[Frontiers in Bioscience 10, 1089-1097, May 1, 2005]

MULTIPLE SIGNALING IS INVOLVED IN ENDOSTATIN-MEDIATED APOPTOSIS IN ECV 304 ENDOTHELIAL CELLS

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TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Materials and Methods
- *3.1. Expression and purification of human endostatin in E. Coli*
- *3.2. Cell culture*
- *3.3. Detection of cell viability*
- *3.4. Annexin V-FITC/PI analysis for apoptosis*
- *3.5. Fluorescence microscopy of apoptotic nuclear features*
- *3.6. DNA gel electrophoresis for specific ladder band*
- *3.7. Measurement of cytosolic calcium and magnesium concentration*
- *3.8. Caspase-3 activity assay*
- *3.9. Detection of Bcl 2 and p53 protein expression*
- *3.10. Statistical analysis*

4. Results
- *4.1. rhEndo affected the viability of the ECV 304 endothelial cells*
- *4.2. rhEndo induced endothelial cell apoptosis*
- *4.3. Intracellular caspase-3 activity increased in rhEndo-treated ECV 304s*
- *4.4. rhEndo increased intracellular calcium and magnesium concentration*
- *4.5. Change of Bcl 2 and p53 expression*

5. Discussion
6. Acknowledgements
7. References

1. ABSTRACT

Apoptosis of vascular endothelial cells is associated with the regression of angiogenesis. Endostatin is a potential anti-angiogenic drug, but the effects of endostatin on apoptotic machinery in endothelial cells largely remain unclear. In the present study, human endostatin was expressed in *E. Coli* to induce apoptosis in endothelial cells. It was found that the expressed human endostatin specifically affected the viability of the ECV 304 in a dose-dependent manner. Endostatin induced apoptosis in these cells in a caspase-dependent manner, and endostatin-mediated apoptosis is associated with several apoptotic signaling pathways including overloading of intracellular magnesium and calcium, as well as regulation of p53 and Bcl 2 expression.

2. INTRODUCTION

The formation of new blood vessels from preexisting capillaries, or angiogenesis is a series of events essential in a broad array of physiologic and pathologic processes. Normal tissue growth, such as embryonic development, wound healing, and the menstrual cycle, is characterized by its dependence on new blood vessel formation for the supply of oxygen and nutrients as well as the removal of waste products (1). Excessive angiogenesis is involved in the pathology of many diseases such as cardiovascular diseases (atherosclerosis), chronic inflammation, diabetes, psoriasis and cancers, etc. In different types of neoplasia, the modulation of angiogenic activity is associated with tumor regression in animals. Major effort has been focused on identification of mediators that normally inhibit the angiogenic switch for therapeutic purposes.

It is known that tumor angiogenesis is regulated by the balance of stimulators, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), and inhibitors such as thrombospondin-1 (TSP-1), endostatin, angiostatin, and angiostatic steroids (2, 3). Angiogenesis plays a critical role in the growth and metastasis of malignant tumors. Several lines of experimental studies suggest that primary tumor invasiveness and metastasis require angiogenesis. However, normal angiogenic response requires a delicate balance between stimulators of angiogenesis, such as VEGF, and the inhibitors of angiogenesis, such as TSP-1

(4, 5), endostatin, etc. Growth of solid tumors is dependent on the induction of angiogenesis to provide adequate oxygen and nutrients to promote the proliferation of cells (6-8). Targeting angiogenesis may provide an alternative or additional treatment for solid tumor. Alternately, inhibition of endothelial cell proliferation and induction of endothelial cell apoptosis are important endpoints for efficacy of antiangiogenic agents.

Endothelial cell apoptosis induced by a variety of mechanisms (including growth factor deprivation) is responsible for inhibiting angiogenesis, thereby preventing the growth of primary tumors and their metastases (9). It has been shown that apoptosis of microvascular endothelial cells leads to regression of neoplastic tissue and involution of normal tissue (10). Understanding the effects of antiangiogenic therapeutic and the apoptotic machinery of endothelial cells is essential for developing logical antiangiogenic regimens for treating human cancers.

Endostatin was found to stop the growth of Lewis lung carcinoma experimental metastases in mice without signs of toxicity, and it has been shown to specifically inhibit the growth of several primary tumors (11, 12). A variety of antiangiogenic agents have been shown to exert potent antiangiogenesis activity and therapeutic potential in tumor models (13). Recently, endostatin therapy has been shown to be effective in a liver cancer model (14-16). In vitro studies have demonstrated that endostatin inhibits proliferation and migration of endothelial cells (17, 18). Moreover, endostatin treatment has been confirmed to induce a block in cell cycle progression, leading to apoptosis in endothelial cells (19-22). The anti-angiogenic activity of endostatin is attributable at least in part to the apoptosis of vascular endothelial cells mediated by this angiogenesis inhibitor. However, relevant molecular signaling machinery is poorly defined and understanding the *in vitro* effects and signaling pathway is beneficial to understanding the cellular and molecular basis of angiogenesis and development of useful anti-angiogenesis drugs in the prevention and treatment of various types of tumors. Our present study demonstrates that the recombinant human endostatin (rhEndo) obtained by using *E. Coli* expression system specifically affects the viability, and proliferation rates of ECV 304 endothelial cells stimulated by bFGF and in basic media *in vitro*. Addition of bFGF made the endothelial cells more sensitive to endostatin treatment. The apoptosis mediated by this recombinant endostatin involves in caspase-3 activation and calcium and magnesium overloading, as well as the regulation of endogenous expression of Bcl 2 and p53, indicating multiple intracellular signaling is associated with the apoptotic endothelial cells induced by endostatin.

3. MATERIALS AND METHODS

3.1. Expression and purification of human endostatin in E. Coli

The rhEndo was expressed in L 21(DE3) SI *E. Coli* using the expression vector of rhEndo pET/ENDO, and purified, and soluble biologically active rhEndo was obtained.

3.2. Cell culture

Human ECV 304 cells (derived as a spontaneous transformant of human umbilical vein endothelial cells), NIH3T3 and HeLa cell lines were maintained in RPMI medium 1640 (GIBCO BRL) supplemented with 10% fetal bovine serum (FBS, Hyclone), 100 units/mL penicillin, 100 µg/mL streptomycin, and 2 mM/L-glutamine. The cells were incubated in a humidified environment at 37 in the presence of 5% CO_2. Serum starvation of endothelial cells was done by switching to the RPMI 1640 media containing 2% FBS for overnight, and 3 ng/mL bFGF (Sigma Chemical) was added into the media containing 2% FBS in bFGF-stimulation groups.

3.3. Detection of cell viability

The rhEndo was evaluated for its ability to affect the viability or growth of ECV 304 and non-endothelial cell HeLa and NIH3T3 by the MTT method. Cell viability was analyzed as described (23) according to TACS™ MTT Assay instruction pamphlet (Trevigen, Inc.). Briefly, after treatment with rhEndo, the cells were incubated continuously for 48 hours followed by addition of freshly prepared MTT for another 4 hours at 37 . Subsequently, cells were lysed with DMSO and the cell viability was analyzed with Universal Microplate Reader (Bio-Tek Instruments ELx 800) at wavelength of 570 nm with reference wavelength at 630 nm. The relative viability or growth was analyzed when the cells were treated with various doses of the endostatin.

The influence of rhEndo in ECV 304 proliferation cycle was detected by propidium iodine (PI) staining and Fluorescence Activated Cell Sorting (FACS) as previously published report (20) by using a Coulter® EPICS XL™ Flow Cytometer. Data was processed with MultiCycle DNA Analysis Software (Phoenix Flow System).

3.4. Annexin V-FITC/PI analysis for apoptosis

Annexin V, a calcium-dependent phospholipid-binding protein with a high affinity for phosphatidylserine (PS) and propidium iodine (PI) was used to detect the apoptosis of ECV 304. The cells were treated in reference to previous report (20) before analysis with 10, 20µg/mL rhEndo, and untreated ECV 304s were used as the control. The cells were trypsinized and obtained for the detection of apoptosis with Annexin V-FITC/PI Kit according to manufacturer's protocol (IMMUNOTECH, France). FACS analysis of apoptotic ECV 304 cells after staining by annexin V-FITC/PI was performed on a Coulter® EPICS XL™ Flow Cytometer. The data was analyzed with System II™ Software.

3.5. Fluorescence microscopy of apoptotic nuclear features

The number of apoptotic and necrotic cells were also quantified by a fluorescence Nikon microscope with Spot 2 CCD camera as described by Duke et al (24). In brief, acridine orange (250 µg/mL) and ethidium bromide (250 µg/mL) were added to the medium of cultured cells and cells were visualized under fluorescence microscopy at a magnification of x 200. Acridine orange is a membrane-

permeable dye that intercalates into DNA, staining the nucleus green and allowing visualization of the nucleus and chromatin. Cells were considered apoptotic if they had a shrunken cytoplasm and nucleus and condensed chromatin that appeared as spheres or a peripheral crescent. Ethidium bromide is a membrane impermeable dye that is taken up only by nonviable necrotic cells, staining them orange. A minimum of 400 cells per dish were examined. Differential staining of apoptotic cells in a mixture of acridine orange/ethidium bromide and typical apoptotic morphology was used to determine the percentage of cells undergoing nuclear changes characteristic of apoptosis.

3.6. DNA gel electrophoresis for specific ladder bands

ECV 304 endothelial cells were collected by centrifugation (500 X g , 5 min), washed in PBS, re-suspended in 0.5 ml lysis buffer (10 mM EDTA, 10 mM Tris –HCL pH 8.0, 100 µg/mL proteinase and 0.4% sodium dodecyl sulfate) and incubated at 50 for 3 - 5 h. DNA was extracted and visualized under UV light after staining with ethidium bromide and photographic image or digital image was collected with Eagle Eye II, PIII Computer W/Mini Camera (Stratagene Co.).

3.7. Measurement of cytosolic calcium and magnesium concentration

Cytosolic calcium or $[Ca^{2+}]_i$ concentration in ECV 304 was measured as previously described (25). Cytosolic magnesium or $[Mg^{2+}]_i$ concentration in the cells was assayed according to previously published report (26). Prior to the experiments, ECV 304s were incubated for 30 minutes in Fura 2/AM specifically for calcium or magnesium ions (Sigma Chemical, USA) respectively in serum-free Hanks buffered salt solution (HBSS) at 37 . The cells were then washed in Fura-free HBSS and kept in dark at room temperature for 30 minutes prior to the start of the experiment to allow for deesterification of the fluorescent dye. RT-540 Spectrofluorophotometer (Shimadzu Corporation, Japan) and connected computer were used to assay the concentrations of the cytosolic calcium and magnesium ions. The absorption shift of Fura-2 that occurs upon binding is determined by scanning the excitation spectrum between 344 and 380 nm while monitoring the emission at 510 nm for $[Ca^{2+}]_i$, and for $[Mg^{2+}]_i$ by scanning the excitation spectrum between 330 and 370 nm while monitoring the same emission wave length as in detecting $[Ca^{2+}]_i$. The concentrations of $[Ca^{2+}]_i$ was calculated from the equation: $[Ca^{2+}]$=KDa R-Rmin /Rmax-R), and the concentration of $[Mg^{2+}]_i$ was estimated from the ratio (R) of the fluorescence intensity at 330 and 370 nm with the emission at 510 nm. Triplicate experiments were performed.

3.8. Caspase-3 activity assay

This assay was performed according to the protocol provided by the manufacturer (Sigma). $2x10^6$ cells were seeded and maintained overnight in RPMI medium 1640 with 10% FBS. The following day, the medium was replaced with fresh RPMI 1640 containing 2% FBS along with bFGF (3 ng/mL), rhEndo of 5, 10 and 20 µg/mL was added, and the cells were grown for 24 h. For control cells, the equivalent amount of PBS was added. After 24 hours, the cells were pelleted by centrifugation at 600 x g for 5 minutes at 4^oC. The supernatant was removed by gentle aspiration. The cells were counted and re-suspended in 1x lysis buffer at a concentration of 100 µL per 10^7 cells. A specific caspase-3 inhibitor, Ac-DEVD-CHO, was used as suggested by the manufacturer. The optical absorbance at 405 nm of wavelength was measured in a Universal Microplate Reader. The increase in protease activity (caspase-3) was determined by comparing the results of the rhEndo-induced sample with the PBS control, and the caspase-3 activity in various samples was calculated.

3.9. Detection of Bcl 2 and p53 protein expression

ECV 304s were cultured on coverslips and treated with or without rhEndo (10 µg/mL), and the coverslips were taken out after 24 hours and were washed with RPMI medium 1640 to remove floating cells. The cells were processed for the analysis of Bcl 2 and p53 expression. The expression was detected by immunocytochemistry streptavidin-biotin (S-P) method (Maxim Biotech, Inc., China Branch). Bcl 2 and p53 antibodies (kindly provided by Dr. Gang Meng, Anhui Medical University) were used in the cells remaining on the coverslips with the treatment of rhEndo and the untreated control. Images in eight visual fields (X200) from 500 to 1200 cells in the slides were randomly collected with a Spot 2 CCD camera in Nikon microscope. The expression of the protein were routinely evaluated and quantified with MetaMorph Imaging System (Universal Imaging) (27).

3.10. Statistical analysis

Student's *t* test was used to analyze the results with statistic analysis software - SPSS 8.0 for Windows and $p<0.05$ was considered statistically significant.

4. RESULTS

4. 1. rhEndo affected the viability of the ECV 304 endothelial cells

The MTT assay was used to detect the viability of the ECV 304 endothelial cells, and non-endothelial cells (NIH3T3 and HeLa). It was found that rhEndo showed a dose-dependent influence on the viability or growth of ECV 304 cells. However, even higher concentration of 20 µg/mL of rhEndo did not show significant effects on the growth of non-endothelial cells. After HeLa, NIH3T3, ECV 304 stimulated by bFGF and ECV 304 in basic media were exposed to 20 µg/mL rhEndo for 48 hours, the optical absorption value in HeLa and NIH3T3 groups did not change significantly as compared with the untreated control, while the optical absorption value for endothelial cells decreased markedly, up to 3.5- and 2.2-fold, respectively, when compared with the untreated control (Table 1), indicating that the endostatin significantly and specifically affected the viability of the endothelial cells. Interestingly, the endostatin showed a much more potent influence on the viability or growth of the endothelial cells in bFGF-stimulated groups when compared with the cell groups grown in basic media (Figure 1-A). The survival rate of the cells treated with 10 and 20 µg/mL rhEndo for 48 h decreased from 100% (untreated control)

Table 1. Effects of recombinant human endostatin on ECV 304 and non-endothelial cell viability expressed with optical absorbance value (mean ± SD)

Cell lines (well)	No. samples Blank control	rhEndo	(20 microgram/mL)
HeLa	16	0.277±0.022	0.232±0.031
NIH3T3	16	0.285±0.030	0.237±0.026
ECV 304 stimulated by bFGF	8	0.328±0.018	0.095±0.012
ECV 304 in basic culture media	8	0.247±0.019	0.112±0.035

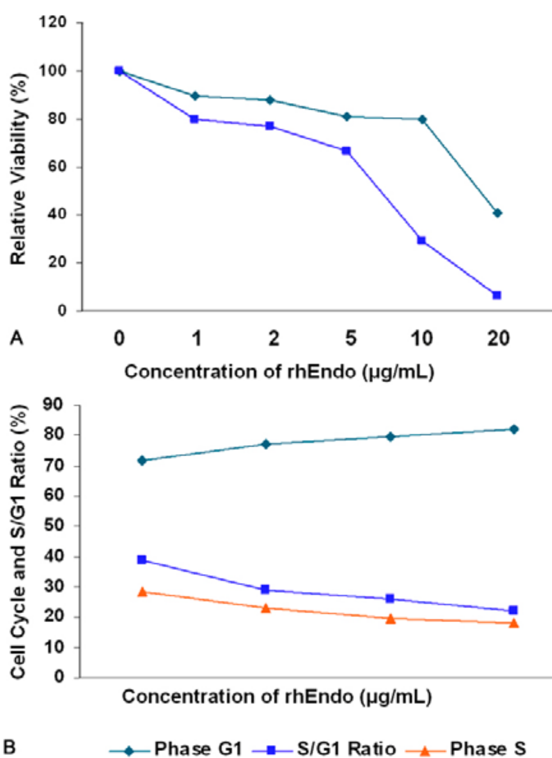

Figure 1. Effects of recombinant human endostatin on the viability of ECV 304 cells in different conditions. ■ECV 304 cultured in bFGF-stimulated conditions ECV 304 cultured with RPMI 1640 containing 10% FBS. They both were treated with different concentrations of the endostatin as indicated for 48 hours. **A.** rhEndo affected the survival in ECV 304 cells in a does-dependent manner, the relative survival rate decreased from 100% (untreated control) to about 6% (20 µg/mL) in the cells stimulated with bFGF and from 100% (untreated control) to about 40% (20 µg/mL) in the cells grown in basic media. Results are representative of three separate experiments. **B.** Influence of recombinant human endostatin on ECV 304 cell cycles. After treatment with different concentrations of rhEndo for 24 hours, the proportion of G1 phase ECV 304 increased from about 71% to 82%, and the ratio of S/G1 decreased from 39% (untreated) to 29% (5 µg/mL), 24% (10 µg/µL) and 22% (20 µg/µL) .

to about 29% and 6% in bFGF-stimulated cells as compared to a decrease from 100% (untreated control) to about 79% (10 µg/mL rhEndo) and 40% (20 µg/mL rhEndo) in the cells grown in basic media, respectively.

Additionally, when ECV 304 cells cultured in RPMI 1640 containing 2% and stimulated with bFGF were treated with rhEndo, we found that rhEndo affected the cycle progression. In the group treated with rhEndo (20 µg/mL), the percentage of cells in S phase decreased by approximately 10% while those in G1 phase cells increased by approximately 10% as compared with untreated control, and therefore the S/G1 ratio decreased significantly (Figure 1-B). This implies that endostatin may affect not only the proliferation but also the synthesis of the DNA in the endothelial cells.

4.2. rhEndo induced endothelial cell apoptosis

Given the importance of apoptosis of endothelial cells in the suppression of angiogenesis, several methods were used to investigate if recombinant human endostatin mediates the apoptosis of ECV 304 endothelial cells.

FACS analysis with Annexin V-FITC showed that rhEndo indeed induced apoptosis in ECV 304 stimulated with bFGF, the percentage of cells that stained positively with Annexin V-FITC was 38.3%, and 56.9% respectively in 10, 20 µg/mL group after treatment with endostatin for 24 hours, and only 4.29% in untreated control (Figure 2-A). After ECV 304 were treated with 10 µg/mL rhEndo for 12 and 24 hours, the apoptotic rate increased from 16.1% to 30.5%, respectively, while the apoptotic index in the control was only 3.5% (Figure 2-B). Agarose gel electrophoresis of DNA isolated from ECV 304 cells treated with rhEndo (10, 20 µg/mL) for 48 hours showed a typical DNA fragmentation during apoptosis (Figure 2-C). Fluorescence microscopy also confirmed that rhEndo induced the apoptosis of the endothelial cells in a dose-dependent manner even when the cells were cultured with RPMI 1640 media containing 10% FBS after treatment with various concentrations of the recombinant endostatin (Figure 2-D).

4.3. Intracellular caspase-3 activity increased in rhEndo-treated ECV 304s

Caspase-3 is a key protease that is activated during the early stages of apoptosis. The activation of this protease is closely associated with the apoptotic process in most of the cell types. Endostatin has previously been shown to activate caspase-3 in bovine pulmonary artery vascular endothelial cells (20). In ECV 304 endothelial cells the effect of rhEndo on the activity of caspase-3 was assayed by colorimetric assay (Sigma Chemical, USA). Our results demonstrated that the caspase-3 activity increased significantly in ECV 304 groups treated with rhEndo of 5, 10 and 20 µg/mL for 24 hours when compared with un-induced control, respectively up to about 1.5-, 1.6- and 1.8-fold increase in enzymatic units (Figure 3). There is a statistically significant difference in the elevation of activity of caspase-3 when compared with the untreated control ($p<0.01$), suggesting that endostatin-mediated apoptosis is caspase-dependent.

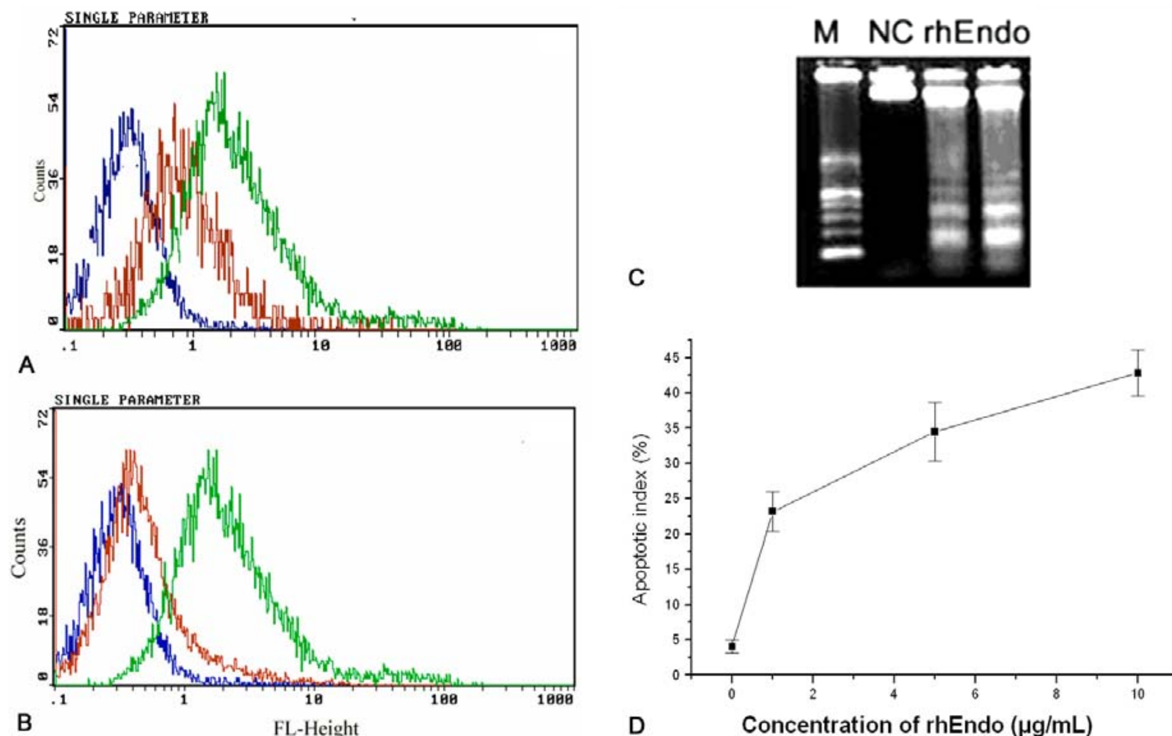

Figure 2. FACS analysis of recombinant human endostatin-induced apoptosis. A. ECV 304 treated with 10 μg/mL (red, middle), 20 μg/mL (green, right) recombinant human endostatin and untreated control (blue, left). The percentage of cells that stained positively with Annexin V-FITC was 38.3%, 56.9% respectively in 10, 20 μg/mL group and 4.29% in untreated control. B. ECV 304 treated with 10 μg/mL recombinant human endostatin for 12 h (red, middle), 24 h (green, right) and untreated (blue, left) for 24 h. The apoptosis index in these cells was found to be up to 16.1% and 30.5% respectively while the apoptosis index in the control was only 3.5%. Results are representatives of three separate experiments. C. Agarose gel electrophoresis of DNA isolated from ECV 304 cells treated with rhEndo (10, 20 μg/mL) and untreated control for 48 h. The DNA ladder was formed obviously. M, DNA marker; NC, Untreated control; rhEndo, 10, 20 μg/mL rhEndo treatment. D. Fluorescent microscopic assay of apoptosis induced by recombinant human endostatin. After treatment with different concentrations of rhEndo for 48 h, ECV 304 in basic culture was 23.21 ± 2.77%,34.49 ± 4.15%,and 42.81 ± 3.26%,while that of the control was 4.05 ± 0.93%. Two to five hundred ECV 304 cells per sample were examined, and triplicate experiments were performed.

4.4. rhEndo increased intracellular calcium and magnesium concentration

The elevated levels of intracellular Ca^{2+} and magnesium ions ($[Ca^{2+}]_i$ & $[Mg^{2+}]_i$) are a prominent feature of apoptosis (28, 29). A sequential activation of a Ca^{2+}/Mg^{2+}-dependent nuclease leads to the fragmentation of the genome into discrete nucleosomal-sized fragmentations of DNA, subsequently followed by the fragmentation of the nucleus itself (i.e., apoptosis), and eventually with the complete digestion of the nucleosomal oligomers into component nucleotides (i.e. DNA loss). It has been reported that endostatin induced an increase in the $[Ca^{2+}]_i$ in endothelial cells(18, 30). However, intracellular magnesium concentrations in endothelial cells treated with endostatin has not been studied. Therefore we investigated the effects of rhEndo on both $[Ca^{2+}]_i$ and $[Mg^{2+}]_i$. Both $[Ca^{2+}]_i$ and $[Mg^{2+}]_i$ concentration were increased after the cells were treated with rhEndo at the concentrations of 1, 5 and 10 μg/mL (Table 2 & 3). Highly significant statistical differences were found between rhEndo treatment groups and untreated control ($p<0.01$). These results suggest that rhEndo-mediated endothelial cell apoptosis is associated with the overload of calcium, and the overloading of intracellular magnesium also contributes to the apoptosis induced by this recombinant endostatin.

4.5. Change of Bcl 2 and p53 expression

As shown in our study and results from Dr. Dhanabal et al, endostatin caused the endothelial cells to arrest in G1 phase and become apoptotic (20). We hypothesized that endogenous expression of p53 may play a crucial role in this process. Growth factors and members of the Bcl-2 family have also been shown to play an important role (31) in this respect, and the cross-talk between survival signaling and apoptotic signaling may be involved in the regulation of apoptosis mediated by endostatin. Therefore, we investigated the influence of endostatin on endogenous p53 and Bcl 2 expression in ECV 304 endothelial cells by immunocytochemistry. Our results demonstrated that the expression of p53 increased and that of Bcl 2 decreased after treatment with rhEndo for 24 hours. The preliminary study by immunocytochemical streptavidin-biotin (S-P) detection showed that staining intensity of samples changed after treatment with rhEndo (10 μg/mL). MetaMorph Imaging System analysis revealed that the average optical density significantly increased in

Table 2. Effects of recombinant human endostatin on $[Ca^{2+}]_i$ in ECV 304 (mean ± SD)

Concentration (microgram/mL)	n	$[Ca^{2+}]_i$ (nM)
0.10	12	136.41±12.52
1.0	13	138.36±4.92
5.0	10	253.21±48.35
10.0	11	372.50±76.46
Control	5	109.25±17.49

Table 3. Effects of recombinant human endostatin on $[Mg^{2+}]_i$ in ECV 304 (mean±SD, n = 11)

Concentration(microgram/mL)	R value of the samples
1.0	0.979 ± 0.010
5.0	2.224 ± 0.305
10.0	3.923 ± 0.154
Control	0.945 ± 0.025

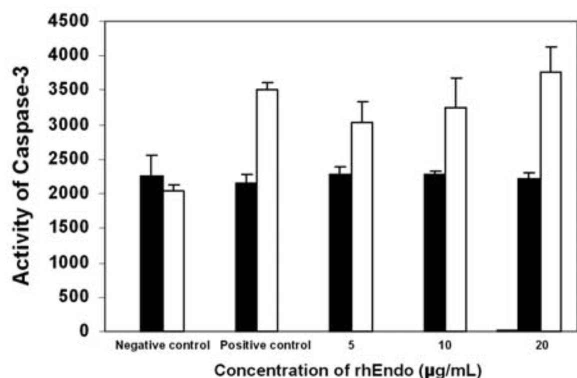

Figure 3. Recombinant human endostatin increased the activity of caspase-3 in ECV 304. Samples from control and treated cells were processed, and caspase-3 activity was detected by reading the samples in a Universal Microplate Reader at 405 nm. The positive control from Sigma was used during the detection procedure. The optical density (OD) was read in the presence and absence of the inhibitor (Ac-DEVD-CHO). p -Nitroaniline calibration curve of OD values versus the concentrations of the p-Nitroaniline solutions and the activity of caspase-3 was calculated based on the formula provided by the manufacturer, represented as black box (add inhibitor) and blank box (no inhibitor). The error bars represent mean ± SD. Fold increases of caspase-3 activity ($P< 0.01$) are found in the recombinant human endostatin induced samples compared with the uninduced control. The triplicate experiments were performed.

groups using mouse anti-human p53 as the first antibody detection but the average optical density decreased in groups using mouse anti-human Bcl 2 as the first antibody when compared with the untreated control. There was a statistical significant increase in p53 expression and a decrease in Bcl 2 expression in rhEndo treated groups when compared with the untreated control (Figure 4). The densitometry result implied that the expression of endogenous p53 was up-regulated whereas that of endogenous Bcl 2 was down-regulated, suggesting Bcl 2 and p53 signaling pathway may be associated with endostatin-mediated anti-angiogenetic effects.

5. DISCUSSION

The natural angiogenesis inhibitor, endostatin has entered phase II clinical trials for cancer treatment (32). However, its clinical effects remain to be determined and remarkably little is known about the mechanistic aspects of this proteolytic product of the nonfibrillar collagen XVIII. At present, recombinant forms of endostatin have been expressed in prokaryotic and eukaryotic cells (19, 33-35). Some *in vitro* studies have shown that endostatin specifically induces the apoptosis of endothelial cells but has little effects on tumor cells or non-endothelial cells (19-20). For ECV 304 cells, it has been reported that *soluble* rhEndo expressed in a yeast expression system inhibited the migration of these bFGF-stimulated cells in a dose-dependent manner (19) but they did not study the effects of endostatin on growth of this cell line. In our previous study, we confirmed that recombinant human endostatin inhibited the proliferation of ECV 304 but not that of primary rabbit aortic endothelial cells (37). In the present study we extend our investigation on the cellular and molecular mechanisms of the endostatin activity in ECV 304 endothelial cells.

In endostatin-treated bovine pulmonary artery endothelial cells, endostatin-induced apoptosis is related to the decreased expression of Bcl 2 and increased activity of caspase-3 (20). In murine brain endothelial cells and porcine aortic endothelial cells endostatin binds to the endothelial cell surface via heparan-sulfated proteoglycans, and directly or indirectly induces tyrosine kinase activity, which may cause apoptosis, depending on the proliferative state of the cells (21). Our results demonstrate that rhEndo affects the viability of the human ECV 304 endothelial cells, arrests the cells in G1 phase, and moreover decreases the synthesis of DNA. Interestingly, though this angiostatic factor did not show obvious effect on the proliferation of primary rabbit aortic endothelial cells grown in media containing 10% FBS (37), it significantly affects the survival or growth of the ECV 304 endothelial cell grown in media containing 10% FBS. Moreover, a more significant effect on growth was found in the endothelial cells stimulated with bFGF. Therefore, we focused on the signaling pathways of endostatin-mediated apoptosis in the cells cultured with low serum and bFGF.

In addition, our study reveals that endostatin affects the DNA synthesis and damages cellular DNA. It is known that the p53 gene is a key player in the cellular response to DNA damage. DNA damage activates p53, which in turn induces either growth arrest primarily in phase G1 of the cell cycle or apoptosis (38, 39). The halting of the cell cycle facilitates surveillance of the genome and allows repair of the damage. If the cells are not prepared to repair the damage, they are alternatively channeled into apoptosis (31). Therefore, we hypothesized that p53 might be involved in the effects of rhEndo on endothelial cells.

In fact, p53 decreases the expression of endogenous Bcl 2(40), as shown in our study, and it may in

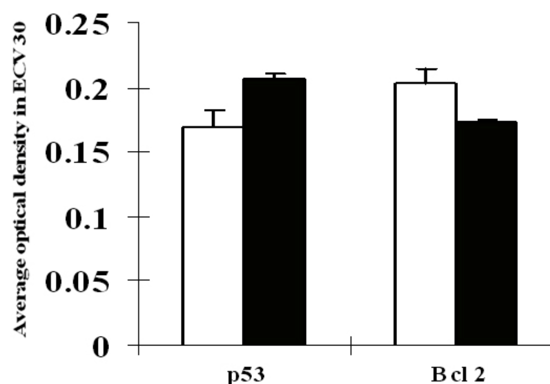

Figure 4. Effects of recombinant human endostatin on endogenous p53 and Bcl 2 expression in ECV 304. Comparison of average optical density in PBS control (blank box) with 10 μg/mL recombinant human endostatin treatment for 24 h (black box) (n = 8, X200; The error bars represent mean ± SD; P<0.01 & P <0.05, respectively in p53 and Bcl 2 versus the control).

turn accelerate the apoptotic process of the endothelial cells. In fact, the endogenous p53 over-expression in ECV 304 mediated by endostatin may be a protective mechanism to prevent cell injury from endostatin activity (41). The rhEndo-mediated apoptosis might be partially attributed to both the increased expression of p53 and decreased expression of anti-apoptotic protein Bcl 2. Additionally, wild type p53 mediates antiangiogenic effects through up-regulation of the thrombospondin (42). Our results suggest that endostatin may modulate viability of endothelial cells through up-regulation of endogenous p53 expression in endothelial cells and down-regulation of endogenous Bcl 2.

Endostatin elicits Ca^{2+} transient elevation in primary bovine aortic endothelial cells and human microvascular endothelial cells grown on coverslips. Prolonged exposure to endostatin attenuates acute Ca^{2+} signaling in response to subsequent treatment with proangiogenic growth factors (30). Endostatin also induced a sustained increase in the $[Ca^{2+}]_i$ in a dose-dependent manner (18). However, our results demonstrate that not only intracellular calcium but also the intracellular magnesium ions are involved in the apoptosis mediated by endostatin, and the exact mechanism of these ions in the regulation of endostatin function remains to be investigated in the future study.

Caspase-3 is an effector of a family of caspases, and it can process procaspase-2, -6, -7 and -9, and specifically cleaves most of caspase related substrates, including the nuclear enzyme poly (ADP-ribose) polymerase (PARP), the inhibitor of caspase-activated deoxyribonuclease (ICAD) (43). It has been implicated as a key protease that is activated during the early stages of apoptosis (44). Our results have shown that caspase-3 activity was elevated in ECV 304 after 24 hours of treatment with various concentrations of rhEndo. The activation of caspase-3 is possibly caused by intrinsic mitochondrial apoptotic pathway through generating active caspase-9 that directly activates the caspase-3, and its activation also results from the activation of caspase-8 through surface receptor apoptotic pathway directly or indirectly via activation of the intrinsic pathway (45). Does endostatin induce endothelial cell apoptosis indirectly through death receptor signaling by binding to endostatin receptors and/or directly activating mitochondria pathway? The exact machinery and associated signaling pathways remain to be clarified.

In summary, rhEndo-mediated apoptosis of ECV 304 is a complicated process associated with multiple signaling pathways. The over-expression of Bcl 2 has been demonstrated to block the translocation of cytochrome c into cytosol (46, 47). In our study, the decreased expression of Bcl 2 is possibly associated with the elevation of endogenous p53 expression. This might also result in the release of cytochrome c and triggers apoptosis, which remains to be elucidated. The interaction of $[Ca^{2+}]_i$ and $[Mg^{2+}]_i$ overload and regulation of the expression of p53 and Bcl 2 may be associated with rhEndo-mediated apoptosis in ECV 304 endothelial cells in a caspase-dependent manner. Further elucidation of endostatin-mediated apoptotic pathways in endothelial cells may yield new insights into the pathogenic mechanisms of this process and clues to block the process in the endothelial apoptosis mediated by nature occurring angiogenic inhibitors.

6. ACKNOWLEDGEMENTS

The project is supported by National Natural Science Foundation of China (39870324) and a grant for Outstanding Young Faculty from Ministry of Education, P.R. China (99044312), Fund for High Level University Construction from Chinese Academy of Science and NIH K08 CA88965-01 A1 (S Parangi). We are grateful to Dr. Roya Khorsravi-Far and Dr. Jack Lawler at Department of Pathology, Harvard Medical School for critical reading of the manuscript, and Yan-qiu Dong and Xuejuan Jiang for technical help.

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Key Words: Recombinant Human Endostatin, Endothelial Cells, Proliferation, Apoptosis, Molecular Signaling, p53 Expression, Intracellular calcium and magnesium

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