

The Antiangiogenic Agent Neovastat (Æ-941) Induces Endothelial Cell Apoptosis¹

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Abstract

Neovastat (Æ-941), a naturally occurring multifunctional antiangiogenic agent, has been shown to inhibit key components of the angiogenic process, including matrix metalloproteinases and vascular endothelial growth factor-mediated signaling events. In this study, we report the presence of a proapoptotic activity within this compound. Neovastat treatment of bovine aortic endothelial cells caused cell death with characteristics of apoptosis, including chromatin condensation and DNA fragmentation. Neovastat markedly induced caspase-3, caspase-8, and caspase-9 activities, at similar levels to those measured in cells treated with tumor necrosis factor- α . Activation of caspases by Neovastat appears to be essential for its proapoptotic effects because all apoptotic features were blocked by zVAD-fmk, a broad-spectrum caspase inhibitor. The activation of caspases was correlated with the cleavage of the nuclear substrate poly(ADP-ribose) polymerase, and by a concomitant release of cytochrome c from mitochondria to the cytoplasm. Neovastat-induced apoptosis appears to be specific to endothelial cells because treatment of other cell types such as U-87, COS-7, NIH-3T3, and SW1353 did not result in increased caspase-3 activity. These results demonstrate that Neovastat contains a proapoptotic factor that specifically induces the activation of caspases in endothelial cells and the resulting apoptosis of these cells.

Introduction

There is compelling evidence that tumor-induced neovascularization, *i.e.*, tumor angiogenesis, represents a central process involved in the aggressive growth of tumors and of their metastases (1). This strict requirement for angiogenesis for sustained tumor growth has led to the development of alternative strategies for treating cancer based on the selec-

tive interference with the growth of tumor microvessels (1). The usefulness of this approach was exemplified in recent studies showing that the treatment of mice bearing tumors with antiangiogenic molecules such as angiostatin and endostatin results in tumor regression (2–4). These observations have thus driven considerable interest in identifying novel angiostatic proteins and molecules that particularly inhibit endothelial cell proliferation, migration, and vessel formation (5–8). However, notwithstanding the specific inhibitory effects of these compounds toward endothelial cell functions, the mechanisms involved in their antiangiogenic effects remain poorly understood.

Apoptotic cell death is a complex, tightly regulated process that involves drastic structural changes in cell morphology, including chromatin condensation, disassembly of the nuclear and cytoplasmic networks, DNA fragmentation, and membrane blebbing (9), resulting in the fragmentation of the cell into apoptotic bodies that are rapidly phagocytosed by neighboring cells (10). It is now well accepted that the activation of a unique family of cysteine proteases named caspases plays a central role in the signaling and execution phases of apoptosis induced by a variety of stimuli, including cytokines like TNF³- α , radiation, and chemotherapeutic drugs (11). Caspases exist in cells as inactive zymogens that are activated by proteolytic cleavage on appropriate apoptotic signals, leading to the degradation of a variety of critical target proteins, thereby disabling important cellular processes and promoting cell death (12).

Caspase activation during apoptosis occurs through a caspase cascade that is initiated by a subset of caspases containing long NH₂-terminal domains that mediate interaction with caspase-activating factors. For instance, procaspase-8 becomes activated after its recruitment to death receptor complexes (13, 14), whereas caspase-9 is activated through its interaction with Apaf-1 and cytochrome c, in the presence of ATP or dATP (15). Activated caspase-8 and caspase-9 in turn cleave and activate other caspases such as caspase-3, -6, and -7, which represent the main caspase activity in apoptotic cells (15, 16). Caspase-8 induces downstream activation of executioner caspases either directly or indirectly, through activation of the BH3-domain-only subset of proapoptotic members of the bcl-2 family, leading to cytochrome c release from mitochondria and the activation of caspase-9 (17).

³ The abbreviations used are: TNF, tumor necrosis factor; BAEC, bovine aortic endothelial cell; DAPI, 4',6-diamino-2-phenylindole; HUVEC, human umbilical vein endothelial cell; MMP, matrix metalloproteinase; PARP, poly(ADP-ribose) polymerase; rfu, relative fluorescence unit; TIMP, tissue inhibitor of MMP; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; EGF, epidermal growth factor; VEGF, vascular endothelial growth factor; zVAD-fmk, z-Val-Ala-Asp-fluoromethylketone; AFC, amino-4-trifluoromethylcoumarin; AMC, amino-4-methylcoumarin; CHX, cycloheximide; COX, cytochrome oxidase.

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Several recent reports have suggested that the induction of endothelial cell apoptosis may represent a common feature of antiangiogenic molecules. Indeed, the incubation of endothelial cells with thrombospondin (18–20), angiostatin (21, 22), endostatin (23), canstatin (24), antithrombin (25), or kringle 5 (26) all resulted in the generation of a number of apoptotic figures such as phosphatidylserine translocation and DNA fragmentation. However, except for thrombospondin-induced endothelial apoptosis, in which the involvement of kinase-dependent caspase activation was suggested (19), very little information is available regarding the apoptotic pathways activated by antiangiogenic molecules nor on the involvement of caspase activities in their effects.

Cartilage was the first tissue reported to contain biological inhibitor(s) of angiogenesis. Moreover, it has long been recognized as an abundant source of angiostatic molecules (27), such as TIMP-1 and TIMP-2 (28, 29), troponin-1 (30), SCF-2 (31), thrombospondins (32), and metastatin (33). Recently, Neovastat, a naturally occurring inhibitor of angiogenesis derived from marine cartilage (dogfish), has been tested in Phase II clinical trials in non-small cell lung cancer and in renal cell carcinoma (34).⁴ It is currently undergoing Phase III clinical trials for the treatment of refractory renal cell carcinoma and nonresectable small cell lung cancer in addition to a Phase II pivotal clinical trial for the treatment of recurrent multiple myeloma (35).

There is now considerable evidence that the clinical benefits observed upon Neovastat treatment rely on the presence of multiple angiogenesis inhibitors within the compound (34, 36). Neovastat inhibits chick embryo vascularization and Matrigel-induced angiogenesis *in vivo* (37) as well as tumor growth of the DA3 breast adenocarcinoma (38, 39), the HGD human glioblastoma (40), and Lewis lung carcinoma metastasis in mice (37). At the molecular level, the antiangiogenic activity of Neovastat has been correlated with the inhibition of metalloproteinases (MMP-2, MMP-9, MMP-12) and serine elastases (41, 42). Neovastat also inhibits several functions of endothelial cells that are mediated by VEGF, including capillary sprouting, tubulogenesis, and hyperpermeability, possibly through interference with VEGF receptor-2 function (43). Neovastat has also been found to induce the activity of the tissue-type plasminogen activator, which generates the accumulation of angiostatin, an endogenous inhibitor of angiogenesis (44).

In this work, we sought to determine whether the inhibitory effect of Neovastat on cell proliferation could be related to a possible induction of endothelial cell apoptosis. Our results indicate that this compound indeed contains an endothelial-specific proapoptotic inducer that stimulates cell death through the activation of caspase-3, -8, and -9. To the best of our knowledge, these results represent the first demonstration of a proapoptotic activity in cartilage and strengthen the notion that extracts from this tissue contain multiple angiostatic activities.

⁴ J. Latreille, G. Batist, F. Laberge, P. Champagne, D. Croteau, P. Falardeau, C. Levinton, C. Hariton, W. K. Hariton, and É. Dupont. Phase I/II trial on the safety and efficacy of Neovastat (Æ-941) in the treatment of non-small cell lung cancer, submitted for publication.

Materials and Methods

Materials and Antibodies. Neovastat (Æ-941) was obtained from Æterna Laboratories (Quebec City, Quebec, Canada; 45). zVAD-fmk, etoposide, and Casputin reagent were from BIOMOL Research Laboratories (Plymouth Meeting, PA). Acetyl-Asp-Glu-Val-Asp-7-AFC (Ac-DEVD-AFC), acetyl-Ile-Asp-Thr-Asp-7-AFC (Ac-IETD-AFC), and acetyl-Leu-Glu-His-Asp-7-AMC (Ac-LEHD-AMC) were purchased from BIOSOURCE International (Camarillo, CA). Recombinant human TNF- α was purchased from Calbiochem (La Jolla, CA). DAPI nucleic acid stain was purchased from Molecular Probes (Eugene, OR). Anti-PARP monoclonal antibody (clone C-2-10) was purchased from Clontech (Palo Alto, CA). Anti- β -actin was purchased from Sigma (St. Louis, MO). Anti-Bax monoclonal antibody (clone 6A7) was from BIOSOURCE International. Anti-Bcl-2 monoclonal antibody (clone 7) was purchased from Transduction Laboratories (San Diego, CA). Anti-cytochrome c was purchased from PharMingen (San Diego, CA). Anti-COX IV antibody was purchased from Molecular Probes.

Cell Culture. BAECs obtained from Clonetics (San-Diego, CA) were cultured in low glucose DMEM containing 10% heat-inactivated calf serum (MediCorp, Montreal, Quebec, Canada), 100 units/ml penicillin G, 100 μ g/ml streptomycin, and 1 ng/ml basic fibroblast growth factor (Upstate Biotechnology, Lake Placid, NY). U-87 MG cell line (American Type Culture Collection) was cultured in MEM containing 10% heat-inactivated FCS (MediCorp) and antibiotics. HUVECs, obtained from Clonetics, were cultured in M199 medium containing 20% heat-inactivated FCS, 40 μ g/ml endothelial cell growth supplement (Upstate Biotechnology), 90 μ g/ml heparin (Life Technologies, Inc., Burlington, Ontario, Canada), and antibiotics. Human dermal microcapillary endothelial cells transformed with SV40 large T antigen (Ref. 46; HMEC-1) were cultured in MCDB 131 medium containing 10% heat-inactivated FCS, 10 ng/ml EGF, 1 ng/ml hydrocortisone, and antibiotics. COS-7, NIH-3T3, and SW1353 cell lines (all from American Type Culture Collection) were cultured in DMEM containing 10% heat-inactivated calf serum and antibiotics. All of the media and antibiotics were from Life Technologies, Inc.

Treatment of Cells with Neovastat, Etoposide, and TNF- α . Cells were grown to about 80% confluence and treated with Neovastat (85 μ g of protein/ml) or with other inducers of apoptosis, such as etoposide (10 μ M) or TNF- α (25 ng/ml), and cycloheximide (10 μ g/ml). HUVEC and human microcapillary endothelial cells were treated with Neovastat in the presence of cycloheximide (10 μ g/ml). When required, zVAD-fmk was added 1 h before treatment at a final concentration of 25 μ M.

Cell Viability Assays. Cells, grown to ~80% confluence in 12-well plates, were treated for various periods of time. Adherent and nonadherent cells were collected and viability was assessed by mixing aliquots of cell suspensions with an equal volume of 0.4% trypan blue (Life Technologies, Inc.). Cells that picked up the dye were considered to be dead.

Fluorimetric Caspase-3, Caspase-8, and Caspase-9 Assays. Cells treated with Neovastat, etoposide, or TNF- α /CHX were collected and washed in cold PBS. Cells were

lysed in Apo-Alert lysis buffer (Clontech, Palo Alto, CA) for 20 min at 4°C, and the lysates were clarified by centrifugation at $16,000 \times g$ for 20 min. Caspase activities were determined by incubation with 50 μM fluorogenic peptide substrates acetyl-Asp-Glu-Val-Asp-7-AFC (Ac-DEVD-AFC; caspase-3-specific) or acetyl-Ile-Asp-Thr-Asp-7-AFC (Ac-IETD-AFC; caspase-8-specific) or 250 μM acetyl-Leu-Glu-His-Asp-7-AMC (Ac-LEHD-AMC, caspase 9 specific) in assay buffer {50 mM HEPES-NaOH (pH 7.4), 100 mM NaCl, 10% sucrose, 0.1% 3-[[3-cholamidopropyl]dimethylammonio]-1-propane-sulfonate, 5 mM DTT, and 1 mM EDTA} on 96-well plates. The release of AFC ($\lambda_{\text{ex}} = 400 \text{ nm}$, $\lambda_{\text{em}} = 505 \text{ nm}$) or AMC ($\lambda_{\text{ex}} = 380 \text{ nm}$, $\lambda_{\text{em}} = 460 \text{ nm}$) was monitored for at least 20 min at 37°C on a SpectraMAX Gemini fluorescence plate reader (Molecular Devices). Caspase activities were expressed as rfu/s/ μg of protein used in the assay.

TUNEL and DAPI Staining. BAECs were grown on glass coverslips and were treated for 18 h. After the treatment, coverslips were washed twice with PBS, fixed for 30 min in 3.7% paraformaldehyde/PBS at room temperature, washed again twice in PBS, and then permeabilized in 0.2% Triton X-100/PBS for 5 min at 4°C. Permeabilized cells were washed in PBS, and the TUNEL assay was performed as described in the *In Situ* Cell Death Detection kit (Roche Diagnostic, Laval, Quebec, Canada). The nuclear morphology of cells was analyzed by the staining of DNA with DAPI (Molecular Probes).

Immunoblot Analysis. Cells were harvested and lysed as described for caspase assays, and the protein concentration was measured using bicinchoninic acid protein assay reagent (Pierce, Rockford, IL) and BSA as the standard. Equal amounts of protein samples in sample buffer [62.5 mM Tris-HCl (pH 6.8), 100 mM DTT, 10% glycerol, 2% SDS, and 0.1% bromophenol blue] were heated at 100°C for 3 min and separated on 0.75-mm-thick SDS-polyacrylamide gels with a MINI-PROTEAN II apparatus (Bio-Rad). Proteins were electroblotted onto 0.45- μm -pore diameter polyvinylidene difluoride membranes (Roche Diagnostics) with a semi-dry apparatus (Millipore) in transfer buffer (96 mM glycine, 10 mM Tris, and 20% methanol) for 1 h at 80 mA/gel. Membranes were blocked overnight at 4°C in Tris-buffered saline [20 mM Tris-HCl (pH 7.5), 137 mM NaCl] containing 0.1% (v/v) Tween 20 and 3% BSA. Blots were incubated with primary antibodies in blocking buffer for 2 h at room temperature, followed by a 1-h incubation with a 1:10,000 dilution of horseradish peroxidase-conjugated donkey antirabbit IgG or goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) in the same incubation medium. Immunoreactive bands were revealed with enhanced chemiluminescence Western blotting kit (Renaissance, NEN Life Science, Boston, MA) and SuperRX films (Fuji).

DNA Degradation Assay. Low-molecular-weight DNA was isolated from cells by a modified Hirt extraction procedure (47). BAECs were grown in 100-mm-diameter plates, treated for 16–20 h, harvested, and washed in cold PBS. Cells were lysed in 0.5 ml of lysis buffer [10 mM Tris-HCl (pH 8.0), 5 mM EDTA, 100 mM NaCl, and 0.5% (w/v) SDS] containing 1 mg of Pronase/ml. Lysates were incubated at 37°C for 2 h, and 150 μl of 5 M NaCl was added (1 M final con-

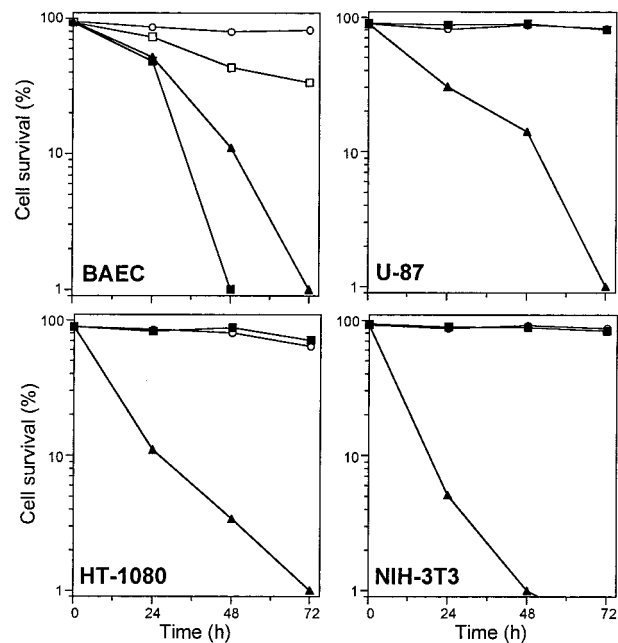


Fig. 1. Neovastat treatment induces endothelial cell-specific cell death. Subconfluent cells were either left untreated (○) or treated with 85 $\mu\text{g}/\text{ml}$ Neovastat (■) or with 25 ng/ml TNF-10 $\mu\text{g}/\text{ml}$ CHX (▲) for 0, 24, 48, and 72 h. BAECs were also treated with Neovastat in the presence of 25 μM zVAD-fmk (□). Adherent and nonadherent cells were collected, and viability was assessed by trypan blue exclusion. Cell death is expressed as the percentage of cells incorporating the dye relative to the total amount of cells.

centration). Samples were incubated overnight at 4°C and centrifuged at $16,000 \times g$ for 30 min. DNA was precipitated from the supernatant with ethanol. Samples were centrifuged at $16,000 \times g$ for 30 min and pellets were resuspended in Tris/EDTA [10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA]. Nucleic acids were treated with 10 μg of RNase A (Sigma) for 1 h at 37°C and then were analyzed on 1% agarose gels stained with ethidium bromide.

Preparation of Mitochondria and Cytosol for Measurement of Cytochrome c. Cells were harvested and washed in cold PBS. Cell pellets were resuspended in homogenization buffer, and mitochondrial and cytosolic extracts were prepared by the method described previously (48).

Results

Neovastat Induces Endothelial Cell Death. Neovastat is a natural, multifunctional antiangiogenic agent produced from cartilage extracts. In the course of studies aimed at identifying activities contributing to its anticancer properties, Neovastat was tested for its ability to induce cell death in endothelial cells. Endothelial and nonendothelial cells were incubated with Neovastat (85 $\mu\text{g}/\text{ml}$), and, at various times, cell viability was measured by exclusion of trypan blue. Fig. 1 shows that Neovastat induced 50% cell death of BAECs at about 24 h of treatment and that by 48 h, virtually all of the cells were dead. The viability of nonendothelial cell lines U-87 (glioblastoma), HT-1080 (fibroblasts), and NIH-3T3 (fibroblasts), however, was not altered by Neovastat, which sug-

gested an endothelial cell specificity. In contrast, all four of the cell lines were highly sensitive to TNF- α /CHX, a well-characterized inducer of death-receptor-mediated cell apoptosis (11, 49). Importantly, Neovastat-induced BAEC cell death was greatly reduced by the presence of zVAD-fmk, a broad-spectrum inhibitor of caspases, which indicated that the cell death is caspase dependent. Endothelial-specific and caspase-dependent induction of cell death by Neovastat was also observed using other methods (WST-1 cell proliferation assay, fluorescence-activated cell sorting (FACS) analysis using annexin V), which further supported its proapoptotic action on endothelial cells (data not shown).

Neovastat Induces DNA Fragmentation and Chromatin Condensation in the Nucleus of Endothelial Cells. To determine whether Neovastat-induced endothelial cell death was caused by programmed cell death rather than by necrosis, we first looked at fundamental characteristics of apoptotic cells: DNA condensation and fragmentation. We used two complementary methods to examine DNA fragmentation in the nuclei of apoptotic cells: TUNEL assay, in which DNA fragmentation is visualized after incorporation of Fluorescein-dUTP; and the DNA ladder assay, in which low-molecular-weight DNA is extracted from cells and visualized on agarose gels in the presence of ethidium bromide. DAPI staining was used to show chromatin condensation, another hallmark of apoptosis. As shown in Fig. 2A, BAECs treated with Neovastat were stained by the TUNEL assay, whereas labeling was not observed when Neovastat-treated cells were cultured in the presence of zVAD-fmk. Nuclei from untreated cells were weakly and uniformly stained with DAPI, whereas the shape of nuclei from Neovastat-treated cells appeared irregular. Moreover, the nucleus of Neovastat-treated cells contained bright fluorescent spots that are characteristic of condensed chromatin. Low-molecular-weight DNA extracted from cells treated with Neovastat, as well as that from cells treated with etoposide or TNF- α /CHX, showed degradation patterns typical of cells undergoing apoptosis (Fig. 2B). This degradation was diminished when cells were treated in the presence of zVAD-fmk. These results show that Neovastat treatment induced apoptosis in endothelial cells and indicate that caspases are involved in the process.

Neovastat Increases Intracellular Activities of Caspase-3 and Caspase-8. Most cell-death pathways involve the activation of caspases, leading to the degradation and inactivation of key cellular proteins such as DNA repair, signaling, and structural proteins. We used fluorogenic peptide substrates to measure caspase-3 activities (Fig. 3A) and caspase-8 activities (Fig. 3B) after 0, 3, 6, and 24 h of incubation with Neovastat. BAECs were also treated with TNF- α /CHX as a positive control for activation of these caspases. Comparable levels of caspase-3 and -8 activities were detected in cell extracts after 24 h of treatment with Neovastat or TNF- α /CHX. However, although caspase-3 and -8 activities were readily detectable after 3 h of treatment with TNF- α /CHX, the activation of these caspases after treatment with Neovastat was much slower, which suggested a different mechanism of induction. No caspase activity was detected in extracts from cells treated in the presence of 25 μ M zVAD-

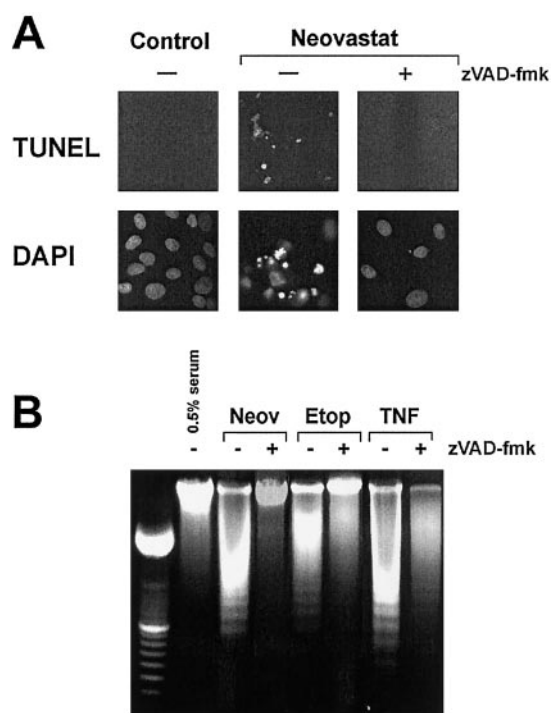


Fig. 2. Neovastat treatment induces DNA fragmentation. **A**, BAECs grown on coverslips were either left untreated (*Control*) or treated with 85 μ g/ml Neovastat in the absence or in the presence of 25 μ M zVAD-fmk. Fragmented DNA was detected by the TUNEL assay, and DNA condensation was detected by staining with DAPI, as described in "Materials and Methods." **B**, BAECs were either left untreated or treated with 85 μ g/ml Neovastat (*Neov*) or 10 μ M etoposide (*Etop*) or 25 ng/ml TNF- α -10 μ g/ml CHX, in the absence or in the presence of zVAD-fmk. Serum concentration was 10% during etoposide and TNF- α /CHX treatments or 0.5% during Neovastat treatment. After 16 h, low-molecular-weight DNA was isolated and then analyzed on 1% agarose gels stained with ethidium bromide.

fmk, confirming that the protease activity measured was attributable to caspases and not to other types of proteases.

Neovastat-induced Activation of Caspases Is Specific to Endothelial Cells. The effect of Neovastat on caspase-3 activity was measured in several nonendothelial and endothelial cell lines (Table 1). Neovastat treatment induced caspase-3 activity in BAECs, HUVECs, and HMEC-1 cells but failed to induce caspase-3 in U-87 glioblastoma, NIH-3T3 fibroblast, MCF-7 human breast cancer, SW1353 chondrosarcoma, and COS-7 simian kidney (Table 1). Neovastat did not induce caspase activities in U-87 (Fig. 4, C and D), whereas these cells were highly responsive to TNF- α /CHX. The specificity of action of Neovastat and TNF- α /CHX on caspase activities was verified with Casputin, a chimeric protein containing domains from human X-linked inhibitor of apoptosis that inhibits caspase-3, -7 and, weakly, caspase-10 but not caspase-1, -2, -6, and -8 (50, 51). DEV-Dase activity (caspase-3) was strongly inhibited by Casputin. In contrast, IETDase activity (caspase-8) was only slightly reduced in the presence of Casputin, which confirmed that caspase-8 activity was measured under our experimental conditions.

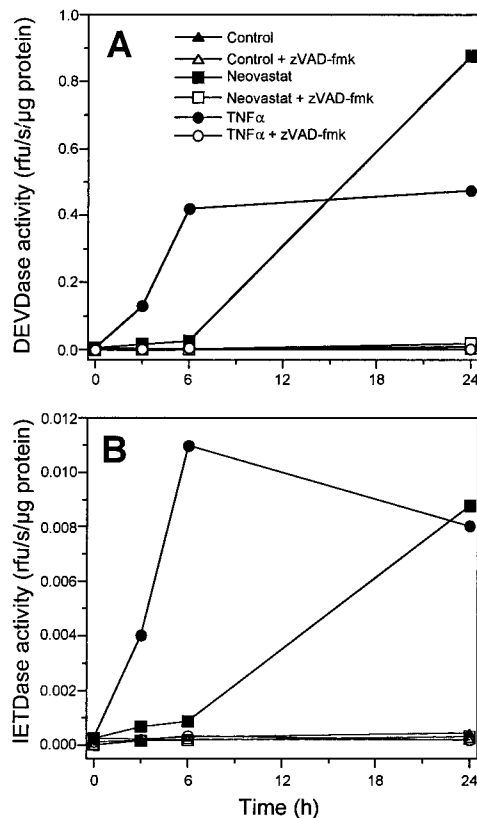


Fig. 3. Time course of caspase-3 and caspase-8 induction in BAECs. Subconfluent cells were either left untreated (\blacktriangle , \triangle) or treated with 85 $\mu\text{g/ml}$ Neovastat (\square , \blacksquare) or with 25 ng/ml TNF-10 $\mu\text{g/ml}$ CHX (\bullet , \circ) for 0, 3, 6, and 24 h in the absence (filled symbols) or in the presence (open symbols) of 25 μM zVAD-fmk. Extracts from control and treated cells were used to determine DEVDase (A, caspase-3) and IETDase (B, caspase-8) activities.

Neovastat Induces PARP Cleavage but Does Not Change Bcl-2 and Bax Expression Levels in BAEC. Activated caspase-3 is able to cleave numerous cellular substrates including PARP. As shown in Fig. 5, Neovastat, etoposide, and TNF- α /CHX induced cleavage of PARP into the characteristic M_r 85,000 fragment. Moreover, coinhibition with 25 μM zVAD-fmk completely inhibited PARP cleavage induced by Neovastat and etoposide. However, PARP cleavage induced by TNF- α was only weakly inhibited by zVAD-fmk at this concentration (not shown), possibly because of the very strong apoptotic response induced by this cytokine. However, the addition of 50 μM zVAD-fmk significantly reduced TNF-mediated PARP cleavage (Fig. 5). Expression levels of Bcl-2 and Bax remained unchanged by treatment with any of the apoptosis inducers. In agreement with the previous results showing that caspases are not activated by Neovastat in U-87 cells (Fig. 4, C and D), PARP cleavage was not observed in these cells treated with Neovastat. In contrast, PARP was cleaved in U-87 cells treated with TNF- α /CHX (Fig. 5, lower panel).

Neovastat Treatment Induces Cytochrome c Release in BAEC. To identify which mechanisms are involved in Neovastat-induced activation of caspase-3, we examined

Table 1 Induction of caspase-3 in various cell lines

Cell line	DEVDase activity (rfu/s/ μg protein)		Caspase induction (x-fold)
	Control	Neovastat-treated	
Endothelial			
BAEC	1.011	23.02	22
HUVEC	0.052	0.493	9.5
HMEC	0.051	0.188	3.7
Nonendothelial			
U-87	0.137	0.137	0.0
COS-7	0.160	0.108	0.0
NIH-3T3	3.371	0.784	0.0
SW1353	0.681	1.144	1.7

the effect of Neovastat treatment on cytochrome c release. Cytochrome c released in the cytoplasm forms a complex with Apaf-1, dATP, and pro-caspase-9, leading to the activation of caspase-9 followed by downstream activation of effector caspases such as caspase-3 (15, 16). BAECs were treated with Neovastat and, at various time intervals, cells were subjected to subcellular fractionation. Then, equal amounts of proteins from mitochondria and cytosol fractions were probed for cytochrome c by immunoblotting. β -actin and COX subunit IV were used as a control for the amount of protein loaded in the cytosol and mitochondrial fractions, respectively. Neither their levels nor their translocation were modulated by Neovastat (Fig. 6A). As shown in Fig. 6, A and B, cytochrome c was released in the cytosol of cells treated with Neovastat. Moreover, the levels of cytochrome c detected in the mitochondrial fraction from treated cells were greatly reduced. Because release of cytochrome c is known to result in the activation of caspase-9, we used the fluorogenic substrate LEHD-AMC to detect such an activity in extracts from cells treated with Neovastat and TNF- α /CHX. As shown in Fig. 6C, caspase-9 activity was increased in both Neovastat- and TNF- α /CHX-treated cells. Moreover, the activation of caspase-9 is prevented in cells treated in the presence of zVAD-fmk. Furthermore, this activity was strongly inhibited by the caspase-9 inhibitor LEHD-CHO. These results, thus, strongly suggest that the induction of caspase activities by Neovastat may involve the mitochondrial pathway.

Discussion

Neovastat is an antiangiogenic drug that has reached Phase III clinical trial evaluation. In contrast to other inhibitors of angiogenesis derived from natural sources, there is significant information available about the mechanism of action of Neovastat. We have recently reported that it contains inhibitors of MMPs as well as of serine elastases (41). It has also been reported that Neovastat inhibits endothelial cell proliferation, whereas it had no significant effect on the proliferation of fibroblast and muscle cells nor on several tumor cell lines (37), which suggests that Neovastat acts directly at the endothelial cell level.

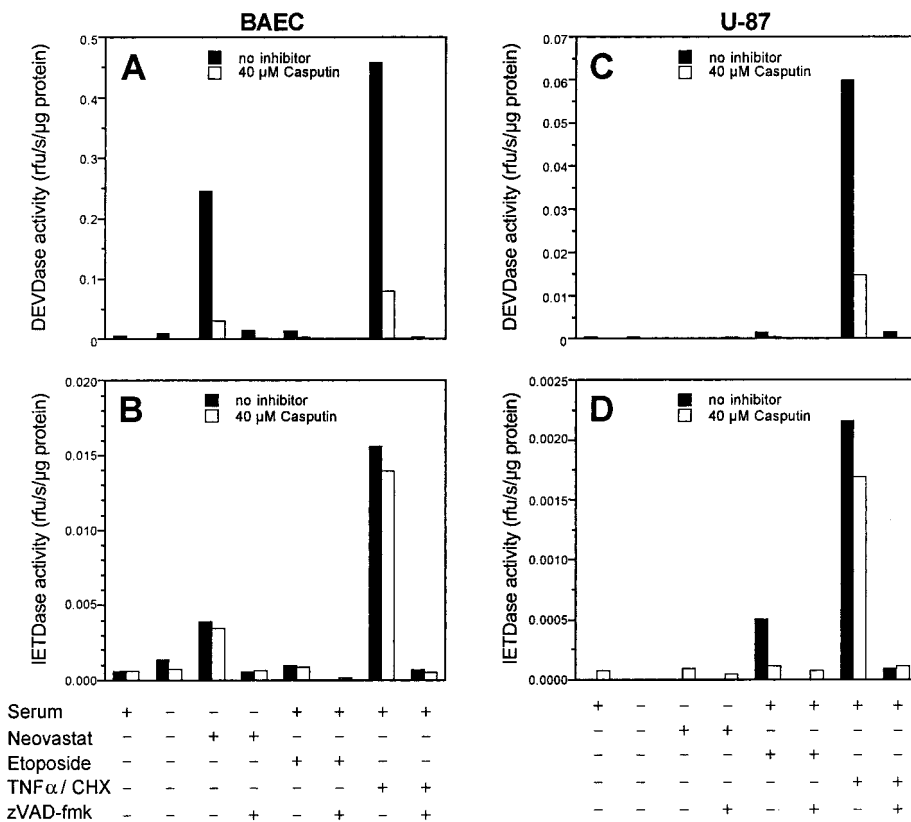


Fig. 4. Neovastat treatment increases caspase-3 and caspase-8 activities in BAECs but not in U-87 glioblastoma cells. Subconfluent cells were either left untreated or treated with 85 μg/ml Neovastat or 10 μM etoposide or 25 ng/ml TNF-α-10 μg/ml CHX for 18 h in the absence or in the presence of 25 μM zVAD-fmk. Extracts from control and treated cells were used to determine DEVDase (caspase-3) and IETDase (caspase-8) activities in the absence (filled bars) or in the presence (open bars) of 40 μM Casputin, as described in "Materials and Methods."

In this work, we provide evidence that Neovastat exerts at least some of its endothelial-specific effects through the induction of apoptosis. Consistent with the presence of a proapoptotic activity within Neovastat as reported for many antiangiogenic molecules (20–24, 26), incubation of endothelial cells with the compound promotes the generation of a number of apoptotic hallmarks, including chromatin condensation and DNA fragmentation. Our results indicate that these alterations in endothelial cell structure are related to the activation of at least three key members of the caspase family, namely caspase-3, caspase-8, and caspase-9. First, we observed that Neovastat induced a marked increase in the activity of these enzymes toward fluorogenic substrates, the extent of activation being similar to that induced by TNF-α/CHX, a prototypic apoptotic inducer. This increase in caspase activities is crucial for the proapoptotic activity of Neovastat because DNA fragmentation, chromatin condensation, and PARP cleavage were abolished by coincubation with zVAD-fmk, a broad-spectrum caspase inhibitor. By contrast, Neovastat did not induce detectable apoptosis nor increased caspase activities in many other cell types, which indicated that its action is specific to endothelial cells.

The mechanisms by which Neovastat induces caspase-3 and -8 activities remain to be established. Caspase-8 is an initiator caspase that is activated on stimulation of death receptors and subsequently activates the executioner caspases either directly or through a Bid-dependent cytochrome c release from mitochondria (17, 52). It is, thus, tempting to spec-

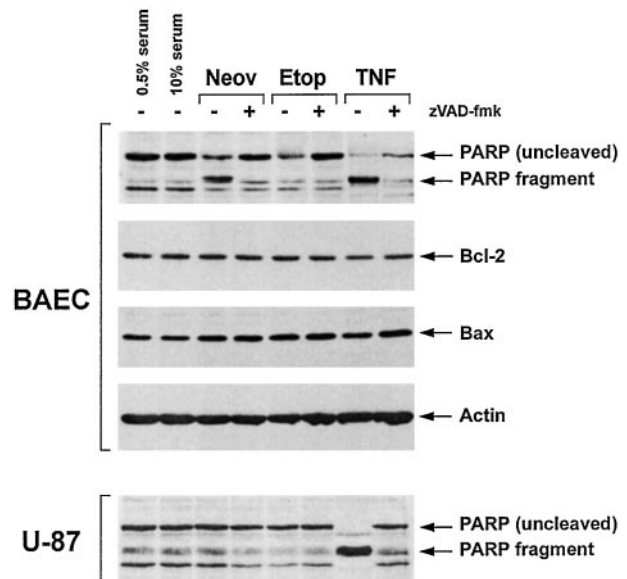


Fig. 5. Western blot analysis of BAECs and U-87 cells for proteins involved in apoptosis. BAECs and U-87 cells were either left untreated or treated with 85 μg/ml Neovastat (Neov) or 10 μM etoposide (Etop) or TNF-α/CHX, in the absence or in the presence of 25 μM (for Neovastat and etoposide) or 50 μM zVAD-fmk (for TNF-α/CHX). After 18 h, cells were harvested, and cell extracts were prepared. Twenty μg of protein was subjected to SDS-PAGE followed by electrotransfer onto polyvinylidene difluoride. Immunoblot analysis was performed with antibodies directed against PARP, Bcl-2, Bax, and β-actin.

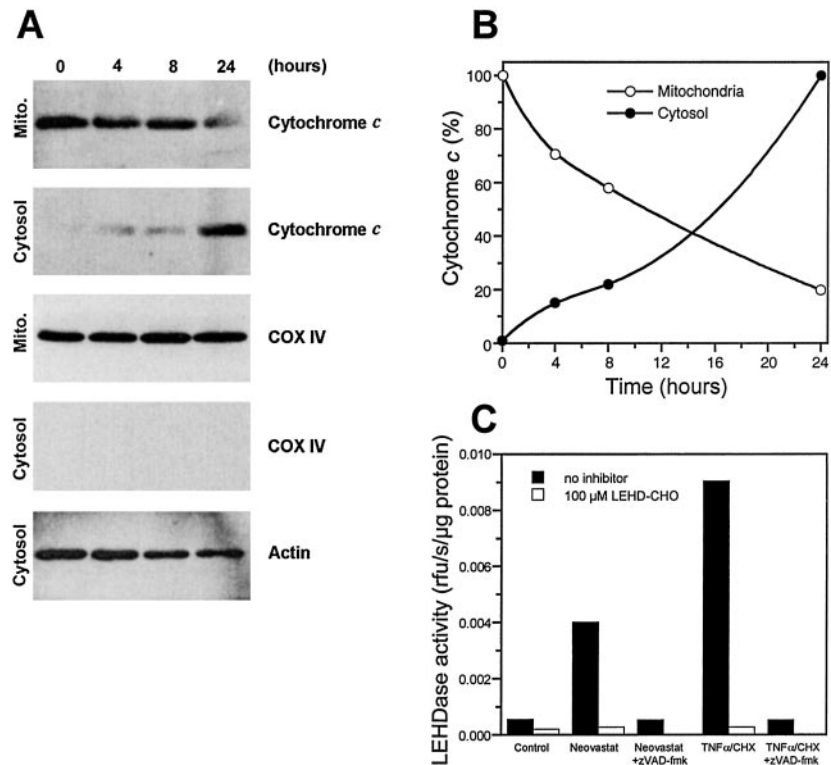


Fig. 6. Neovastat treatment induces release of mitochondrial cytochrome *c* and caspase 9 activity in BAEC. **A**, BAECs were either left untreated or treated with 85 $\mu\text{g/ml}$ Neovastat. At the indicated periods of time, cells were harvested, fractionated into mitochondrial (*Mito*) and cytosolic (*Cytosol*) fractions, and then analyzed by Western blot for cytochrome *c*, COX IV, and β -actin. **B**, cytochrome *c* detected in mitochondrial (\circ) and cytosolic fractions (\bullet) was quantified using IPlab Gel Image Processing Software (Signal Analytic), and the results were expressed as percentage. **C**, BAECs were either left untreated or treated with Neovastat or TNF- α /CHX for 18 h in the absence or in the presence of zVAD-fmk. Extracts from control and treated cells were used to determine LEHDase (caspase-9) activity in the absence (filled bars) or in the presence (open bars) of 100 μM LEHD-CHO, as described in "Materials and Methods."

ulate that a similar pathway may be involved in Neovastat-induced apoptosis. In this respect, we observed that the incubation of cells with Neovastat resulted in a time-dependent release of cytochrome *c* from mitochondria, in a manner similar to that observed after incubation with TNF- α . However, in a number of cell types, caspase-8 has been suggested to function as an executioner caspase, being activated by caspase-9 (53). The exact apoptotic pathways involved in Neovastat-induced apoptosis are currently under investigation.

The presence within Neovastat of an endothelial-specific proapoptotic factor may, thus, significantly contribute to the antiangiogenic activity of the compound. Given the recent observations of antiprotease as well as anti-VEGF activities in Neovastat (41), the activity of Neovastat appears to act through multiple targets. Its efficiency against several crucial steps of the angiogenic cascade closely linked to tumor progression contrast from other natural or chemical antiangiogenic agents, in which only one activity was targeted. Moreover, Neovastat's multiple mechanism of action could reflect the presence of several components that could synergistically act together to control neovascularization. The findings reported in this paper may, thus, be of significant importance to our understanding of the mechanisms by which Neovastat elicits its antiangiogenic, antitumoral, and antimetastatic effects.

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