Endostar Induces Apoptotic Effects in HUVECs through Activation of Caspase-3 and Decrease of Bcl-2

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Abstract. *Background: Endostar is a novel recombinant human endostatin expressed and purified in Escherichia coli with the N-terminal modified. It has been shown that endostar inhibited the proliferation of human umbilical vein endothelial cells (HUVECs) through the membrane surface receptor KDR/flt-1(VEGFR-2, vascular endothelial growth factor receptor-2) by exerting anti-angiogenesis effects. But the molecular mechanism remained unclear. Materials and Methods: The apoptotic effects induced by endostar in the serum-deprived situation were investigated by 4'-6-diaminidino-2-phenylindole (DAPI) staining and the Annexin V-fluorescein isothiocyanate (FITC) binding assay. The mechanism of action was explored by Western blotting assay. Results: Endostar induced remarkable apoptosis in HUVECs. The expressions of apoptosis-related proteins showed that caspase-3 was activated, but caspase-8, a marker of the non-mitochondria-mediated apoptosis signal pathway, was not. Further investigation revealed that cellular Bcl-2 decreased in the endostar-treated groups, while the level of Bax was almost unchanged. Conclusion: Endostar induces apoptotic effects in HUVECs through the activation of caspase-3 and a decrease of the Bcl-2 to Bax ratio.*

Endostatin, the 20-kDa *C*-terminal fragment of collagen XVIII, is a potent endogenous angiogenesis inhibitor (1). It has been proven that endostatin can effectively suppress endothelial cell proliferation (2) and migration (3, 4) *in vitro* and inhibit tumor growth in different types of animal model *in vivo*. Endostatin demonstrated such pronounced anti-angiogenesis and antitumor effects that it was quickly pushed into clinical trials (5-7). However, it was apparently not successful in the clinical trials because of problems with the technique for recombinant human endostatin production (8, 9). Reduced solubility of the recombinant endostatin prepared from *Escherichia coli* led to failure in clinical therapy. A soluble form of endostatin is available from a yeast system that has a relatively low yield and high cost, but which has made it difficult to produce endostatin in quantities sufficient for extensive clinical evaluation (10). Despite much investigative effort, thus far, this problem still exists.

Endostar, a recombinant human endostatin which is expressed and purified in *E. coli*, was approved by the Chinese State Food and Drug Administration (SFDA) in 2005 for the treatment of non-small cell lung cancer. Compared with endostatin, endostar has an additional nine-amino acid sequence (MGGSHHHHH) added to the *N*-terminal of the protein, which results in the formation of a six-histidine tag and enhancement of affinity with metal ions (11). These changes have simplified the purification and improved the stability of the protein. It is reported that endostar had a potent effect in animal tumor models and the half-life of endostar was longer than endostatin (12). The antiangiogenic efficacy of endostar has been demonstrated (13).

Basic fibroblast growth factor (bFGF) is a member of the fibroblast growth factor (FGF) family, which is widely involved in the proliferation of many cells, the inhibition of some stem cells and the development of mesoderm in early embryos. In normal tissue, it is present in basement membranes and in the subendothelial extracellular matrix of blood vessels. Some data have shown (14, 15) that bFGF exhibits multiple functions in angiogenesis and tumor growth of various carcinomas. It has been proven (16) that bFGF was able to suppress the apoptotic effects caused by many factors such as serum-deprivation-induced apoptosis.

Apoptosis is an important process during normal development for the maintenance of tissue homeostasis and

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the elimination of unwanted or damaged cells from multicellular organisms. When the regulation of apoptosis is disordered, it leads to the occurrence of many human diseases, especially tumorigenesis. Several lines of evidence have indicated that endothelial cell apoptosis may limit angiogenesis and actively lead to vessel regression in adult neovascularization(17). Especially in tumor tissues, endothelial cell apoptosis efficiently prevents nutrient supply through blocking the formation of new blood vessels. Therefore, clarification of the mechanism of apoptosis could be important for preventing and treating tumors (18). Whether or not programmed cell death contributes to the inhibition of proliferation of endothelial cells with endostar treatment was investigated by 4'-6-diaminidino-2-phenylindole (DAPI) staining, Annexin V-fluorescein isothiocyanate (FITC) binding assay and by Western blot.

Materials and Methods

Materials. Endostar, expressed and purified in *E. coli*, was provided by the corporation of Simcere (Nanjing, China). Recombinant human bFGF was purchased from Chemicon International Inc (Temecula, CA, USA). The primary antibodies for Bax, Bcl-2, caspase-3, -8 and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). IRDyeTM800 (a fluorescent dye, Ex: 780 nm, Em:820 nm) conjugated secondary antibodies were obtained from Rockland Inc (Gibertsville, PA, USA). The Annexin V-FITC apoptosis detection kit was provided by KeyGEN Biotech. Co. Ltd (Nanjing, China).

Cell culture. Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord veins by collagenase treatment as described previously (19). The harvested cells were maintained at 37˚C in a 5% CO_2 humid incubator and cultured using medium 199 (Gibco, Grand Island, NY, USA) containing 20% heat-inactivated fetal calf serum (Gibco), endothelial cell growth supplement (ECGS, 30 μg/ml; Sigma, St. Louis, MO, USA), epidermal growth factor (EGF 10 ng/ml; Sigma), 100 U/ml penicillin, and 100 μg/ml streptomycin. After 3-5 passages, the HUVECs were collected for use. In all the experiments, the HUVECs were incubated in starvation medium supplemented with 1% FBS and were treated with or without various concentrations of endostar; 5 ng/ml bFGF was added to each treatment except the control.

DAPI staining assay. DAPI, a nuclear dye for detecting changes of nucleic acid and DNA, is known to form fluorescent complexes with natural double-stranded DNA, showing fluorescence specificity for AT (adenine-thymine), AU (adenine-uracil) and GC (guanine-cytosine) clusters. When DAPI binds to DNA, its fluorescence is strongly enhanced. The HUVECs were seeded in 6-well plates at a seeding density of 105 cells/ml. When all the cells were adhered, various concentrations of endostar (5, 25 and 125 μg/ml) and 5 ng/ml bFGF were simultaneously added. The additional of bFGF mimicked the *in vivo* situation. After 24 h incubation, the cells were fixed with 4% paraformaldehyde for 20 minutes and permeabilized by incubation in 0.1% sodium citrate containing 0.1% Triton® X-100 for 2 minutes at 4˚C, then labeled with DAPI (300 nM). After labeling, the apoptotic cells were visualized using an Olympus IX51 (Olympus, Japan) microscope using a filter designed for DAPI fluorescence (Chroma, 82000 series; Olympus). Digital imaging was performed with an Olympus DP71 digital camera and Olympus DP Controller Application software and analyzed by DP manager. The cells were considered to be apoptotic when they showed either fragmented or condensed (pyknotic) nuclei. The apoptotic cells were defined morphologically by cytoplasmic and nuclear shrinkage and chromatin condensation or fragmentation. A total of 500 cells were counted in five fields per sample. The percentage of apoptotic cells was calculated as the number of apoptotic cells over the total number of cells counted (20).

Annexin V- FITC binding assay. The Annexin V-FITC binding assay was performed according to manufacturer's instruction. The cells were seeded and treated with endostar as for the DAPI staining assay. After 24 h incubation, the cells were trypsinized, washed in PBS and resuspended in binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). The cells were incubated with Annexin V-FITC (100 ng/ml) in the dark for 10 min and 10 ml propidium iodide (PI) was added to each group before flow cytometric analysis. Positive Annexin V staining indicated apoptosis, while positive PI indicated necrosis. For each group, a minimum of 10,000 cells were counted. Data analysis was performed with standard Cell Quest software.

Western blotting analysis. The HUVECs were seeded and cultured in culture plates until 60-70% confluence and then incubated with various concentrations of endostar for different periods of time with bFGF simultaneously added except for the control. The cells were lysed in lysis buffer (50 mM Tris-Cl, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% (m/v) Nonidet P40 (NP-40), 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM NaF and 1.0 mM dithiothreitol-deoxynucleoside triphosphate (DTT)) and the lysates were clarified by centrifugation at 4˚C for 15 min at 13,000 g. The concentration of protein in the supernatants was detected using the BCA (bicinchoninic acid) protein assay with a Varioskan multimode microplate spectrophotometer (Thermo, Waltham, MA, USA). Then equal amounts of proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, USA). After blocking with 10% nonfat milk in PBS for 1 h at 37˚C, the membranes were incubated for 1 h with the indicated primary antibodies at 37˚C followed by IRDyeTM800 conjugated second antibody for 1 h at 37˚C. Detection was performed by the Odyssey Infrared Imaging System (LI-COR Inc., Lincoln, USA). All the blots were stripped and reprobed with polyclonal anti-β-actin antibody to ascertain equal loading of proteins.

Statistical analysis. All the results shown represent the mean ± S.E.M. from triplicate experiments performed in a parallel manner unless otherwise indicated. Statistical comparisons between groups were performed using one-way ANOVA followed by Student's *t*-test, and significant differences were indicated as $*p<0.05$ and $**p<0.01$.

Results

The chromatinic changes of HUVECs treated with endostar. The apoptotic nuclei were filled with condensed or fragmented chromatin, which showed enhanced fluorescence with DAPI staining. Marked morphological changes and chromatin

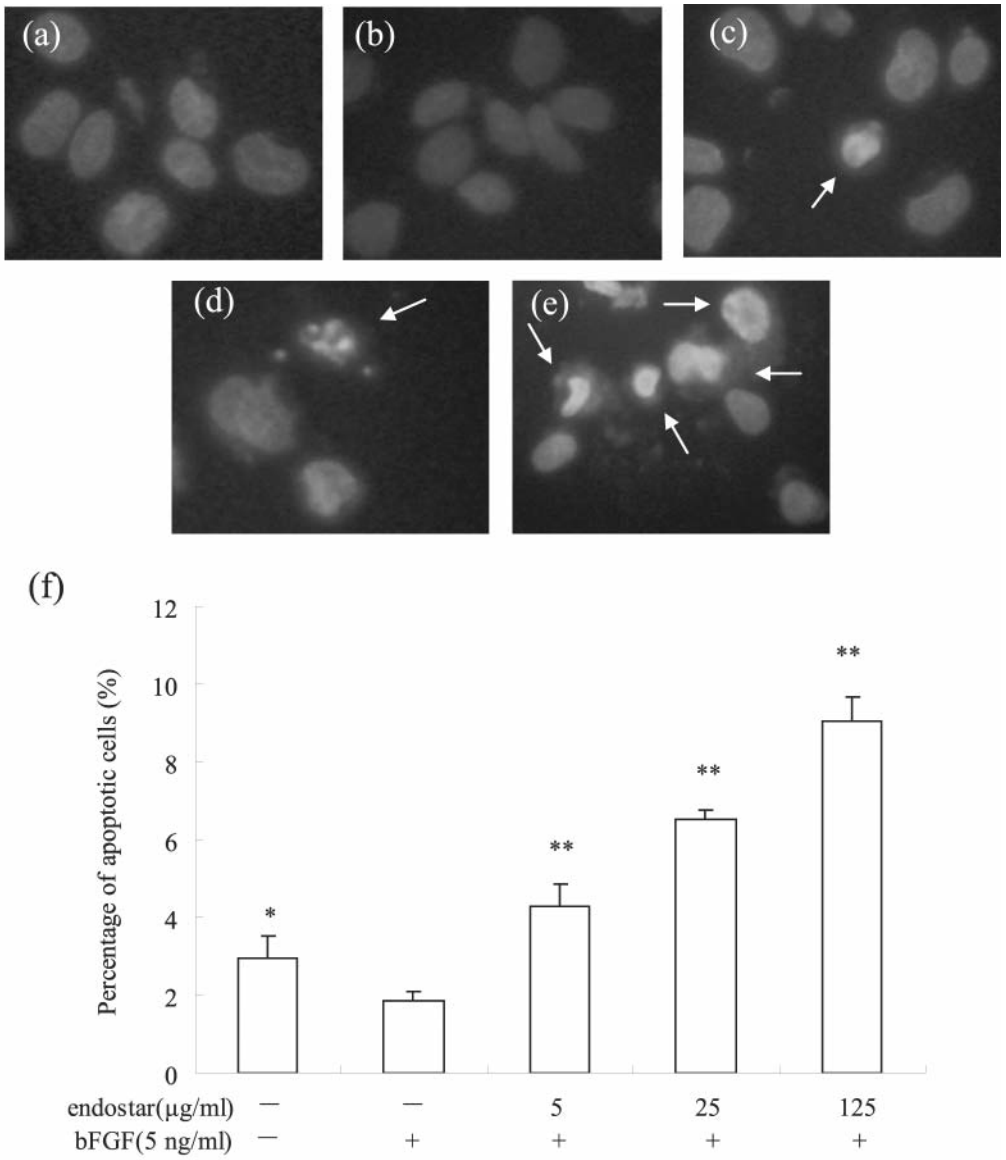

Figure 1. *The morphological changes of HUVECs shown by DAPI staining: (a) control; (b) bFGF alone; (c) endostar 5 μg/ml with bFGF; (d) endostar 25 μg/ml with bFGF; (e) endostar 125 μg/ml with bFGF. Quantification of the chromatin morphological changes with DAPI staining (f). Data are mean±S.E.M. of three independent experiments. *p<0.05, **p<0.01 compared with bFGF alone. Arrows: apoptotic cells.*

condensation were seen in the adherent and detached cells in the endostar-treated groups. Notable apoptosis was seen in the cells treated with endostar (125 μg/ml) compared with the control group (Figure 1). There was a concentration-dependent increase in cells staining with DAPI after treatment with endostar.

The induction of apoptosis in HUVECs by endostar. As shown in Figure 2, a significant induction of apoptosis was shown by endostar treatment, which gradually increased as the concentrations rose from the 5 μg/ml to 125 μg/ml. Cells incubated in 1% FBS medium without bFGF also displayed apoptosis. There were necrotic cells detected in this investigation.

The activation of caspase-3 in HUVECs by endostar. Western blot analysis showed that the expression of caspase-8 was not affected by the 24 h endostar treatment (Figure 3a). However, the level of cleaved caspase-3 gradually increased as the concentration of endostar rose from 5 μg/ml to 125 μg/ml (Figure 3b). The additionally endostar treatment activated caspase-3 from 12 to 48 h (Figure 3c).

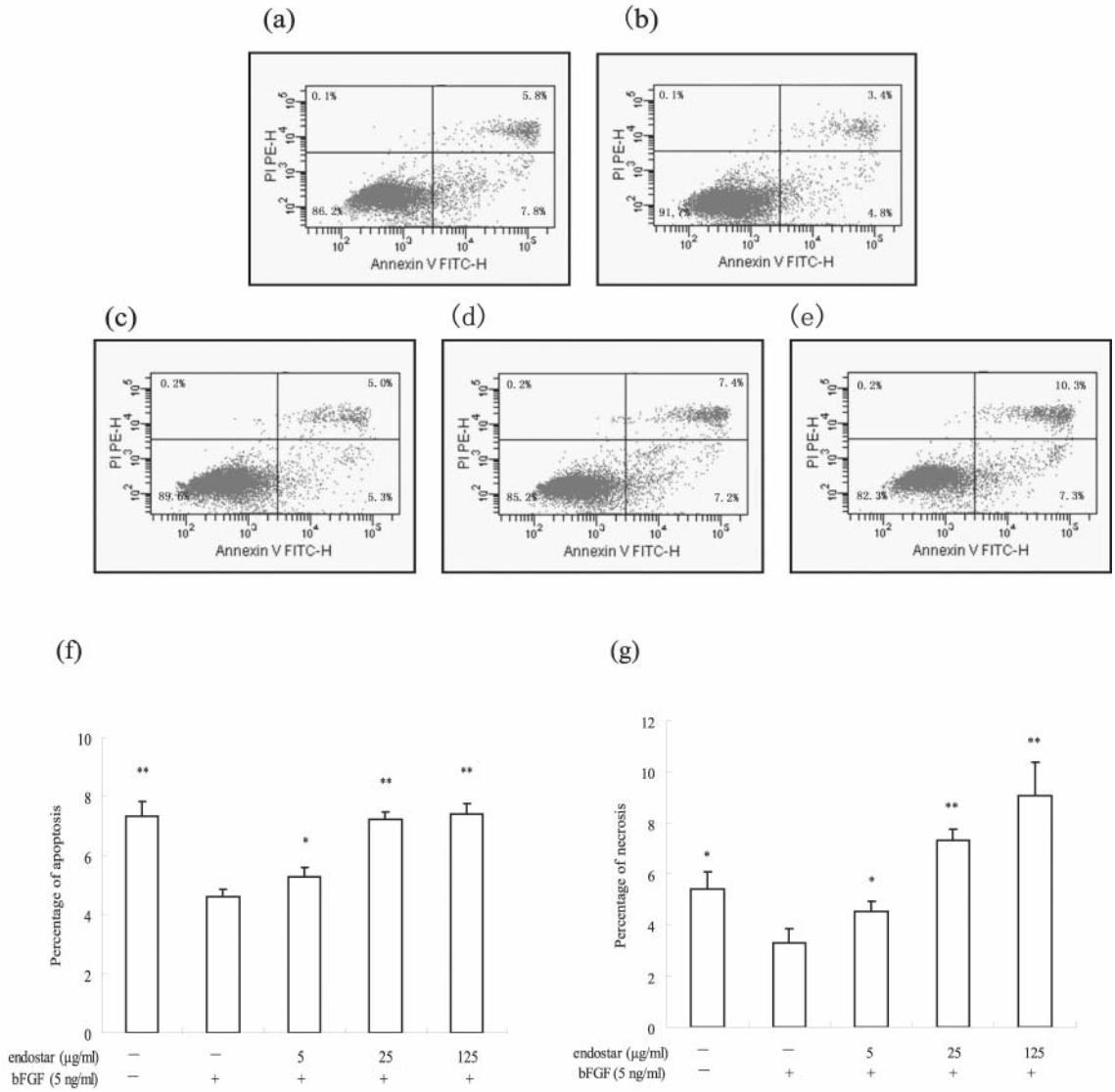

Figure 2. *Apoptosis and necrosis in HUVECs as shown by Annaxin V / PI. Flow cytometry of (a) control, (b) bFGF alone, (c) endostar 5 μg/ml with bFGF, (d) endostar 25 μg/ml with bFGF, (e) endostar 125 μg/ml with bFGF, (f) the percentage of apoptotic cells in all groups, (g) the percentage of necrosis in all groups. Data are mean±S.E.M. of three independent experiments. *p< 0.05; **p< 0.01 compared with bFGF alone.*

The effect of endostar on the expression of Bcl-2 and Bax. As shown in Figure 4, there was an evident decrease in the expression of Bcl-2 after the treatment with endostar. Therefore the ratio between Bcl-2 and Bax decreased, though the expression of Bax showed no change with endostar treatment.

Discussion

In the present study, endostar induced apoptosis in the HUVECs as shown by both DAPI staining and Annexin V-FITC binding assay. However, necrosis, as another form of cell death due to severe change in the surrounding environment, was also detected as the dosage rose. This was obviously associated with the dosage of endostar and probably induced after serum deprivation and endostar treatment for 24 h.

The caspases are a family of cysteine proteases whose activation induces cellular apoptosis. Caspases participate in a cascade triggered by pro-apoptotic signals and culminates in the cleavage of a set of proteins, resulting in disassembly of the cells (21). Specifically, caspase-3, the active form of pro-caspase-3, is a key molecule for identifying the activation of apoptosis (22). However, several different factors act upstream

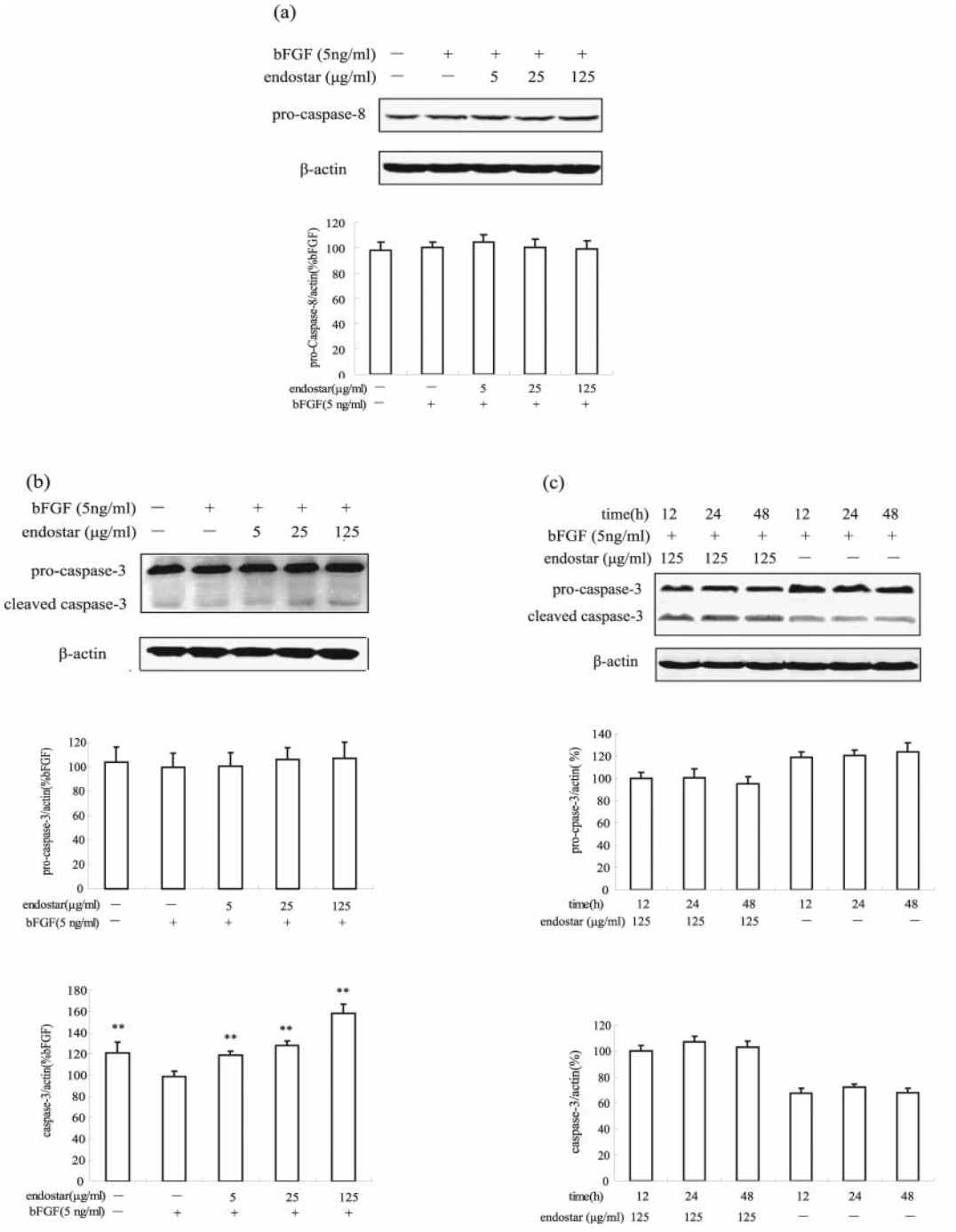

Figure 3. *Effect of endostar on the expression of caspase-3 and caspase-8. (a) The expression level of caspase-8 after 24 h treatment. (b) The expression level of caspase-3 after 24 h treatment. (c) Effect of treatment time on caspase-3 expression. Data are mean±S.E.M. from three independent experiments. **p<0.01 compared with bFGF alone.*

of caspase-3. Caspase-3 can be activated by caspase-8, caspase-9, caspase-10, CPP32 (caspase-3)-activating protease and granzyme B (23). All the activators are classified into two main pathways: one, the death receptor-mediated pathway (non-mitchondria-mediated) correlates with caspase-8, -10 (24); the other, the mitochondrion-mediated pathway, involves caspase-9. Caspase-3 is a critical protein downstream of both signal pathways (25). In the present study endostar activated

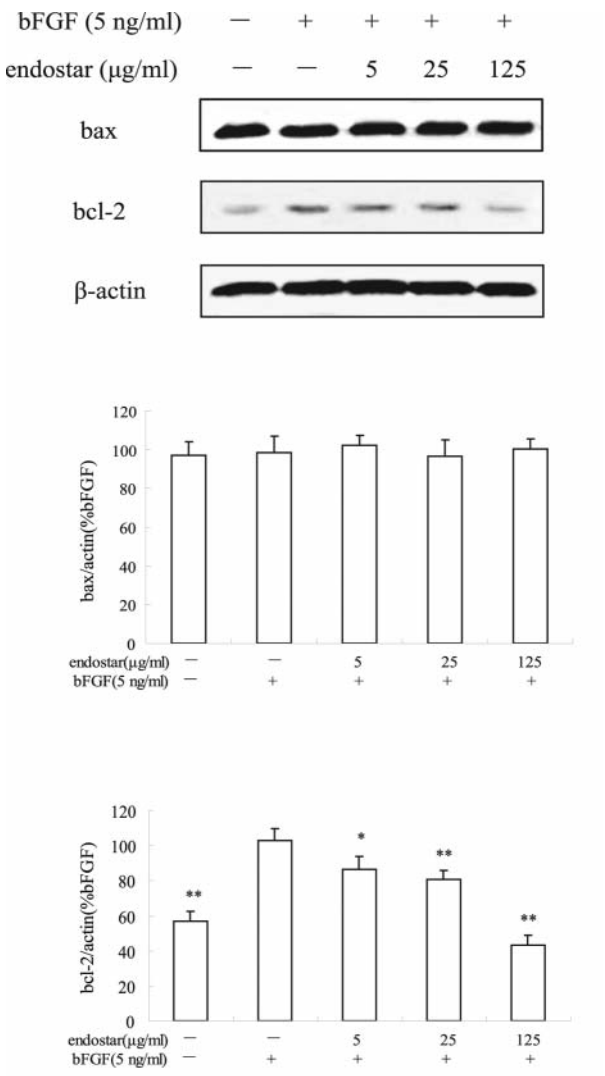

Figure 4. *Western blotting analysis of Bcl-2 and Bax after 24 h treatment with or without endostar. Data are mean±S.E.M. of three independent experiments. *p<0.05, **p<0.01 compared with bFGF alone.*

caspase-3 but not caspase-8. Therefore, the induction of apoptosis by endostar depended on the activation of the mitochondrial apoptotic pathway rather than the death receptor-mediated pathway.

The Bcl-2 family is an important apoptosis-regulating family and includes proteins such as Bcl-2, which is an anti-apoptotic molecule, and Bax, which is pro-apoptotic (26). A decrease of Bcl-2/Bax is sufficient to promote apoptosis in mammalian cells and induce cell death by directly activating the mitochondrial apoptotic pathway in upstream of caspase-9 and caspase-9 naturally activates caspase-3 (23, 27, 28). Therefore the change of Bcl-2/Bax is crucial for the activation of caspase-3 in the mitochondrion-mediated pathway. In the present study, Bcl-2/Bax decreased and caspase-3 was activated with the endostar treatment. Thus, the decrease of Bcl-2/Bax played an important role in the induction of apoptosis by endostar.

bFGF was used to promote proliferation of the serum-deprived endothelial cells in the present experiments. bFGF has been found to inhibit the endothelial cell apoptosis induced by growth factor deprivation (29), which was also the case in the present study. Furthermore, bFGF has been shown to induce the expression of anti-apoptotic proteins, such as Bcl-2 and survivin (30). The overexpression of Bcl-2 in endothelial cells prevents apoptosis induced by serum and growth factor deprivation, whereas it has no effect on bFGF-induced endothelial cell proliferation (29). The present results showed no effect on the expression of Bax with the endostar treatment, although the ratio of Bcl-2/ Bax decreased. The levels of Bcl-2 and Bax suggested that the induction of Bcl-2 by bFGF was probably partial blocked by the treatment with endostar.

Some angiogenesis inhibitors have previously been reported to cause apoptosis of endothelial cells (31-33). This suggests there are some associations between survival, apoptosis and angiogenesis in endothelial cells. However, such a hypothesis has yet to be demonstrated and warrants further investigations.

In summary, this is the first report showing that endostar causes the apoptosis of endothelial cells *in vitro* through the activation of caspase-3 and decrease of Bcl-2, which supports the use of endostar in further clinical application.

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