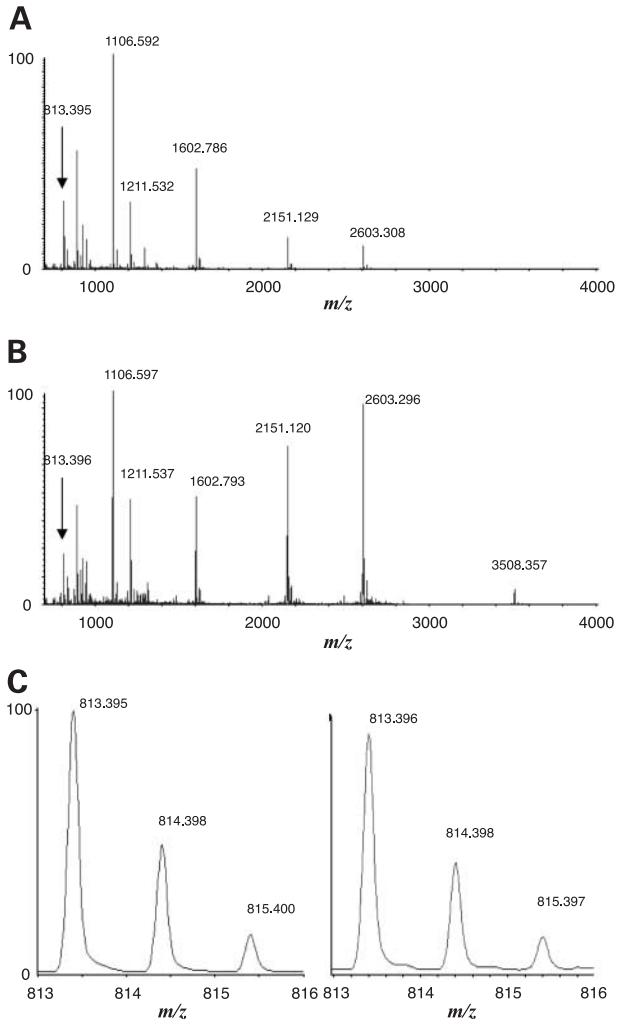

Figure 2. MALDI-QqTOF mass spectra of tryptic digests of endostatin from Calbiochem (**A**) and EntreMed (**B**). Panel **C** illustrates the observed $[M + H]^{1+}$ ions corresponding to the peptide IFSFDGK (theoretical monoisotopic mass = 812.4146 Da) from the Calbiochem (*left panel*) or the EntreMed (*right panel*) endostatin. On the basis of the accurate mass from this experiment, amino acid 104 in each is unequivocally observed to be aspartic acid.

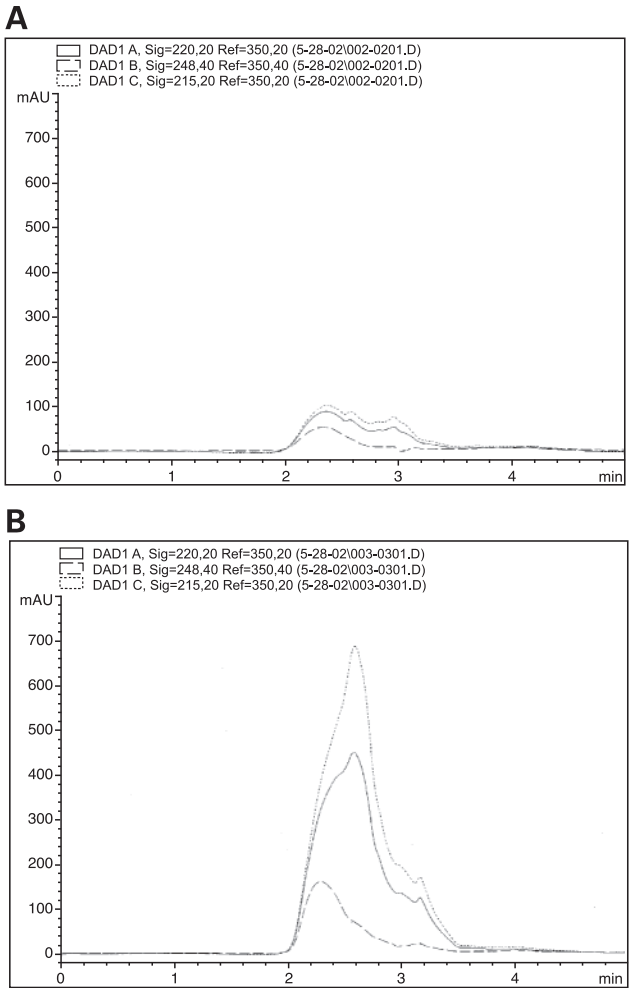

Figure 3. HPLC analysis of Calbiochem (**A**) and EntreMed (**B**) endostatin samples. Identical profiles are observed in the chromatogram for both samples with the exception that the Calbiochem (**A**) sample is less concentrated.

Rat Aortic and Human Saphenous Vein Ring Assays of Angiogenesis

In a previous study (21), we reported that the anti-angiogenic activity of endostatin appeared to be species specific because murine endostatin but not human endostatin had activity in a rat aortic ring assay, despite the high degree of sequence conservation between human and murine endostatin (25). Herein we report results that refute this. Human endostatin purchased from Calbiochem inhibits vessel outgrowth in a dose-dependent manner in the human saphenous vein angiogenesis assay, but this inhibition is sensitive to the timing of treatment, delayed treatment resulting in marked decrease in efficacy (Fig. 4A). Likewise, the same endostatin was able to inhibit vessel outgrowth in the rat aortic ring angiogenesis assay (Fig. 4B).

Endostatin ELISAs

To determine the amount of exogenously administered endostatin that gets degraded by treated endothelial cell

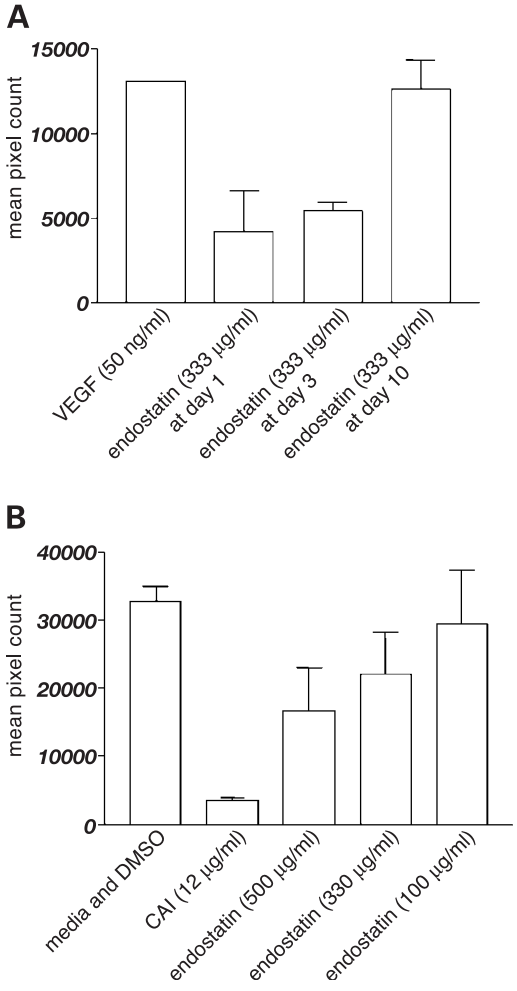

Figure 4. Anti-angiogenic activity of human endostatin (Calbiochem) in aortic ring assays. **A,** timing of treatment influences the efficacy of human endostatin in a human saphenous vein angiogenesis assay. **B,** effect of human endostatin (Calbiochem) in a rat aortic ring assay of angiogenesis.

cultures, ELISAs were performed using an antibody preparation that recognizes total endostatin. Endostatin levels measured at 1, 24, and 48 h time points following initial endostatin treatment did not reveal a decrease in detected total endostatin over time.[1]

Immunoblotting

Both Calbiochem endostatin-treated and untreated PC3 cells produced HIF-1α (Fig. 5). However, HIF-1α production was induced above the normoxia level in cells exposed to 16 h of hypoxia (Fig. 5). There was no observable change in HIF-1α protein production by PC3 cells in response to endostatin at any of the concentrations tested in normoxia or hypoxia (Fig. 5).

[1] G. R. Macpherson, S. L. Forbes, and W. D. Figg, unpublished data.

Immunocytochemistry

In both LNCaP cells (Fig. 6A) and HUVEC (Fig. 6B), HIF-1α levels and nuclear localization increased in hypoxia compared with normoxia. HIF-1α is excluded from the nucleoli, consistent with the results of a previous report describing HIF-1α localization in various human and murine cell lines (26). This increase in HIF-1α was accompanied by increased production of VEGF in both cell types, although to a greater extent in LNCaP cells (Fig. 6). Calbiochem endostatin did not reduce HIF-1α levels or disrupt HIF-1α nuclear localization in LNCaP cells (Fig. 6A) or HUVEC (Fig. 6B) at any of the concentrations tested. VEGF levels likewise appeared not to be reduced in LNCaP or HUVEC in response to endostatin (Fig. 6). Rather, HIF-1α and VEGF levels appear slightly higher in LNCaP cells and HUVEC treated with higher endostatin concentrations relative to untreated controls (Fig. 6, compare II to IV). Interestingly, localized clustering of VEGF is observed in LNCaP cells (Fig. 6A) and to a lesser extent in HUVEC (Fig. 6B) and the degree of clustering appears to increase in response to endostatin treatment in LNCaP cells but not in HUVEC (Fig. 6).

Luciferase Reporter Gene Assay

Calbiochem endostatin did not inhibit HIF-1 transactivational activity of HRE-luciferase at any concentration tested as assessed by measurement of luciferase activity. Interestingly, cells treated with lower concentrations of endostatin had increased HIF-1 activity in a dose-dependent manner relative to untreated controls (Fig. 7).

Discussion

Advanced tumor growth is angiogenesis dependent (27) and endothelial cell organization into vascular networks is a hallmark of the angiogenic process (28). Such organization involves the mobilization of endothelial cells from a quiescent state to form a quasi-mature vasculature that can supply the growing mass with nutrients and oxygen (28). Inhibition of endothelial cell tube formation by endostatin may be due (at least in part) to blockade of the endothelial cell's mobilization stimuli, although it is unclear whether this inhibition requires direct interaction with the endothelial cell or other cells that provide the required stimulatory

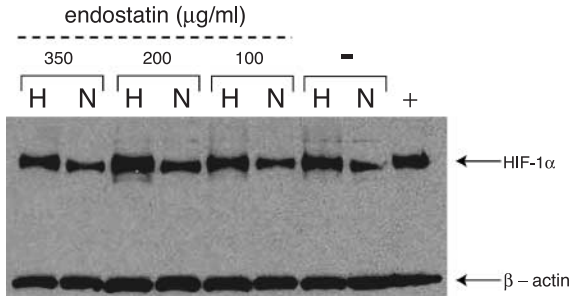

Figure 5. Immunoblot detection of HIF-1α protein lysates from PC3 cells incubated in normoxia or hypoxia and treated with various concentrations of Calbiochem endostatin. Hypoxia (*H*), normoxia (*N*), untreated control (−), and HeLa cells treated with $CoCl_2$-positive control (+).

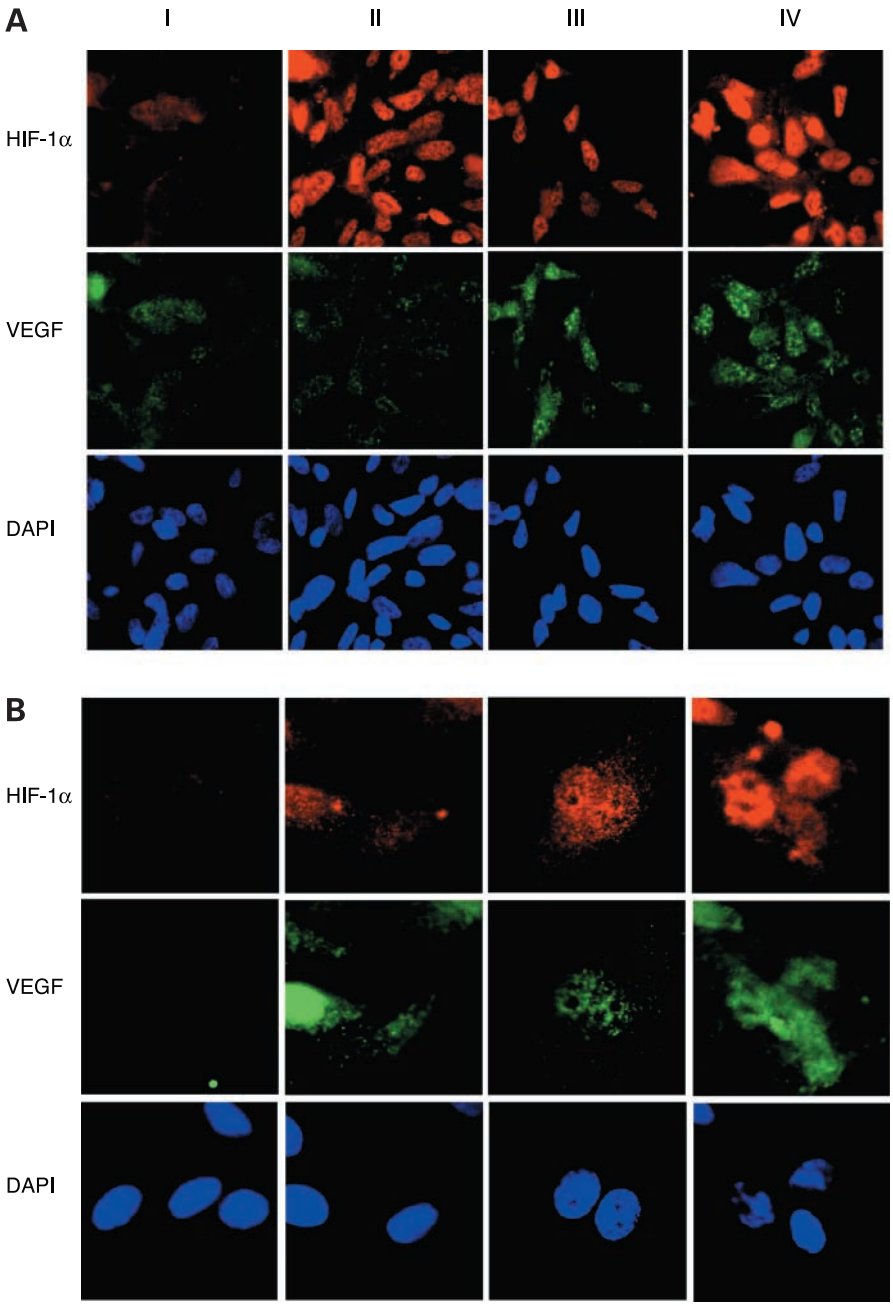

Figure 6. Immunofluorescence detection of HIF-1α and VEGF in LNCaP (**A**) and HUVEC (**B**). Untreated cells incubated in normoxic conditions (*I*); untreated cells incubated in hypoxic conditions (*II*); Calbiochem endostatin (10 μg/ml)-treated cells incubated in hypoxic conditions (*III*); and Calbiochem endostatin (350 μg/ml)-treated cells incubated in hypoxic conditions (*IV*). Corresponding DAPI-stained cell nuclei are shown.

cytokines. Surprisingly, endostatin obtained from EntreMed or Calbiochem had different efficacy profiles in our endothelial cell tube formation assays. This observation was unexpected given that EntreMed produces human endostatin in a *P. pastoris* expression system and supplies it to Calbiochem for packaging and sale. Follow-up experiments were conducted to assess the primary sequences and structural integrity of the endostatin samples. Mass spectral and HPLC analyses verified that the proteins (*a*) were identical with regard to primary amino acid sequence and (*b*) have the same HPLC profile. These data indicate that the difference observed with regard to the efficacy of the two products may be the result of differences in storage or handling conditions such as concentration, temperature, and/or pH rather than a breakdown event or inherent difference in primary amino acid sequence. Changes in tertiary structure resulting from chemical alterations that are below the resolving power of mass spectrometry and HPLC may have hindered efficacy of the EntreMed samples. Clearly proper handling conditions for this drug should be established and consistently maintained so as to avoid false-negative results.

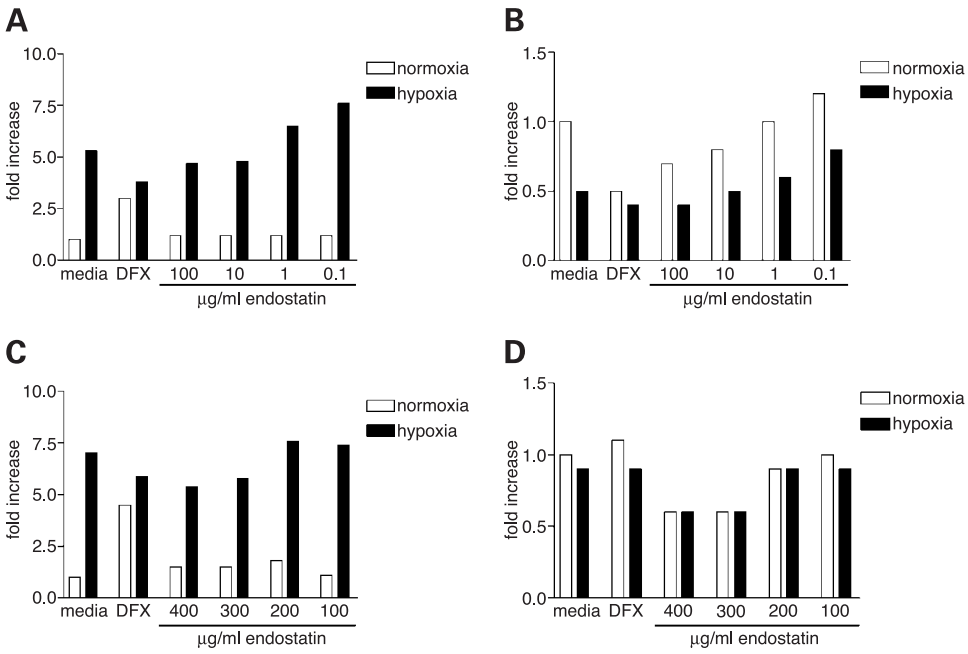

Figure 7. Effect of Calbiochem endostatin on HIF-1-dependent transactivation of luciferase activity in normoxia and hypoxia. Effect of Calbiochem endostatin on HIF-1-dependent activation of luciferase activity in U251-HRE cells (**A** and **C**). Effect of Calbiochem endostatin on HIF-1-independent (constitutive) activation of luciferase in U251-pGL3 cells (**B** and **D**).

Previous data reported by our group suggested that the anti-angiogenic activity of endostatin is species specific because no inhibition of vascular sprout outgrowth was observed in a rat aortic ring angiogenesis assay in response to treatment with the recombinant human form of the drug (21). Murine endostatin, however, did have dose-dependent anti-angiogenic activity in this assay. Likewise, human endostatin had an effect in the rat aortic ring model. Human endostatin was able to inhibit angiogenesis in a human saphenous vein angiogenesis assay (21). Due to limited availability of saphenous vein, we had not been able to test murine endostatin in the human model. Here we show that the anti-angiogenic activity of human endostatin in the human saphenous vein assay is influenced by the timing of treatment — delayed treatment resulting in markedly decreased efficacy. This is a strong indication that endostatin's activity is due to an inhibition of cell function(s) that occur at an early stage of angiogenesis, consistent with (*a*) the suggestion that endostatin's anti-angiogenic activity involves the blockade of cellular mobilization stimuli and (*b*) its observed ability to bind integrins (12) and affect MMP activity (10). Experiments conducted in our lab confirmed that endostatin is not degraded over a 48-h period when administered to proliferating endothelial cells *in vitro*. Taken together, these data suggest that early endostatin treatment and prolonged endostatin half-life synergistically afford effective and sustained inhibition of angiogenesis *in vitro*. Consistent with this are recent clinical data that show poor efficacy in treatment of advanced cancer with endostatin (29) and the hypothesis that sustained endostatin treatment would be expected to maximize its clinical efficacy, particularly if administered in combination with a cytotoxic agent (30). Conceivably, cytotoxic intervention would reduce tumor load and endostatin treatment would inhibit the angiogenic potential of recurring tumors. The requirement for early endostatin treatment in our saphenous vein assay suggests that current clinical trial design, which involves primarily the treatment of late stage disease, underrepresents the true efficacy of endostatin in the treatment of early-stage disease. A recent *in vivo* study with human endostatin is in agreement with our observation that endostatin's anti-angiogenic efficacy is greater when treating early-stage disease (31). These data suggest that future clinical trials should be modified to adequately test therapeutic agents in early-stage disease and include combination trials with chemotherapy and anti-angiogenic agents. We also show in this study that the Calbiochem stock of human endostatin inhibits angiogenesis in the rat aortic ring assay in a dose-dependent manner. Therefore, the activity does not appear to be species specific as was previously suspected. The reported inactivity of human endostatin in the rat ring model may have been the result of suboptimal handling and/or storage conditions in which that stock of human endostatin was maintained, underscoring the requirement for careful establishment and consistent maintenance of the product in suitable conditions.

Hypoxia is an angiogenic phenomenon in solid tumors (32) that provides a selective environment in which clonal progression of malignant cells can occur (33). To determine whether endostatin's activity is at least partly explained through an effect on hypoxia-driven up-regulation of VEGF via HIF-1, experiments were conducted to assess (*a*) Calbiochem endostatin's effect on HIF-1α protein production, (*b*) its effect on HIF-1-dependent transactivation of a HRE-luciferase reporter gene, and (*c*) production and cellular localization of HIF-1α and VEGF in response to Calbiochem endostatin treatment. Immunoblot results showed no inhibition of HIF-1α protein

production by PC3 cells in hypoxia or normoxia in response to treatment with Calbiochem endostatin. As expected given previous studies (34), significant production of HIF-1α was observed in PC3 cells in normoxia, and noticeably increased in hypoxia. Also, no effect on cellular production or localization of HIF-1α was observed in Calbiochem endostatin-treated LNCaP cells or endothelial cells, except for a possible increase in abundance at higher drug treatment concentrations. Interestingly, in hypoxic conditions, endostatin-treated LNCaP cells appear to have an increase in localized accumulations of VEGF relative to untreated cells, a phenomenon not observed in endothelial cells. The significance of this apparent change in cellular localization of VEGF is not known, but indicates that endostatin can affect molecular changes in cancer cells as well as endothelial cells, perhaps reducing the availability of VEGF for secretion by stimulating overproduction of an as yet undiscovered VEGF sequestering protein. Increase in VEGF production in tumors derived from lung cancer cells has been shown to occur in response to human endostatin, although hypoxic up-regulation of the growth factor following endothelial cell death was assumed to have produced this effect (20). Interestingly, simultaneous presence of abundant HIF-1α, VEGF, and endostatin in partially obstructed rat bladder (35) is consistent with our suggestion that endostatin can have an anti-angiogenic effect without reducing HIF-1α or VEGF levels, possibly by reducing VEGF availability. Given that pathological angiogenesis frequently involves mosaic vasculature comprised of cancer cells and endothelial cells (36), the ability of endostatin to alter the cellular localization of an important angiogenic growth factor in cancer cells is worth noting. Endostatin did not inhibit, but rather slightly increased HIF-1α transactivation of luciferase in hypoxia, particularly at lower concentrations. Although not yet verified *in vivo*, these data indicate that endostatin inhibits angiogenesis via a mechanism that is independent of hypoxia-driven VEGF via HIF-1, thereby potentially excluding an important angiogenic pathway on which the drug might be acting. Future experiments in our lab will include *in vivo* angiogenesis assays to determine if endostatin is similarly HIF-1/VEGF independent in that setting, and to assess the role of the stroma in determining levels of these factors. It is not yet known whether other endogenous inhibitors are similarly HIF-1 independent. The true action mechanism of endostatin therefore remains unclear, but likely involves inhibition of endothelial cell proliferation and migration mediated by integrin binding with downstream apoptotic signals (12), and/or MMP inhibition (10), and possibly via a sequestering effect on VEGF in tumor cells that precludes its release and subsequent recruitment of new vasculature.

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