Endostatin induces autophagic cell death in EAhy926 human endothelial cells

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Summary. Endostatin, a proteolytic fragment of collagen XVIII, is a potent inhibitor of angiogenesis and suppresses neovascularization and tumor growth. However, the inhibitory mechanism of endostatin in human endothelial cells has not been characterized yet. Electron microscopic analysis revealed that endostatin induced formation of numerous autophagic vacuoles in endothelial in 6 to 24 h after treatment. Moreover, there was only a 2- to 3-fold increase in intracellular reactive oxygen species after endostatin treatment. Endostatin-induced cell death was not prevented by antioxidants (vitamin C, vitamin E, or propyl gallate) or caspase inhibitors, suggesting that the increase of oxidative stress or the activation of caspases may not be the crucial factors in the anti-angiogenic mechanism of endostatin. However, the cytotoxicity of endostatin was significantly reduced by 3-methyladenine (a specific inhibitor of autophagy) and serine and cysteine lysosomal protease inhibitors (leupeptin and aprotinin). Taken together, these results suggest that in human endothelial cells: (1) endostatin predominantly causes autophagic, rather than apoptotic, cell death, (2) endostatin-induced autophagic cell death occurs in the absence of caspase activation and through an oxidative-independent pathway, and (3) endostatin-induced 'autophagic cell death or 'type 2 physiological cell death' is regulated by serine and cysteine lysosomal proteases.

Key words: Endostatin, Human endothelial cell, Autophagic cell death, Lysosomal protease

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Introduction

Angiogenesis plays an important role in physiological processes such as embryonic development, wound healing, and menstrual cycle (Risau, 1997; Augustin, 1998; Ferrara and Alitalo, 1999). However, it is also required for the growth and development of solid tumors and their metastasis, tumor mass being limited to 1-2 mm^3 unless the tumor recruits additional vascular network (Folkman, 1996; Hanahan and Folkman, 1996). To stimulate angiogenesis in order to promote their survival, tumors upregulate the production of a variety of angiogenic factors, such as fibroblast growth factors (aFGF and bFGF) and vascular endothelial growth factor/vascular permeabilty factor (VEGF/VPF) (Clement et al., 1999; Bruns et al., 2000). Extensive angiogensis is, therefore, linked to aggressive tumor growth and poor prognosis (Folkman, 1995, 1996; O'reilly et al., 1997; Risau, 1997).

The concept of combating tumors by cutting off their blood supply using angiogenesis inhibitors or antiangiogenic proteins has received considerable interest after the discovery of several endogenous antiangiogenesis proteins (eg, angiostatin, endostatin, antithrombin fragment and canstatin) (Harris, 1998). Endostatin, in particular, has attracted tremendous attention and has recently been used in human clinical trial for cancer therapy (Bergers et al., 1999; Oehler and Bicknell, 2000). It has been isolated from conditioned media of a murine hemangioendothelioma cell line and identified as a 20 kDa C-terminal proteolytic fragment of the extracellular matrix protein, collagen XVIII (O'reilly et al., 1997). In vitro studies, both mouse and human endostatin specifically inhibit the proliferation and migration of endothelial cells, and potently suppress the neovascularization and tumor growth (Chen et al., 1999; Moulton et al., 1999; Perletti et al., 2000). Analysis and comparison of the human and murine endostatin sequences revealed a high level of conservation (>90% amino acid identity) (Saarela et al., 1998). Most remarkably, in mice bearing various types of tumors, repeated treatments with recombinant endostatin led to

tumor dormancy but did not induce resistance (Harris, 1998; O'reilly et al., 1997; Yokoyama et al., 2000). Moreover, endostatin causes apoptosis of rat or cow pulmonary artery endothelial cells by down-regulating cell proliferation genes and anti-apoptotic proteins (Bcl-2, Bcl-X$_L$), then increasing the activation of intracellular protease caspase 3 (Dhanabal et al., 1999; Shichiri and Hirata, 2001). Currently, the mechanism of endostatin-mediated cell death of human endothelial cell lines is still unclear.

Apoptosis, a complicated event, is induced by various stimuli or pharmacological agents. Recently, oxidative stress has been recognized as an important factor involved in the mechanism of apoptosis. Increased levels of intracellular free radicals impair mitochondrial functions, including ATP synthesis, release of cytochrome c and cleavage of caspases, then initiate the cell death program (Brunk and Svensson, 1999; Olejnicka et al., 1999). Our previous observations indicate that endostatin induces changes in respiratory rate and decreases ATP synthesis in human endothelial cells, suggesting that it affects mitochondrial functions and depletes the energy pool of endothelial cells (unpublished data). Since it is not known whether oxidative stress is involved in the mechanism of endostatin-mediated apoptosis, in the present study we tested whether it was involved in endostatin-mediated cell death in endothelial cells. Here, we present evidence that endostatin predominantly causes autophagic, rather than apoptotic, cell death. Moreover, endostatin-induced cell death is independent of oxidative stress and the caspase pahway, and regulated by serine and cysteine lysosomal enzymes.

Materials and methods

Cell culture

Human vascular endothelial cell line, EAhy926, was cultured in 5% CO_2 at 37 °C in Dulbecco's modified Eagle medium containing 10% fetal calf serum, HAT (100 μM sodium hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine), 2 mM glutamine, and 100 U/ml of penicillin and of streptomycin (Gibco BRL, Rockville, MD).

Cloning, expression and purification of recombinant endostatin

Total RNA, isolated from mouse liver using the Trizol reagent (Gibco BRL, Rockville, MD), was used for RT-PCR cloning of mouse endostatin cDNA. The sequences of the PCR primers used to clone endostatin (5'-GCGCATATGCACAGCCACCGCGACTTCCAGC-3'; 5'-GGGGGATCCCTACTTGGAGGCAGTCATGAAGCTG-3') were designed using the mouse collagen XVIII cDNA sequence (GenBank accession number: L22545). After DNA sequencing, the endostatin cDNA was subcloned into the *NdeI* and *Bam*HI sites of the GST-2TKcs vector (Amersham Pharmacia Biotech; UK) to yield the GST-2TKcs-endostatin plasmid. For expression and purification, the GST-2TKcs-endostatin plasmid was transformed into BL-21 cells (pLysS; Novagen Inc., Madison, WI), which were then grown at 37 until log phase (OD600nm approximately 0.5-0.9). IPTG (1 mM) was then added to induce protein expression and culture continued for another 3 hr at 30 °C. After centrifugation at 5,000 g for 10 min at 4 ºC, the cell pellet was harvested and resuspended in NETN buffer (20 mM Tris pH 8.0, 0.5% NP-40, 100 mM NaCl, 1 mM EDTA) containing 1 mM PMSF, 10 μg/ml of aprotinin, and 10 μg/ml of leupeptin, then homogenized by sonication or French press. After centrifugation at 14,000 g for 20 min at 4 , the supernatant was collected and incubated for 30 min at 4 °C with GST-Sepharose 4B resin (Amersham Pharmacia Biotech; UK) to bind the GST-fused recombinant protein. Following several washes with NETN buffer, the recombinant protein was eluted with NETN containing 20 mM reduced glutathione (Sigma, St. Louis, MO) and applied to a Sephadex G-25 gel filtration column (Amersham Pharmacia Biotech, UK) to remove salts. The recombinant protein was further passed through Detoxi-Ggel (Pierce; Rockford, Illinois, USA) to remove endotoxin and then analyzed by western blotting. Under this purification scheme, the recombinant endostatin was at least >90% in purity (data not shown).

Cell death assay

Approximately 5 x 10^3 EAhy926 cells were seeded in each well of 96-well tissue culture plates and cultured for 24 h, then incubated for 24 h with various concentrations (0.001-10 μg/ml) of GST or GST-endostatin before cell viability was determined using the MTT assay. In brief, 10 μl of a 5 mg/ml stock solution of MTT was added to each well and incubation continued for another 4 h at 37 °C. The insoluble formazan product was dissolved at 37 °C for 30 min in 100 μl of DMSO and the absorbance measured at 570 nm using a microplate reader (Tecan, Austria). For antioxidant and protease inhibitor studies, EAhy926 cells were pretreated for 3 h with various drugs [antioxidants: ascorbic acid (vitamin C, 25 μM), α-tocopherol (vitamin E, 10 mM), or propyl gallate (PG, 5 μM); caspase inhibitors: Ac-DEVD-CHO or Ac-YVAD-CHO (100 μM); endosomal trafficking inhibitor: brefeldin (BFA, 100 mM); autophagy inhibitor: 3-methyladenine (3-MA, 10 mM); lysosomal proteinase inhibitors: leupeptin (100 μM) and pepstatin (100 μM)], then coincubated with the drugs and endostatin for a further 3, 6, 12, or 24 hrs before cell viability was evaluated using the MTT assay. Data are presented as the mean ± SD for at least three sets of independent experiments, each experiment being carried out in duplicate. Differences between different groups were examined using one-way ANOVA with the Scheffe's test. A p value less than 0.05 was considered statistically significant.

Acridine Orange (AO) Staining

5×10^4 EAhy926 cells cultured on coverslides in 24-well plates were used. For AO staining, cells were exposed to various concentrations of endostatin for 3, 6, 12, or 24 h, then fixed with methanol : glacial acetic acid (3:1, v/v), stained for 5 min with 0.5 ml of AO solution (10 μg/ml in PBS), and examined using an Olympus BH-2 microscope with a fluorescence attachment.

Transmission electron microscopy

After two washes in PBS, untreated, GST-treated, or GST-endostatin-treated cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4, 4 °C) for 30 min. Following osmification and dehydration, the cells were embedded in Epoxy-Araldite resin and sections cut on a Reichert ultramicrotome (Ultracut E), mounted on grids, and double-stained with uranyl acetate and lead citrate. The sections were viewed and photographed on a Joel-2000 electron microscope.

Measurement of intracellular reactive oxygen

Species (ROS) Production. Free radical production was monitored by flow cytometry using 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Molecular probe, Eugene, OR). Briefly, the cells (5×10^5) were incubated for 60 min at 37 ºC with 50 μM DCFH-DA in the presence or absence of GST or GST-endostatin, then centrifuged, resuspended in ice-cold PBS, and subjected to FACScan flow cytometry (Becton Dickinson).

Flow cytometric analysis

Untreated, GST-treated, or GST-endostatin-treated cells were washed with ice-cold PBS, then fixed for 1 hour at -20 ºC in 70% ethanol. After PBS washes, they were incubated for 30 min at 37 ºC with 0.5 ml of 0.5 % Triton X-100 in PBS containing 1 mg/ml of RNase A, and stained for 10 min at RT with 0.5 ml of 50 μg/ml of propidium iodide (PI). The intensity of the fluorescence emitted by the PI-DNA complex was quantified after laser excitation of the fluorescent dye using FACScan flow cytometry (Becton Dickinson).

Immunocytochemical staining

5×10^4 cells plated on coverslips in 24-well plates were used to study the expression of lysosomal-associated membrane protein-1 (LAMP-1). Briefly, untreated, GST-treated, or GST-endostatin-treated cells were fixed for 10 min with cold 4% paraformaldehyde in PBS (pH 7.4), washed several times with PBS, then incubated for 2 h at 37 °C with anti-LAMP-1 antibody (diluted 1:200, Transduction Lab., San Diego, CA). After washing for 30 min with PBS, the cells were incubated for 2 h at RT with horseradish peroxidase (HRP)-conjugated secondary antibodies diluted 1:200, then bound antibody was visualized using diaminobenzidine (DAB) and 0.003% H_2O_2. For light microscopy, the stained cells were dehydrated using a graded alcohol series, then mounted on glass slides with Entellan (Merck, Germany).

Western Blot analysis

Whole cell extracts from untreated, GST-treated, or GST-endostatin-treated cells were prepared at various times. Briefly, cells in 6-well culture dishes were washed twice with ice-cold PBS, scraped off, and collected by centrifugation (4 ºC, x800 g for 10 min), then lysed for 30 min at 4 ºC with gentle agitation in lysis buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 10 mM EDTA, 10% NP-40, 0.1% SDS, 1 mM PMSF, 10 μg/ml of aprotonin, and 10 μg/ml of leupeptin]. After centrifugation (4 °C, 15,000 g for 10 min), the supernatants were collected and stored at –80 ºC as whole cell extracts. Protein concentrations were determined using the Bradford assay (Bio-Rad., Hercules, CA) and 100-200 μg samples of protein separated by 12% SDS-PAGE. The separated proteins were transferred for 2 h at 200 V to Immobilon-P membranes (Millipore, Bedford, MA) in a Trans-Blot Electrophoretic Transfer cell, then the membranes were blocked with 5% skimmed milk in PBS-0.2 % Tween 20 (PBST) for 1 h, incubated for 2 h at RT with caspase-3 and caspase-8 antibodies (diluted 1:500 in PBST, Transduction Lab. San Diego, CA) respectively, washed for 1 h at RT in PBS-0.2 % Tween 20, then incubated at RT with horseradish peroxidase-conjugated secondary antibody (diluted 1:2000 in PBST). Bound antibody was detected using the ECL Western blotting reagent (Amersham, Piscataway, NJ), chemiluminescence being detected using Fuji Medical X-ray film (Tokyo, Japan). Induction levels were quantified using either densitometry or gel image analyses on a Bio-Bad Gel Doc 1000 system.

Results

Endostatin induces cell death in EAhy926 cells

The effect of endostatin on the viability of EAhy926 cells was evaluated using the MTT assay. More than 90% of cells were killed after 24 h treatment with 10 μg/ml of endostatin (Fig. 1A). As shown in Fig. 1A,B, endostatin-mediated cell death was dose- and time-dependent. Surprisingly, treatment with 10 μg/ml endostatin for 1-2 h was sufficient to reduce cell viability (Fig. 1B). To determine whether endostatin induced apoptosis, cells treated for 24 h with 10 μg/ml of endostatin were examined using AO staining. Our results demonstrated that only 15-30% of endostatin-treated cells showed relatively intensive fluorescence with AO staining (Fig. 2). Consistenly, cell-cycle analysis confirmed that sub-G1 hypodiploid cells (apoptotic cells) only made up 15-30% of the cells after 24 h exposure to 10 μg/ml of endostatin (Fig. 3). Taken

together, these results indicated that endostatin induces both apoptosis and non-apoptotic cell death in the EAhy926 cell line.

Induction of lysosomal vacuoles in EAhy926 cells by endotastin

In contrast to untreated or GST-treated cells, some endostatin-treated cells showed a typical morphology of
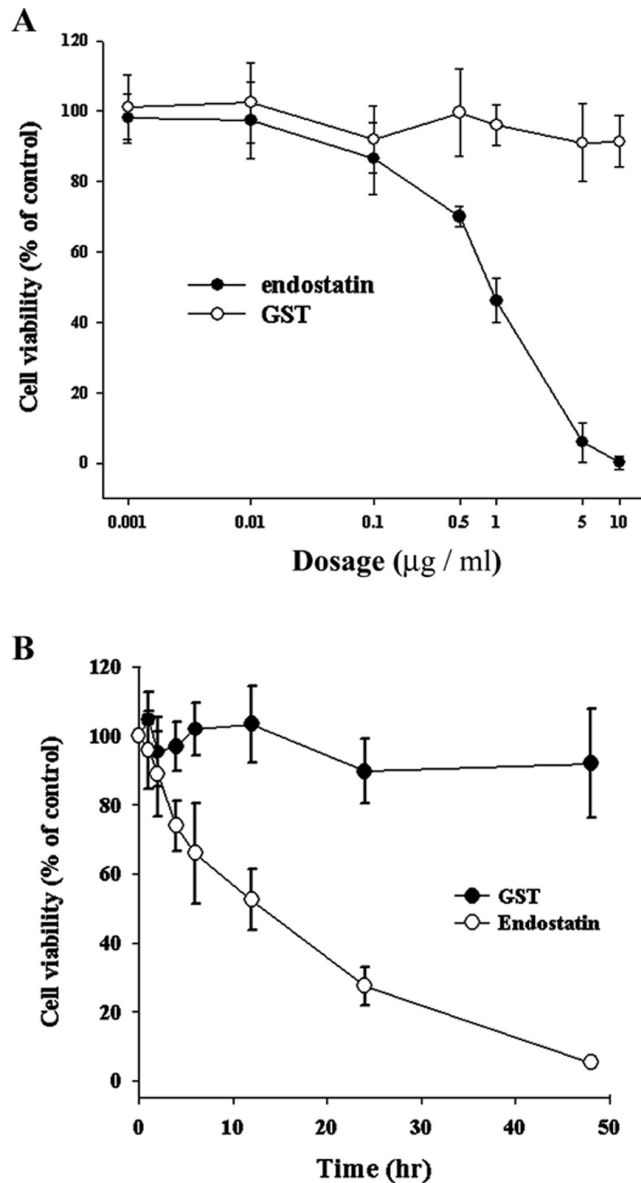

Fig. 1. Concentration- **(A)** and time- **(B)** dependent effects of endostatin on the viability of human endothelial EAhy926 cells. Cells (5 x 10³) were seeded in 96-well culture plates for 24 h. After 24 h with various concentration of GST or GST-endostatin **(A)** or after treatment with 10 μg/ml of GST or GST-endostatin for the indicated times **(B)**, cell survival was determined using MTT assay. Data are expressed as mean ± SD of three measurements.

apoptotic cells with a shrunken cytoplasm and nuclear chromatin condensation, but most had a relatively large cytoplasm with abundant vacuoles (Fig. 4). After 3 h treatment with 10 μg/ml endostatin, numerous vacuoles were seen, the size of which gradually increased in a time-dependent manner (Fig. 4C-F). Electron microscopic analysis indicated that these vacuoles were autophagosomes, originating from the rough endoplasmic reticulum (RER), with dilated cisternae (Fig. 5). These nascent autophagic vacuoles then enlarged by fusion with primary lysosomes to form autolysosomes. This was confirmed by immunostaining with anti LAMP-1 antibody, which specifically stained the membrane of the vacuoles (Fig. 6A). These results suggest that the endostatin-induced cell death of EAhy926 endothelial cells may occur via autophagy.

Measurement of oxidative stress induced by endostatin

Oxidative stress is frequently involved in the apoptotic mechanism. To determine whether it was involved in the mechanism of endostatin-mediated cell death of EAhy926 cells, we measured the production of intracellular ROS in endostatin-treated cells using DCFH-DA and flow cytometry. Endostatin treatment increased H_2O_2 production 2- to 3-fold within 2 h (Fig. 6B). Addition of antioxidants, such as vitamin C, vitamin E, or propyl gallate, failed to inhibit endostatin-induced cytoplasmic vacuolization or cell death (Fig. 7). Taken together, these results suggested that endostatin induced a small increase in oxidative stress in endothelial cells, but that oxidative stress may not play a causal factor in the cytotoxic mechanism of endostatin in EAhy926 cells.

Lack of caspase activation during endostatin-induced cell death

Caspases play a central role not only in apoptosis, but also in some forms of necrosis or autophagic degeneration. However, since activation of the caspase cascade is not always found in autophagic cell death, we examined whether it was involved in endostatin-mediated cell death. Western blot analysis demonstrated that there was no significant change in caspase-3 and caspase-8 in endostatin-treated cells (data not shown). In addition, three caspase inhibitors (Ac-DEVD-CHO, Ac-YVAD-CHO, and z-VAD-FMK) failed to suppress endostatin-induced cell death (Fig. 8A), further supporting the fact that caspase was not involved in the mechanism of endostatin-induced autophagic cell death in EAhy926 cells.

Endostatin-induced autophagic cell death is inhibited by 3-MA, a specific autophagic inhibitor, and by lysosomal serine and cysteine proteinase inhibitors

To determine whether endocytosis and lysosomal enzymes were involved in endostatin-induced cell death,
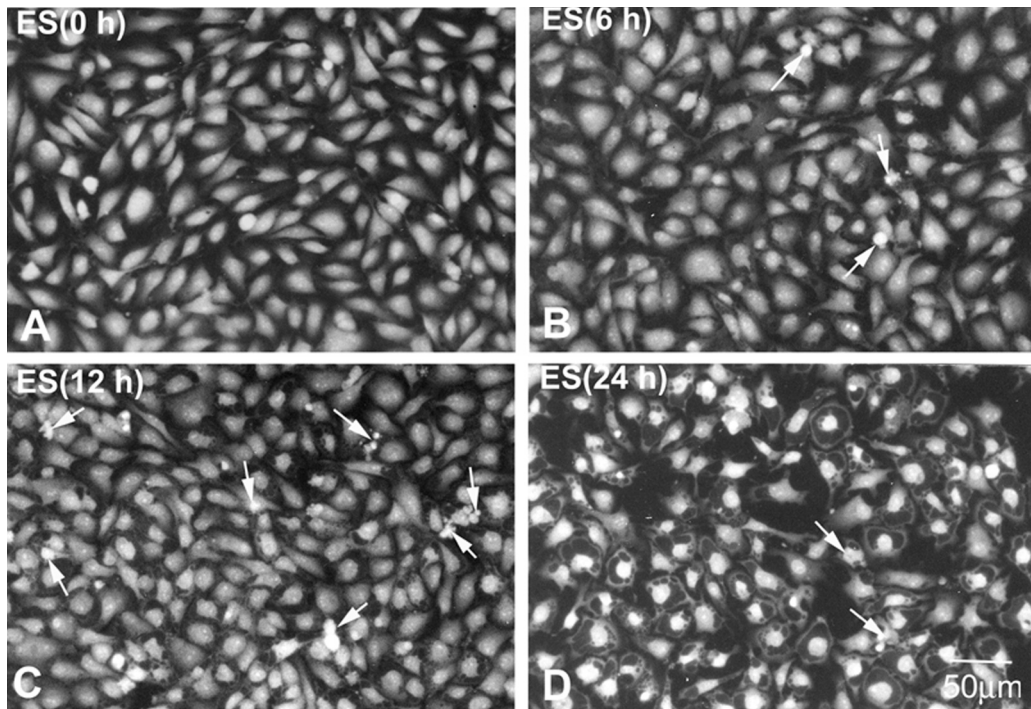
Fig. 2. Analysis of apoptotic cell death in EAhy926 cells after endostatin treatment. EAhy926 cells, grown on coverslides, were treated with 10 μg/ml of endostatin for 0 h **(A);** 6 h **(B)**; 12 h **(C)** or 24 h **(D)**, then fixed with methanol : glacial acid (3:1) for 10 min at 4 °C. Following several PBS washes and AO staining, the cells were examined using an Olympus fluorescence microscope.
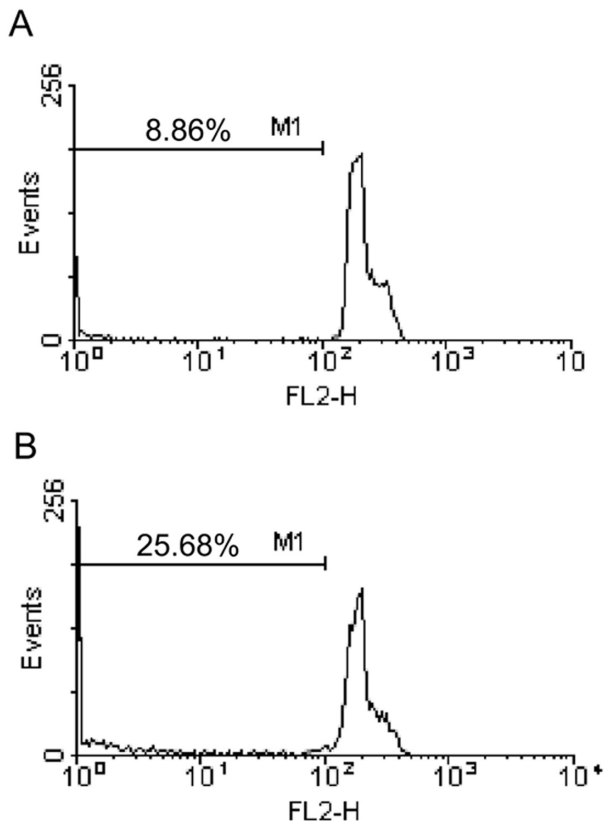

Fig. 3. Flow cytometric analysis of endostatin-treated EAhy926 cells. EAhy926 cells were treated for 24 h with vehicle **(A)** or 10 μg/ml of endostatin **(B)**, then fixed in cold 70 % ethanol, stained with propidium iodide (PI) and analyzed by flow cytometry as described in Materials and Methods.

cells were pretreated for 3 h with an inhibitor of endosomal trafficking (brefeldin A), an inhibitor of autophagy (3-MA), or three lysosomal proteinase inhibitors (leupeptin, pepstatin and aprotinin) prior to endostatin treatment. As shown in Fig. 8B, the autophagic inhibitor and the lysosomal serine and cysteine inhibitors (leupeptin and aprotinin), but not the endosomal trafficking inhibitor (brefeldin A) or the lysosomal aspartic acid proteinase inhibitor (pepstatin A), provided partial protection for endostatin-treated cells. These results suggest that the endostatin-induced cell death of EAhy926 cells occurs via the autophagic, rather than the endocytic, pathway, and is regulated by lysosomal serine and cysteine proteinases, but not by the lysosomal aspartic acid proteinase.

Discussion

Endostatin induced different types of cell death in human endothelial (EAhy926) cells

Although the human endothelial EAhy926 cell line is a hybrid resulting from fusion of human umbilical vein endothelial cells (HUVEC) with human lung adenocarcinoma, A549 cells, EAhy926 cells possess numerous characteristics of human endothelial cells (Edgell et al., 1983; van Oost et al., 1986) and are currently used in cardiovascular studies (Haynes et al., 2000; Muller et al., 2000; Stula et al., 2000). Since endostatin arrests cellular proliferation and induces apoptosis of rat or bovine endothelial cells (Dhanabal et al., 1999; Gasparini, 1999; Dixelius et al., 2000), we first

examined its effect on the viability of EAhy926 cells. Our results show that endostatin caused cell death of EAhy926 cells in a dose- and time-dependent manner (Fig. 1). Both fluorescent microscopic and flow cytometric results showed that it induced apoptotic cell death in 15-30% of cells and autophagic cell death in the remaining 70-85%. Apoptotic cells showed morphological alterations consisting of shrinkage of the cytoplasm and nuclear condensation and intensive fluorescence with AO staining (Figs. 2, 4). In contrast, autophagic cells contained numerous autophagic vacuoles identified by positive LAMP-1 immunostaining (Fig. 6A). Autophagy is generally accepted as a normal catabolic pathway for lysosomal degradation of the intracellular membrane, cytosolic organelles and secretory products, but can be increased under pathological conditions, such as starvation, oxidative stress or mitochondrial dysfunction (Aubert et al., 1996;
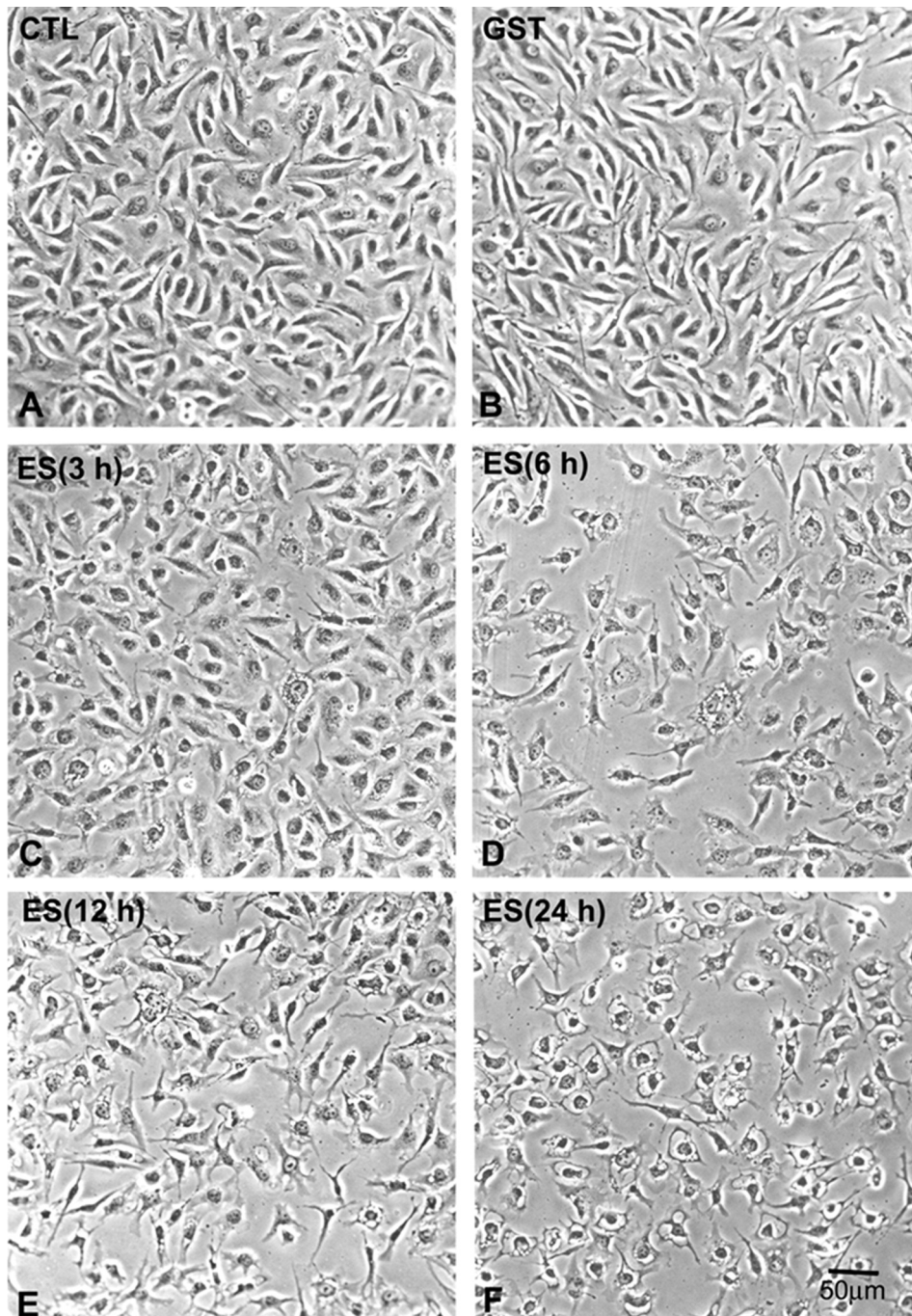

Fig. 4. Phase-contrast microscopy of EAhy926 cells following endostatin treatment. Cells (5×10^4), grown on coverslides in 24-well culture plates for 24 h, were treated with PBS **(A)** or 10 μg/ml of GST (B) for 24 h, or with 10 μg/ml of GST-endostatin for 3 h **(C)**; 6 h **(D)**; 12 **(E)**, or 24 h **(F)** and observed on a Nikon phase-contrast microscope. Note that numerous vacuoles are seen within cells after treatment with 10 μg/ml endostatin for 3 h **(C)**, the sizes of vacuoles gradually increasing with time **(C-F)**.

Nilsson et al., 1997; Isahara et al., 1999). In addition, autophagy has recently been linked to neurodegenerative disease, cardiomyopathy (Danon's disease) and tumor development (Petersen et al., 2001; Larsen and Sulzer, 2002; Yue et al., 2002). Recent evidence showed that the expression of beclin 1 (a mammalian autophagy gene) protein that can inhibit tumorigenesis is frequently low in human breast epithelial carcinoma cell lines and tissue, suggesting that the decreased expression of autophagic proteins may contribute to the development of breast and other human malignancies (Liang et al.,

1999). Apoptosis and autophagic degeneration are recognized as two different types of physiological cell deaths responsible for the elimination of surplus cells during embryogenesis and maintenance of tissue cell number (Bursch et al., 2000). Apoptotic cell death is characterized by nuclear condensation, DNA fragmentation, shrinkage of the cytoplasm, and activation of the caspase cascade, whereas autophagic degeneration is always accompanied by cytoplasmic vacuolization and activation of lysosomal enzymes (Afford et al., 2000; Bursch et al., 2000; Strasser et al.,
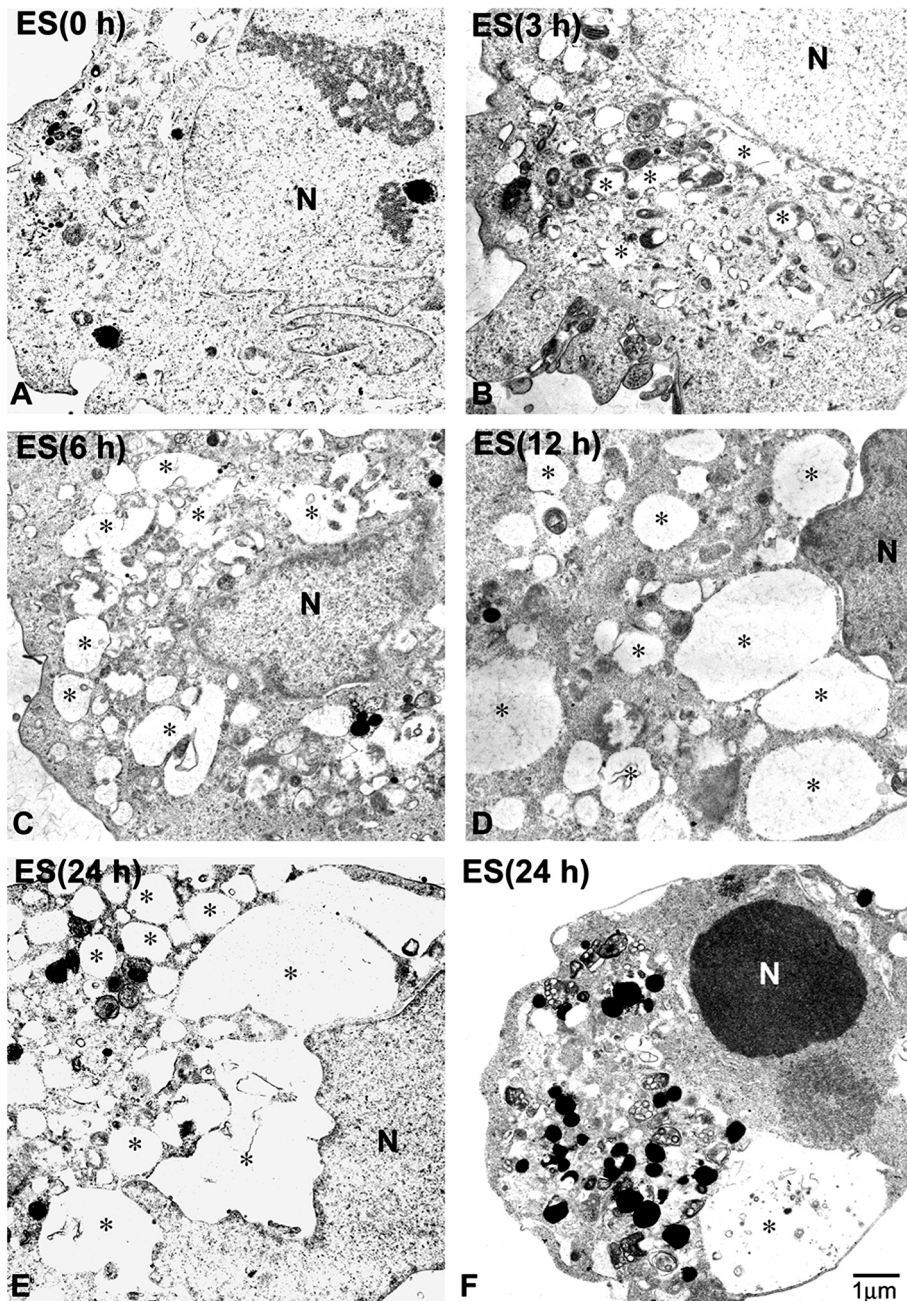

Fig. 5. Ultrastructural analysis of endostatin-treated EAhy926 cells. EAhy926 cells ($5x 10^4$) were cultured on cultured plates for 24 h, then treated with 10 μg/ml of GST-endostatin for 0 h (**A**), 3 h (**B**), 6 h (**C**), 12 h (**D**), and 24 h (**E and F**) before being fixed with 4 % paraformaldehdye in 0.1 M phosphate buffer, postfixed with 1% osmic tetroxide, dehydrated with series alcohol and embedded in Epon-Araldite resin. Thin sections, cut using an ultramicrotome, were mounted on grids and double-stained with uranyl acetate and lead citrate as described in Materials and Methods. Electron microscopy shows endostatin-induced autophagy in both non-apoptotic (**B-E**) and apoptotic cells (**F**). Numerous autophagic vacuoles (*), originating from the rough endoplasmic reticulum (RER) with dilated cisternae (**B-F**) are seen in the endostatin-treated cells. Some autophagic vacuoles with digested cytoplasmic content are observed. The sizes of the autophagic vacuoles increases with time (**B-E**). Note that endostatin-induced autophagy is also observed in an apoptotic EAhy926 cell (**F**) characterized by condensed chromatin.

2000). Although the mechanism of autophagy is still unclear, it is seen in both apoptotic and non-apoptotic cells after serum deprivation, mitochondrial damage, or TNFα treatment (Reipert et al., 1995; Jia et al., 1997; Lemasters et al., 1998; Isahara et al., 1999). In addition, there is an indication that early stages of autophagy are required in the process of TNFα-induced apoptosis (Jia et al., 1997). In this study, we observed numerous autophagic bodies in both apoptotic and non-apoptotic cells, suggesting that endostatin-induced cell death in EAhy926 cells may occur via the autophagic pathway and be regulated by lysosomal enzymes.

Endostatin induces cell death of EAhy926 cells via a ROS- and caspase-independent pathway

Oxidative stress-induced caspase activation is a common event in both apoptotic and non-apoptotic cell death. An increase in oxidative stress caused by various stimuli or anticancer drugs induces DNA damage, decreases the mitochondrial membrane potential, activates the caspase cascades and subsequently initiates cell death (Nilsson et al., 1997; Bunk and Svensson, 1999). However, it is not known whether oxidative stress increases during endostatin-induced cell death. In the present study, we showed that ROS production only increased by a small amount (2-fold) in endostatin-treated cells, whereas, under the same experimental conditions, an up to 5-fold increase in the intensity of DCF-DA fluorescence was seen in the H_2O_2-treated positive control (Fig. 6B). In addition, pretreatment of antioxidants and caspase inhibitors had no significant protective effect on endostatin-induced cell death, suggesting that it occurred via a ROS- and caspase-independent pathway (Figs. 7, 8A).

Involvement of lysosomal enzymes in endostatin-induced cell death

Morphological and immunocytochemical observations showed that increased autophagic activity was, in some way, associated with the process of endostatin-induced apoptotic and autophagic cell death (Figs. 4-6A). Autophagy, which usually occurs in normal cells to maintain cellular homeostasis, consists of the sequestration of intracellular components and degradation by lysosomal enzymes. It is also induced by various starvation conditions and is necessary for the cells to degrade and for reused cytosolic macromolecules for survival. The autophagic machinery is highly conserved in organisms as diverse as plants, animals and yeast. The process includes at least four discrete steps: the induction and the formation of the autophagosome, fusion of the autophagosome with the
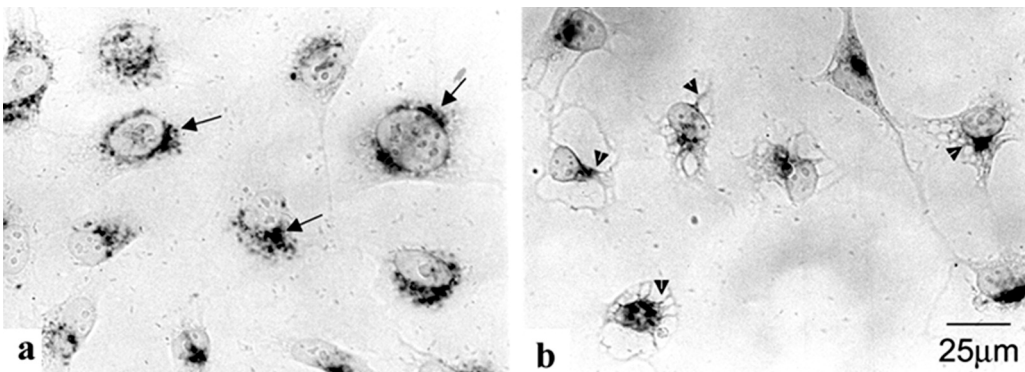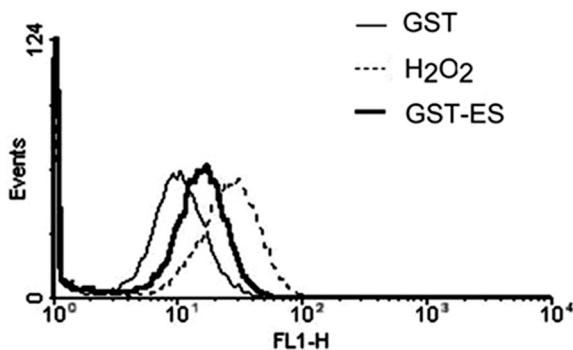

6B

Fig. 6. Immunostaining of LAMP-1 (lysosome associated membrane protein-1) and Measurement of ROS generation in EAhy926 cells following endostatin treatment. **A.** EAhy926 cells, grown on coverslides in 24-well plates, were treated with 10 μg/ml of GST-endostatin for 0 h **(a)** or 12 h **(b)**. After several PBS washes, the cells were fixed, then incubated with anti-LAMP-1 antibody followed by horseradish peroxidase (HRP)-conjugated secondary antibodies. Bound anti-LAMP-1 was visualized using diaminobenzidine (DAB) and 0.003% H_2O_2 as substrate. Lysosomes (arrows) in cells are immunostained with anti-LAMP-1 antibody. The arrowheads indicate that LAMP-1 immunostaining is specifically localized on the membrane of autophagic vacuoles in endostatin-treated cells. (B) Intracellular hydrogen peroxide generation in GST-treated, H_2O_2-treated or GST-endostatin-treated cells was detected by flow cytometry using a peroxide-sensitive dye, DCFH-DA. Note that only a small increase in H_2O_2 is seen in EAhy926 cells after treatment with endostatin compared to in H_2O_2-treated positive control cells.

lysosome or vacuole, and autophagic vacuole breakdown (Klionsky and Emr, 2000). Autophagic vacuoles are known to form by the fusion of endocytotic vesicles (endosomes) and lysosomes, indicating the convergence of the autophagic and endocytic pathways (Liou et al., 1997). Active endostatin uptake is detected in murine brain endothelial cells, indicating that endostatin internalization occurs and is possibly mediated by a cell surface receptor. However, it is still unclear whether the endocytic/autophagic pathways are involved in the mechanism of endostatin-mediated cell death. The effects of brefeldin A, an endocytic trafficking inhibitor, and 3-MA, an anti-autophagic drug, were tested, and 3-MA, but not brefeldin A, was found to reduce endostatin-induced cell death (Fig. 8B), supporting our idea that the internalized endostatin did not provided any cytotoxic effect on endothelial cells because internalized endostatin in endosomes is rapidly degraded (Dixelius et al., 2000). Autophagy, a major pathway for the degradation of cellular proteins, is regulated by lysosomal proteinases (Klionsky and Emr, 2000). Lysosomal serine proteases and cysteine protease have
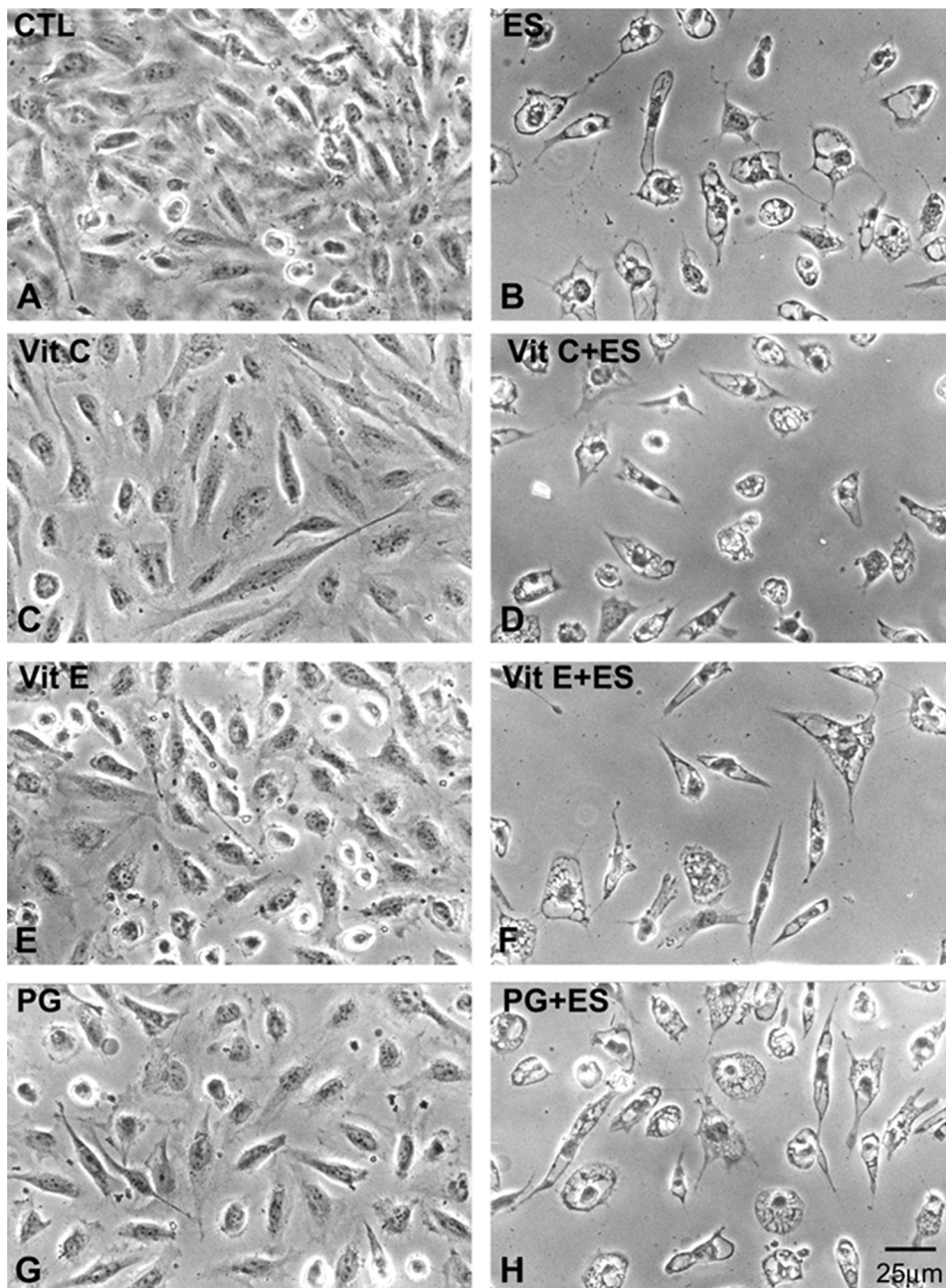

Fig. 7. Effects of various antioxidants on endostatin-induced cell death in EAhy926 cells. Cells (5 x 10⁴) were seeded in 96-well culture plates for 24 h. Following a 3-h pretreatment with various antioxidants (vitamin C, vitamin E or propyl gallate), EAhy926 cells were coexposed to antioxidant and 10 μg/ml of GST-endostatin for 24 h and the cell morphology monitored by phase contrast microscopy. Note that pretreatment with antioxidants fails to inhibit the morphological changes such as cytoplasmic vacuolization, induced by endostatin.

been implicated in the processing and degradation of native collagens in vitro (Starkey, 1977; Maciewicz and Etherington, 1988). The neutrophil serine elastase and the cysteine protease cathepsin K can attack the triple helix of collagen (Kafienah et al., 1998a,b) . Cathepsin L

and the other collagenolytic serine and cysteine proteases cleave within non-helical regions located at either end of native collagens, leading to destabilization of the fibrils (Starkey, 1977; Maciewicz and Etherington, 1988; Kafienah et al., 1998a,b; Zatterstrom et al., 2000). Although aspartic acid lysosomal proteases are not involved in the degradation of native collagen, the aspartic acid protease, cathepsin D, has been demonstrated to be a death factor regulating neuronal cell death under conditions of serum deprivation (Isahara et al., 1999). We therefore examined the roles of lysosomal enzymes in the autophagic process, using three lysosomal proteinase inhibitors; aprotinin (a serine proteinase inhibitor), leupeptin (a serine and cysteine proteinase inhibitor), or pepstatin A (an aspartic acid proteinase inhibitor), respectively, and found that only aprotinin and leupeptin provided partial protection and delayed endostatin-induced cell death. Taken together, these results suggest that the effect of endostatin on EAhy926 cells is mediated via a signaling pathway which upregulates autophagic capacity and promotes the activities of serine and cysteine lysosomal proteinases. In this study, we observed that it is the RER, rather than ribosome-free ER, which is responsible for autophagic sequestration, indicating that protein synthesis in the ER may be perturbed by endostatin. Autophagy is a novel pathway of active cell death, which is regulated by lysosomal proteinases (Kim et al., 2001; Klionsky and Emr, 2000). Inhibition of lysosomal enzymes by protease inhibitors showed that the serine and cysteine lysosomal enzymes, but not aspartic acid lysosomal enzymes, participate in the endostatin-induced autophagic process. Moreover, it is likely that multiple signaling pathways are involved in the anti-angiogenic effect of endostatin. Many studies have shown that endostatin interacts with several cellular molecules such as integrin $\alpha5\beta1$, caveolin-1, MMP-2, and KDR/Flk-1 (Wickstrom et al., 2002; Kim et al., 2002, Lee et al., 2002; Shichiri and Hirata, 2001). Accordingly, it is tempting to speculate that the cellular response to endostatin such as apoptosis and autophagy observed in the present study may be mediated by different signaling pathways.

In summary, we conclude that in EAhy926 human endothelial cells: (1) endostatin causes predominantly autophagic, rather than apoptotic cell death; (2) endostatin-induced autophagic cell death occurs in the absence of caspase activation and through an oxidative-independent pathway; and (3) endostatin inducing 'autophagic cell death' or 'type 2 physiological cell death' is regulated by serine and cysteine lysosomal proteases.
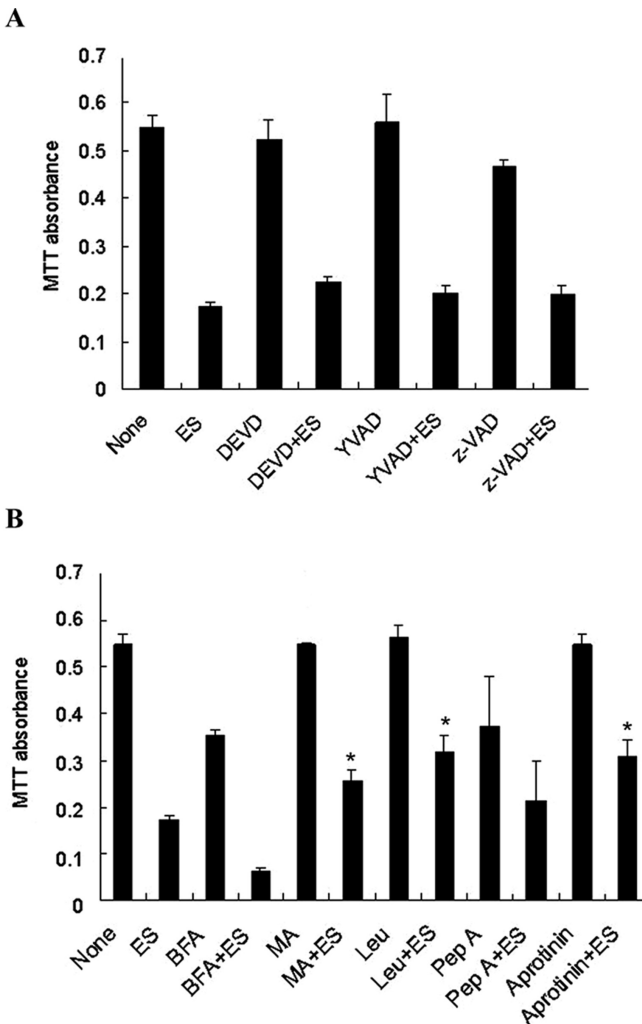
Fig. 8. Effects of caspase inhibitors and protease inhibitors in endostatin-mediated autophagy in EAhy926 cells. **A.** Following a 3-h pretreatment with various caspase inhibitors (z-VAD-FMK, Ac-DEVD-CHO and Ac-YVAD-CHO), EAhy926 cells were coexposed to inhibitor and 10 μg/ml of GST-endostatin for 24 h. The effects of various caspase inhibitors on the survival of endostatin-treated cells were measured by the MTT assay. Data are presented as the mean ± SD (n=6). No significant difference (p<0.05%) is found between the endostatin-treated group and caspase inhibitor / endostatin-treated groups. **B.** The inhibitory effects of 3-MA and serine and cysteine lysosomal inhibitors on endostatin-induced autophagic cell death were examined in EAhy926 cells. Cells were pretreated for 3 h with brefeldin A, 3-MA, leupeptin, pepstatin or aprotinin prior to coexposure to inhibitor and GST-endostatin for 24 h. The survival of endostatin-treated cells in the presence and absence of various lysosomal inhibitors was measured using the MTT assay. Data are presented as the mean ± SD (n=6). A significant inhibitory effect of 3-MA or leupeptin or aprotinin is seen compared with endostatin alone (*p: <0.05%).

References

Afford S. and Randhawa S. (2000). Apoptosis. Mol. Pathol. 53, 55-63.

Aubert S., Gout E., Bligny R., Marty-Mazars D., Barrieu F., Alabouvette J., Marty F. and Douce R. (1996). Ultrastructural and biochemical characterization of autophagy in higher plant cells subjected to carbon deprivation: control by the supply of mitochondria with respiratory substrates. J. Cell Biol. 133, 1251-1263.

Augustin H.G. (1998). Antiangiogenic tumour therapy: will it work?. Trends Pharmacol. Sci. 19, 216-222.

Bergers G., Javaherian K., Lo K.M., Folkman J. and Hanahan D. (1999). Effects of angiogenesis inhibitors on multistage carcinogenesis in mice. Science 284, 808-812.

Brunk U.T. and Svensson I. (1999). Oxidative stress, growth factor starvation and Fas activation may all cause apoptosis through lysosomal leak. Redox Report 4, 3-11.

Bruns C.J., Liu W., Davis D.W., Shaheen R.M., McConkey D.J., Wilson M.R., Bucana C.D., Hicklin D.J. and Ellis L.M. (2000). Vascular endothelial growth factor is an in vivo survival factor for tumor endothelium in a murine model of colorectal carcinoma liver metastases. Cancer 89, 488-499.

Bursch W., Ellinger A., Gerner C., Frohwein U. and Schulte-Hermann R. (2000). Programmed cell death (PCD). Apoptosis, autophagic PCD, or others? Ann. NY Acad. Sci. 926, 1-12.

Chen Q.R., Kumar D., Stass S.A. and Mixson A.J. (1999). Liposomes complexed to plasmids encoding angiostatin and endostatin inhibit breast cancer in nude mice. Cancer Res. 59, 3308-3312.

Clement B., Musso O., Lietard J. and Theret N. (1999). Homeostatic control of angiogenesis: A newly identified function of the liver? Hepatology 29, 621-623.

Dhanabal M., Ramchandran R., Waterman M.J., Lu H., Knebelmann B., Segal M. and Sukhatme V.P. (1999). Endostatin induces endothelial cell apoptosis. J. Biol. Chem. 274, 11721-11726.

Dixelius J., Larsson H., Sasaki T., Holmqvist K., Lu L., Engstrom A., Timpl-Welsh M. and Claesson-Welsh L. (2000). Endostatin-induced tyrosine kinase signaling through the Shb adaptor protein regulates endothelial cell apoptosis. Blood 95, 3403-3411.

Edgell C.J., McDonald C.C. and Graham J.B. (1983). Permanent cell line expressing human factor VIII-related antigen established by hybridization. Proc. Natl. Acad. Sci. USA 80, 3734-3737.

Ferrara N. and Alitalo K. (1999). Clinical applications of angiogenic growth factors and their inhibitors. Nat. Med. 5, 1359-1364.

Folkman J. (1995). Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat. Med. 1, 27-31.

Folkman J. (1996). Tumor angiogenesis and tissue factor. Nat. Med. 2, 167-168.

Gasparini G. (1999). The rationale and future potential of angiogenesis inhibitors in neoplasia. Drugs 58, 17-38.

Hanahan D. and Folkman J. (1996). Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 86, 353-364.

Harris A.L. (1998). Are angiostatin and endostatin cures for cancer?. Lancet 351, 1598-1599.

Haynes M.P., Sinha D., Russell K.S., Collinge M., Fulton D., Morales-Ruiz M., Sessa W.C. and Bender J.R. (2000). Membrane estrogen receptor engagement activates endothelial nitric oxide synthase via the PI3-kinase-Akt pathway in human endothelial cells. Circ. Res. 87, 677-682.

Isahara K., Ohsawa Y., Kanamori S., Shibata M., Waguri S., Sato N.,

Gotow T., Watanabe T., Momoi T., Urase K., Kominami E. and Uchiyama Y. (1999). Regulation of a novel pathway for cell death by lysosomal aspartic and cysteine proteinases. Neuroscience 91, 233-249.

Jia L., Dourmashkin R.R., Allen P.D., Gray A.B., Newland A.C. and Kelsey S.M. (1997). Inhibition of autophagy abrogates tumour necrosis factor alpha induced apoptosis in human T-lymphoblastic leukaemic cells. B. J. Haematol. 98, 673-685.

Kafienah W., Bromme D., Buttle D.J., Croucher L.J. and Hollander A.P. (1998a). Human cathepsin K cleaves native type I and II collagens at the N-terminal end of the triple helix. Biochem. J. 331, 727-732.

Kafienah W., Buttle D.J., Burnett D. and Hollander A.P. (1998b). Cleavage of native type I collagen by human neutrophil elastase. Biochem. J. 330, 897-902.

Kim J., Huang W.P. and Klionsky D.J. (2001). Membrane recruitment of Aut7p in the autophagy and cytoplasm to vacuole targeting pathways requires Aut1p, Aut2p, and the autophagy conjugation complex. J. Cell Biol. 152, 51-64.

Kim Y.M., Hwang S., Kim Y.M., Pyun B.J., Kim T.Y., Lee S.T., Gho Y.S. and Kwon Y.G. (2002). Endostatin blocks vascular endothelial growth factor-mediated signaling via direct interaction with KDR/Flk-1. J. Biol. Chem. 277, 27872-27879.

Klionsky D.J. and Emr S.D. (2000). Autophagy as a regulated pathway of cellular degradation. Science 290, 1717-1721.

Larsen K.E. and Sulzer D. (2002) Autophagy in neurons: a review. Histol. Histopathol. 17, 897-908.

Lee S.J., Jang J.W., Kim Y.M., Lee H.I., Jeon J.Y., Kwon Y.G. and Lee S.T. (2002). Endostatin binds to the catalytic domain of matrix metalloproteinase-2. FEBS Lett. 519, 147-152.

Lemasters J.J., Nieminen A.L., Qian T., Trost L.C., Elmore S.P., Nishimura Y., Crowe R.A., Cascio W.E., Bradham C.A., Brenner D.A. and Herman B. (1998). The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy. Biochim. Biophys. Acta 1366, 177-196.

Liang X.H., Jackon S., Seaman M., Brown K., Kempkes B., Hibshoosh H. and Levine B. (1999). Introduction of autophagy and inhibition of tumorigenesis by beclin 1. Nature 402, 672-676.

Liou W., Geuze H.J., Geelen M.J. and Slot J.W. (1997). The autophagic and endocytic pathways converge at the nascent autophagic vacuoles. J. Cell Biol. 136, 61-70.

Maciewicz R.A. and Etherington D.J. (1988). A comparison of four cathepsins (B, L, N and S) with collagenolytic activity from rabbit spleen. Biochem. J. 256, 433-440.

Moulton K.S., Heller E., Konerding M.A., Flynn E., Palinski W. and Folkman J. (1999). Angiogenesis inhibitors endostatin or TNP-470 reduce intimal neovascularization and plaque growth in apolipoprotein E-deficient mice. Circulation 99, 1726-1732.

Muller L., Valdenaire O., Barret A., Korth P., Pinet F., Corvol P., and Tougard C. (2000). Expression of the endothelin-converting enzyme-1 isoforms in endothelial cells. J. Cardiovasc. Pharmacol. 36, S15-S18.

Nilsson E., Ghassemifar R. and Brunk U.T. (1997). Lysosomal heterogeneity between and within cells with respect to resistance against oxidative stress. Histochem. J. 29, 857-865.

O'reilly M.S., Boehm T., Shing Y., Fukai N., Vasios G., Lane W.S., Flynn E., Birkhead J.R., Olsen B.R. and Folkman J. (1997). Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell 88, 277-285.

Oehler M.K. and Bicknell R. (2000). The promise of anti-angiogenic

cancer therapy. Brit. J. Cancer 82, 749-752.

Olejnicka B.T., Dalen H. and Brunk U.T. (1999). Minute oxidative stress is sufficient to induce apoptotic death of NIT-1 insulinoma cells. APMIS 107, 747-761.

Perletti G., Concari P., Giardini R., Marras E., Piccinini F., Folkman J. and Chen L. (2000). Antitumor activity of endostatin against carcinogen-induced rat primary mammary tumors. Cancer Res. 60, 1793-1796.

Petersen A., Larsen K.E., Behr G.G., Romero N., Przedborski S., Brundin P. and Sulzer D. (2001). Expanded CAG repeats in exon 1 of the Huntington's disease gene stimulate dopamine-mediated striatal neuron autophagy and degeneration. Hum. Mol. Genet. 10, 1243-1254.

Reipert S., Berry J., Hughes M.F., Hickman J.A. and Allen T.D. (1995). Changes of mitochondrial mass in the hemopoietic stem cell line FDCP-mix after treatment with etoposide: a correlative study by multiparameter flow cytometry and confocal and electron microscopy. Exp. Cell Res. 221, 281-288.

Risau W. (1997). Mechanisms of angiogenesis. Nature 386, 671-674.

Saarela J., Ylikarppa R., Rehn M., Purmonen S. and Pihlajaniemi T. (1998). Complete primary structure of two variant forms of human type XVIII collagen and tissue-specific differences in the expression of the corresponding transcripts. Matrix Biol. 16, 319-28.

Shichiri M. and Hirata Y. (2001). Antiangiogenesis signals by endostatin. FASEB J. 6, 1044-1053.

Starkey P.M. (1977). The effect of human neutrophil elastase and cathepsin G on the collagen of cartilage, tendon, and cornea. Acta Biol. Med. German 36, 1549-1554.

Strasser A., O'Connor L. and Dixit V.M. (2000). Apoptosis signaling. Annu. Rev. Biochem. 69, 217-245.

Stula M., Orzechowski H.D., Gschwend S., Vetter R., von Harsdorf R., Dietz R. and Paul M. (2000). Influence of sustained mechanical stress on Egr-1 mRNA expression in cultured human endothelial cells. Mol. Cell. Biochem. 210, 101-108.

van Oost B.A., Edgell C.J., Hay C.W. and MacGillivray R.T. (1986). Isolation of a human von Willebrand factor cDNA from the hybrid endothelial cell line EAhy926. Biochem. Cell Biol. 64, 699-705.

Wickstrom S.A., Alitalo K. and Keski-Oja J. (2002). Endostatin associates with integrin alpha5beta1 and caveolin-1, and activates Src via a tyrosyl phosphatase-dependent pathway in human endothelial cells. Cancer Res. 1, 5580-5589.

Yokoyama Y., Green J.E., Sukhatme V.P. and Ramakrishnan S. (2000). Effect of endostatin on spontaneous tumorigenesis of mammary adenocarcinoma in a transgenic mouse model. Cancer Res. 60, 4362-4365.

Yue Z., Horton A., Bravin M., Dejager P.L., Selimi F. and Heintz N. (2002). A novel protein complex linking the delta 2 glutamate receptor and autophagy: implications for neurodegeneration in lurcher mice. Neuron 35, 921-933.

Zatterstrom U.K., Felbor U., Fukai N. and Olsen B.R. (2000). Collagen XVIII/endostatin structure and functional role in angiogenesis. Cell Struct. Funct. 25, 97-101.