Endostatin Induces Endothelial Cell Apoptosis*

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Endostatin, a carboxyl-terminal fragment of collagen XVIII, has been shown to regress tumors in mice. In this study, we have analyzed the mechanism of endostatin action on endothelial cells and nonendothelial cells. Endostatin treatment of cow pulmonary artery endothelial cells caused apoptosis, as demonstrated by three methods, annexin V-fluorescein isothiocyanate staining, caspase 3, and terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling assay. Moreover, addition of endostatin led to a marked reduction of the Bcl-2 and Bcl-XL anti-apoptotic protein, whereas Bax protein levels were unaffected. These effects were not seen in several nonendothelial cells. Collectively, these findings provide important mechanistic insight into endostatin action.

Angiogenesis is required for tumors to grow beyond a few millimeters in size (1). Advantages of anti-angiogenic therapy include ease of access of drugs to the endothelial cell compartment, targeting of a genetically stable cell population (endothelial versus tumor cells) thereby lessening the chance of drug resistance and “amplification” achieved, because one endothelial cell supports the growth of 50–100 tumor cells (2–7).

Fragments of proteins have recently been described with anti-angiogenic action. Endostatin, a 20-kDa C-terminal fragment of collagen XVIII, is a specific inhibitor of endothelial cell proliferation (8), migration (9), and angiogenesis (8). Endostatin regresses established syngeneic Lewis lung carcinomas, T241 fibrosarcoma, and B16 melanoma tumors in xenograft models (8). Recently, tumor dormancy was induced following repeated cycles of endostatin treatment (10). Administration of recombinant mouse endostatin to tumor-bearing animals increases the apoptotic index of the tumor cells without affecting their proliferation index (8), but no data on endothelial cells has been described.

The mechanism of endostatin action is unknown. In vitro results from this laboratory and others (8, 9) show that endostatin inhibits the proliferation of endothelial cells but has no effect on tumor cells or nonendothelial cell types, including fibroblasts and smooth muscle cells. Thus, the action of endostatin seems to be endothelial cell specific. Because the presence of endostatin in endothelial cell culture affects proliferation (and migration), we asked whether the endothelial cells are undergoing programmed cell death.

Our studies show that soluble yeast-derived mouse endostatin altered the morphology of endothelial cells leading to the characteristic features of cells undergoing apoptosis. Endostatin-treated cells shifted in annexin V-FITC labeling by FACS, and after roughly 12 h, externalization of phosphatidylserine on the cell surface ensued. In addition, endostatin treatment increased the activity of the intracellular protease caspase 3, resulting in DNA degradation in the nucleus, as independently confirmed via the terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL) assay. Western blot analysis showed reduction of the Bcl-2 and Bcl-XL anti-apoptotic proteins without any effect on the level of the Bax pro-apoptotic protein. In nonendothelial cells, viz., NIH3T3 and A10 smooth muscle cells, endostatin did not show any effects in any of the above assays, indicating specificity of endostatin toward endothelial cells.

Experimental Procedures

Reagents

The mouse endostatin protein was produced and purified from yeast, as described elsewhere (9). Some batches contained DNA, which had no effect on apoptosis. Anti-Fade reagent was purchased from Molecular Probes, Inc. Tumor necrosis factor (TNF)-α was obtained from R & D Systems, Inc. 16% methanol-free formaldehyde, used for the fixing solutions, was purchased from Polysciences, Inc. The annexin V-FITC-labeling kit was purchased from CLONTECH.

Cell Lines

Cow pulmonary artery endothelial (C-PAE) cells, obtained from ATCC (Manassas, VA), were maintained in DMEM supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine. The cells were cultured in a humidified environment at 37 °C in the presence of 5% CO₂. Bovine aortic endothelial cells, a gift from C. Ferran, Beth Israel Deaconess Medical Center, Boston, were maintained in DMEM containing 10% fetal calf serum. Bovine adrenal cortex capillary endothelial cells were a gift from Dr. J. Folkman, Children’s Hospital, Boston. HUVE and HMVE-L cells were obtained from Clonetics Corp. (San Diego, CA). IMR-90, lung fibroblast, NIH3T3 fibroblast, A10 smooth muscle cell, H9c2 (2–1)-myoblast cell line, and 786–0 renal clear cell carcinoma cells were all purchased from ATCC.

Annexin V-FITC Assay

Annexin V, a calcium-dependent phospholipid-binding protein with a high affinity for phosphatidylserine (PS) was used to detect early stage apoptosis (11). Briefly, 200,000 cells were plated onto a fibronectin-
coated 6-well plate in DMEM containing 2% FCS and 3 ng/ml bFGF. Different concentrations of recombinant mouse endostatin were added to each well, and cells were harvested and processed 18 h after treatment. For the time course study, 10 μg/ml endostatin was added and cells were processed after 3, 4, 6, 12, and 18 h. Human recombinant TNF-α (20 ng/ml) was used as a positive control. The cells were washed in PBS and resuspended in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂). Annexin V-FITC was added to a final concentration of 100 ng/ml, and the cells were incubated in the dark for 10 min, then washed again in PBS and resuspended in 300 μl of binding buffer. 10 μl of propidium iodide (PI) was added to each sample before flow cytometric analysis. The cells were analyzed using a Becton Dickinson FACStar plus flow cytometer. Electronic compensation was used to eliminate bleed-through fluorescence. In each sample, a minimum of 10,000 cells was counted and stored in listmode. Data analysis was performed with standard Cell Quest software (Becton-Dickinson). The quadrant settings were set so that the negative control allowed less than 1% positivity. Endostatin was added to nonendothelial cells (NIH3T3 and 786–0) at 10 μg/ml, and the cells were processed and analyzed as described above.

Caspase 3 Assay

This assay was performed in a 75-cm² tissue culture flask or on fibronectin-coated 6-well plates. For the 6-well plates, 0.5–1 × 10⁶ cells (75-cm² flask; 2 × 10⁶ cells) were seeded and maintained overnight in DMEM with 10% FCS. The following day, the old medium was replaced with fresh medium (2% FCS), and the cells were incubated overnight at 37 °C. Following starvation, the cells were stimulated with bFGF (3 ng/ml) in DMEM (2% FCS). Along with bFGF, yeast endostatin (10 μg/ml, final concentration) was added, and the cells were grown for 24 h. For the control plate, only the PBS buffer was added. As a positive control, TNF-α was used at a final concentration of 20 ng/ml. After 24 h, the supernatant was centrifuged and collected. The wells (flasks) were trypsinized to collect the attached cells and combined with the supernatant cells. The cells were counted and resuspended in cell lysis buffer (CLONTECH) at a concentration of 4 × 10⁶ cells/ml. The rest of the protocol followed the manufacturer’s instruction (CLONTECH). A specific inhibitor of caspase 3, DEVD-fmk, was used to confirm the specificity as suggested by the manufacturer. The absorbance was measured in a microplate reader (Bio-Rad) at 405 nm. Fold-increase in protein activity (caspase 3) was determined by comparing the results of the induced sample (yeast endostatin or TNF-α) with the uninhibited control. Similarly nonendothelial cells (NIH3T3 and H9c2 (2–1)–myoblast) were used and analyzed as described above.

Microscopic Detection of TUNEL Staining

For Adherent Cells—C-PAE cells were seeded at a density of 5,000 cells/well on fibronectin-coated (10 μg/ml) Lab-Tek chamber slides and grown in 0.4 ml of DMEM with 10% FCS. After 2 days, the old medium was aspirated and fresh DMEM with 2% FCS was added, and the cells were starved overnight. The following day, 0.36 ml of new medium (with 2% FCS) containing 3 ng/ml bFGF was added along with yeast endostatin (10 μg/ml) or TNF-α (20 ng/ml). For control samples, fresh medium (2% FCS) containing bFGF (3 ng/ml) was added. Following induction (24 h), the slides were washed twice with PBS, and subsequently fixed in fresh 4% formaldehyde/PBS at 4 °C for 25 min. The slides were washed in PBS and the cells permeabilized in 0.2% Triton X-100/PBS for 5 min on ice, then washed with fresh PBS twice for 5 min each at room temperature, and the TUNEL assay was performed as described below.

The TUNEL assay was performed as described in the ApoAlert DNA fragmentation assay kit user manual (CLONTECH), except that the final concentration of propidium iodide (Sigma) used was 1 μg/ml. After the assay, a drop of anti-fade solution was added, and the treated portion of the slide was covered with a glass coverslip with the edges sealed with clear nail polish. Slides were viewed immediately under a fluorescent microscope using a dual filter set for green (520 nm) and red fluorescence (>620 nm). The images were captured using a digital microscope (Nikon Microphot-SA) and processed using SPOT software version 1.1.02. For all samples except the positive control (TNF-α; 5 fields), 15 random fields were chosen, and the number of green and red cells per field were counted.

For Cells in Suspension—Floating cells were collected by centrifugation at 300 × g for 10 min at 4 °C. The old medium was aspirated, and the cells were resuspended in 500 μl of PBS (pH 7.4). Cells were centrifuged again, the PBS removed, and the pellet was resuspended in 75 μl of fresh PBS. Resuspended cells were spread on a poly-L-lysine-coated slide (LabScientific, Inc.) using a clean slide. The cells were fixed by immersing the slides in fresh 4% formaldehyde/PBS at 4 °C for 25 min. The rest of the protocol was carried out as explained above.

Western Blot Analysis

C-PAE cells (1 × 10⁶) were seeded in 10-cm Petri dishes precoated with fibronectin (10 μg/ml) in the presence of 2% FCS containing 3 ng/ml bFGF. Endostatin was added at 10 μg/ml, and cells were harvested at 12, 24, and 28 h after treatment. Cells were washed three times in PBS buffer, pH 7.4, and the cells were resuspended in 1 ml of 1× EBC buffer (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 1% Nonidet P-40) containing freshly added complete protease inhibitor tablet (Pierce, Rockford, IL). 100 μg/ml Pefabloc, 1 μg/ml pepstatin. The protein concentration in whole cell lysate was measured by the bicinchoninic acid method. 30 μg of whole cell extract was loaded onto a 4–15% gradient polyacrylamide gel. Transfer was performed using a semi-dry transblot apparatus (Bio-Rad). The membrane was blocked in wash buffer (1× Tris-buffered saline) with 5% nonfat dry milk and incubated at 37 °C for 1 h. Goat antibody directed against human Bcl-2 (N-19) {sc-492-G} was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Affinity purified mouse polyclonal antibody against Bax (B-9) {sc7490} and Bcl-XL (m-8) {sc1690} were purchased from the same manufacturer. Polyclonal anti-actin antibody (Sigma) was used to normalize for protein loading. Secondary antibodies were anti-goat, mouse and rabbit immunoglobulin conjugated to horseradish peroxidase (Amersham Pharmacia Biotech). The immunoreactivity was detected with an enhanced chemiluminescence reagent (Pierce). Images were scanned using a flat bed scanner (ScanJet 4C) and quantitated by the NIH image 1.61 software. Normalization was done by dividing the Bcl-2 signal by that of actin within each experiment.

RESULTS AND DISCUSSION

Endostatin Changes the Morphology of Endothelial Cells—When proliferating, C-PAE cells were treated with mouse en-
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**Fig. 2.** A, annexin V-FITC staining was performed on C-PAE cells treated with increasing concentrations of endostatin (0.1–10 µg/ml). FACS was done to quantify the percentage of cells undergoing apoptosis. Cells were stained with PI just before analysis. Gating was performed to analyze only annexin-V positive and PI negative cells. TNF-α (40 ng/ml) was used as a positive control. Control (black); endostatin, 10 µg/ml (red); 5 µg/ml (yellow); 1 µg/ml (green); TNF-α, 40 ng/ml (blue). FL1-height represents the annexin fluorescence intensity as a log scale. B, flow cytometric analysis of apoptosis induced by endostatin treatment. Endostatin was added at 10 µg/ml and cells were harvested at 3, 4, 6, 12, and 18 h after treatment. Processing of cells was performed as described earlier. The cell populations were gated to analyze only annexin V positive/PI negative cells. Control (black); 3 h (red); 6 h (green); 12 h (blue); and 18 h (orange). FL1-height represents the annexin fluorescence intensity as a log scale. C and D, flow cytometric analysis of apoptosis induced by endostatin on nonendothelial cells. 786–0, renal clear cell carcinoma cells (C) and NIH3T3 fibroblasts (D). FL1-height represents the annexin fluorescence intensity as a log scale. Control (black); endostatin, 10 µg/ml (green); 5 µg/ml (red); 2.5 µg/ml (dark blue); 1 µg/ml (orange); and 0.5 µg/ml (light blue).

dostatin at a concentration of 10 µg/ml, the cells rounded (Fig. 1A) and detached from the fibronectin-coated plate. In the controls, the cells showed intact endothelial cell morphology, whereas endostatin-treated cells appeared rounded and showed membrane blebbing characteristic of apoptotic cells. We have also observed similar effects with endostatin-treated bovine adrenal cortex capillary endothelial and bovine aorta endothelial cells (data not shown), but C-PAE cells showed the most dramatic effect. Interestingly, these effects of endostatin were most pronounced when bFGF was added to subconfluent monolayers (2 × 10⁴/well in a 24-well plate and 0.1 × 10⁶/well in a 6-well plate) when cells were in exponential growth. This,

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**Fig. 3.** A, endostatin treatment increases caspase 3 activity. Samples from control and treated cells were processed, and caspase 3 activity was detected by reading the samples in a microplate reader at 405 nm. The A₄₀₅ reading was plotted in the presence (black box) and absence (open box) of the inhibitor (DEVD-fmk). The error bars represent mean ± S.E. B, caspase 3 activity in nonendothelial cells. NIH3T3 and H9c2 (2–1)-myoblast cells were treated with endostatin at 10 µg/ml. The cells were harvested and processed as described for C-PAE cells.
Endostatin Translocates Phosphatidylserine from the Inner Face of the Plasma Membrane to the Cell Surface—After initiation of apoptosis, most cell types translocate the membrane phospholipid PS from the inner surface of the plasma membrane to the outside (11–13). PS can be detected by staining with an FITC conjugate of annexin V, 38-kDa protein that binds naturally to PS. During programmed cell death, PS externalization typically precedes membrane bleb formation and nuclear condensation with DNA fragmentation.

**Endostatin Translocates Phosphatidylserine from the Inner Face of the Plasma Membrane to the Cell Surface**

With regard to nonendothelial cells, 786–0 and NIH3T3 cells, we failed to see any distinct annexin positivity (Fig. 2, C and D). In addition, we screened other nonendothelial cells (IMR-90, A10, and H9c2 (2–1)-myoblast) and found no effect of PS, we conducted a time course experiment (Fig. 2B). The effect of endostatin was significant \((p = 0.01)\) at 12 h after treatment. Time points before 6 h did not show a difference between control and treated samples.

Morphological examination of FACS-analyzed samples with fluorescence microscopy (Nikon) showed annexin V staining localized to the cell membrane at 12 h with no staining in the cytoplasm (data not shown). During this period, the majority of the cells were negative for PI, implicating the early stage of apoptosis. With increased exposure time (24–36 h), in addition to membrane staining with annexin V some of the cells turned positive for PI (data not shown) (12, 14), consistent with a more advanced stage of apoptosis.

Similar levels of annexin V staining were observed in two other endothelial cell lines studied, namely, bovine aorta endothelial and bovine adrenal cortex capillary endothelial (data not shown). We have also tested the effect of human endostatin on these three bovine endothelial cell lines. We failed to detect annexin V staining in the presence of human endostatin added to these cells, whereas when human endothelial cell lines were used (HUVE and HMVE-L), it resulted in a marked shift in annexin V fluorescence. These data indicate that apoptosis, as assessed by annexin V staining, occurs in diverse endothelial cells in response to mouse and human endostatin.

**Endostatin Translocates Phosphatidylserine**

![Diagram](image)

**Fig. 2.** A, detection of fragmented DNA in adherent C-PAE cells by the TUNEL assay. Adherent cells were fixed with fresh 4% formaldehyde/PBS at 4 °C for 25 min. The cells were permeabilized with prechilled 0.2% Triton X-100/PBS for 5 min on ice. After permeabilization, the TUNEL assay was performed along with propidium iodide dye staining. On staining, pictures were taken immediately using a fluorescent microscope attached to a digital camera and analyzed using the SPOT software. **Panels a, c, e, and g are fields of cells stained with propidium iodide (1 μg/ml). Panels b, d, f, and h are the same fields under green fluorescent light.**

**Summary**: Endostatin causes externalization of PS, which perhaps, mimics the *in vivo* situation, *i.e.* endostatin appears to be selective for tumor vasculature where there is endothelial cell division. Importantly, endostatin at 10 μg/ml on nonendothelial cells, *viz.* A10 and H9c2 (2–1)-myoblast, did not show any effect as was seen in endothelial cells (Fig. 1, B and C).

Moreover, because cells plated efficiently on fibronectin-coated plates even in the presence of endostatin, endostatin’s effect was not simply via altering cell-matrix interactions. Next, we checked for other hallmarks of apoptosis, phosphatidylserine externalization, intracellular protease activation, and nuclear condensation with DNA fragmentation.

**Fig. 2A** shows annexin V-FITC staining in conjunction with PI staining in C-PAE cells treated for 18 h with different concentrations of endostatin. Endostatin at 10 μg/ml showed a distinct shift in annexin fluorescence intensity. The mean fluorescence intensity difference between control and endostatin-treated cell was significant \((p = 0.01)\) at 5 and 10 μg/ml. The shift in fluorescence intensity was similar for endostatin at 10 μg/ml and the positive control TNF-α (40 ng/ml). Concentrations of endostatin below 0.1 μg/ml did not show any significant annexin V positivity (data not shown). To investigate the earliest time point at which endostatin caused externalization of PS, we conducted a time course experiment (Fig. 2B). The effect of endostatin was significant \((p = 0.01)\) at 12 h after treatment. Time points before 6 h did not show a difference between control and treated samples.
endostatin (data not shown). Based on these results, endostatin’s action appears to be selective for endothelial cells.

**Endostatin Increases the Intracellular Activity of Caspase 3**—Caspase 3 (CPP32) is an intracellular protease activated early during apoptosis of mammalian cells and initiates cellular breakdown by degrading specific structural, regulatory, and DNA repair proteins (12, 14–16). This protease activity can be measured spectrophotometrically by detection of the chromophore (p-nitroanilide) after cleavage from the labeled substrate (DEVD-pNA). First, we performed a time course experiment with 10 μg/ml of endostatin and checked for increase in caspase 3 activity. There was no difference in caspase 3 activity between the treated and the control samples at 2, 4, 8, and 14 h (data not shown). However, caspase 3 activity was elevated 24 h after treatment with endostatin over controls. The caspase activity of the endostatin and TNF-α (positive control) treated samples is shown in Fig. 3A. When compared with controls, endostatin-treated cells showed a 1.8-fold increase in caspase 3 activity after 24 h, whereas TNF-α gave a comparable (1.75-fold) increase. When a specific inhibitor of caspase 3 (DEVD-fmk) was included in the same samples, the protease activity was at baseline (comparison of the dark box with the corresponding white box), indicating that the increase in the measured activity was specific for caspase 3. For NIH3T3 cells only, a marginal increase in caspase 3 was seen, whereas in myoblast cells there was no difference in caspase 3 levels between treated and control cultures (Fig. 3B).

**Endostatin Induces DNA Fragmentation in the Nucleus of Endothelial Cells**—Fragmentation of nuclear DNA is one of the distinct morphological changes occurring in the nucleus of an apoptotic cell. A TUNEL assay was performed on endostatin-treated, TNF-α-treated, and control cells. Fig. 4A shows the immunofluorescent pictures of adherent cells on slides. In the presence of the enzyme terminal deoxynucleotidyltransferase, both endostatin- and TNF-α-treated slides showed numerous positive cells under green fluorescence (panels f and h), whereas no positive cells were seen in the control (panel b). Without the enzyme, the endostatin-treated slide showed background cell fluorescence (panel d). Panels a, c, e, and g represent the same field of cells stained with propidium iodide.

We counted the number of apoptotic cells in several fields, and the percent of apoptotic cells (green divided by the number of red cells per field) is plotted in Fig. 4B. The apoptosis rate in the control cells was 1.24%. In the endostatin-treated cells, a 30-fold increase in the apoptosis rate was observed in suspension cells (38.3%), whereas a 15-fold increase was observed in
the attached cells (19.4%). With TNF-α, the apoptosis rate was 6.4%. In contrast, the percent of TUNEL-positive in the angiostatin-treated bovine adrenal cortex capillary endothelial cells was 2% when compared with the control cells (1.2%), a 1.6-fold increase (17), suggesting that endostatin is a stronger apoptotic agent than angiostatin.

**Bcl-2 and Bax Expression by Western Blot Analysis**—Anti-apoptotic members such as Bcl-2 and Bcl-X_L prevent programmed cell death in response to numerous stimuli (18, 19). Conversely, pro-apoptotic proteins such as Bax and Bak can accelerate cell death, and in certain cases, they are sufficient to cause apoptosis independent of additional signals (19). We tested whole cell extract of endostatin-treated and control C-PAE cells for Bcl-2 and Bax expression levels. In growth arrested C-PAE cells, Bcl-2 expression was high. It was relatively constant up to 28 h; in contrast, endostatin-treated cells showed a marked decrease in Bcl-2 (Fig. 5A). Densitometry revealed that the levels of Bcl-2 compared with control was 1.2-, 1.5-, and 3-fold less at 12, 24, and 28 h, respectively, after treatment, with actin levels used as normalization controls. In contrast, Bax expression was similar between control and treated cultures (Fig. 5B).

Bcl-2 protein was not detected in NIH3T3 and IMR-90 cells. Bax expression levels were not affected by endostatin treatment in these cell lines (Fig. 6, A and B). In C-PAE cells, at the early time point (12 h), Bcl-X_L level was reduced by 2-fold; whereas its expression was unchanged in NIH3T3 cell (Fig. 6, C and D). Interestingly, we have detected only the larger pro-apoptotic form of Bcl-X in C-PAE, whereas both smaller and larger forms were detected in NIH3T3.

These findings suggest that endostatin exerts its regulatory activity by altering Bcl-2 expression. Interestingly, vascular endothelial growth factor has been shown to augment Bcl-2 levels in endothelial cells (20). Because endostatin antagonizes VEGF’s proliferative effects (data not shown), Bcl-2 appears to be one of potentially many down-regulated proteins. Recent studies indicate that the Bcl-2 protein binds to other proteins, such as Bax, Bcl X_L, Bik, and Bad, which ultimately enhance cell survival (21, 22). The function of another Bcl-2 homologue, Bax, remains enigmatic (23). Bcl-2 and Bcl-X_L function through heterodimerization with Bax (24, 25) and overexpression of Bax accelerates apoptosis (23). Recently, it was shown that FGF-2 inhibited endothelial cell apoptosis by Bcl-2-dependent and -independent mechanisms (26). In our study, we did see differences in Bcl-2 (and Bcl-X_L) expression in endostatin-treated cultures but no difference in Bax levels. It is possible that Bcl-2 may act independently of Bax, as has been shown for T cells (26). Studies are in progress to delineate the importance of other anti-apoptotic and pro-apoptotic proteins and their phosphorylation status in response to endostatin treatment.

In summary, we have demonstrated that endostatin causes apoptosis of endothelial cells in vitro, but not nonendothelial cells. This finding sheds the first mechanistic insight into the action of endostatin. The intracellular events that trigger the apoptotic response are under current investigation.

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