Endostar Suppresses Invasion Through Downregulating the Expression of Matrix Metalloproteinase-2/9 in MDA-MB-435 Human Breast Cancer Cells

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Endostar, a novel recombinant human endostatin expressed and purified in *Escherichia coli* with an additional nine-amino acid sequence forming another his-tag structure, was approved by the State Food and Drug Administration of China (SFDA) in 2005 for the treatment of non-small-cell lung cancer. However, the molecular mechanism of its potent anticancer activity remains poorly understood and warrants further investigations. In this study, we examined the anti-invasive activities of endostar *in vitro*. The results showed that endostar suppressed MDA-MB-435 cell adhesion to the fibronectin-coated substrate in a concentration-dependent manner. It could inhibit the wound healing migration of MDA-MB-435 cells and invasion of MDA-MB-435 cells through reconstituted ECM (matrigel). Zymography revealed that endostar decreased the secretion of MMP-2 and MMP-9. Endostar could also inhibit the expressions of MMP-2 and MMP-9 in MDA-MB-435 cells. Additionally, endostar exerted an inhibitory effect on the phosphorylation of ERK1/2. Collectively, these data provided a molecular basis for the anti-invasive effects of endostar. Exp Biol Med 233:1013–1020, 2008

Key words: endostar; invasion; MMP-2; MMP-9

Introduction

Tumor invasion and metastasis formation are major obstacles for successful cancer therapy. Metastasis is a complex multistep process that requires sequential interactions between the invasive cell and the extracellular matrix. It is a stepwise process that includes adhesion, invasion, and migration of cancer cell (1). Metastasis necessarily involves penetration of the extracellular matrix (ECM) and basement membrane and is considered to require the action of proteases. There are three major groups of protease known to be involved in tumor invasions: the matrix metalloproteinases (MMPs), the plasminogen activators (PAs), and the cathepsins (CPs). The matrix metalloproteases are a family of zinc metalloenzymes with well-characterized structural and catalytic properties (2–4). These enzymes are all secreted as proenzymes that undergo proteolytic cleavage of an aminoterminal domain during activation and are further defined by sequence similarities, inhibition by metal chelators, and inhibition by tissue inhibitors of metalloproteases (TIMPs) (5). The elevated activities of MMP-2 and MMP-9 have been associated with increasing tumor metastases in various human cancers, suggesting an important functional role for these proteases in the metastatic process (6). In particular, MMP-2 and MMP-9 degrade components of the basement membrane and are strongly implicated in the invasion and metastasis of malignant tumors (7, 8). Therefore, inhibition of MMP activity is important for arresting metastasis, mostly affecting cancer development after carcinogenesis.

Endostatin (9, 10), the 20 kDa internal fragment of the carboxyterminus of collagen XVIII (11, 12), was first identified in the conditioned media of hemangioendothelioma cells as an antiangiogenic molecule in 1997 by Folkman et al. in their laboratory at the Children's Hospital in Boston.

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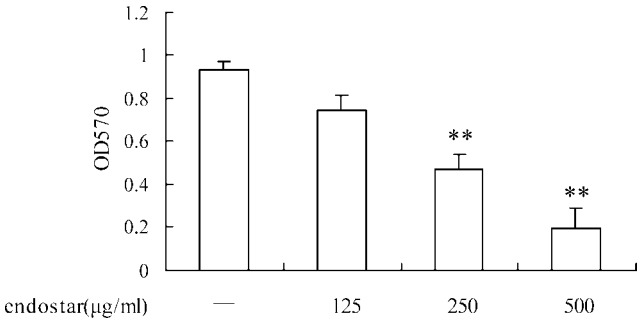

Figure 1. Effect of endostar on MDA-MB-435 cell adhesion to fibronectin. One hundred microliters of cell suspension (5×10^5 cells/ml) was added to the wells coated with fibronectin and the plates were incubated at 37°C for 40 min. Medium was then carefully suctioned out of each well. Each well was washed three times with PBS. Then the MTT assay was used to determine the number of adherent cells. $^*P < 0.05$ compared with control; $^{**}P < 0.01$ compared with control.

It was shown that endostatin strongly inhibited the growth of tumors by suppressing the neovascularization *in vivo* and *in vitro*. Endostatin, thought to be an ideal anticancer weapon, was quickly pushed into clinical trials. However, there were some problems with the production and reproduction of recombinant human endostatin, thus the use of endostatin was restricted. Since then, many efforts have been focused on the production of it.

Endostar, a novel recombinant human endostatin that expressed and purified in *E. coli*, was approved by the State Food and Drug Administration of China (SFDA) for the treatment of non-small-cell lung cancer in 2005. Compared with rh-endostatin reported in previous literature, an additional nine-amino acid sequence (MGGSHHHHH) was added to the N-terminal of the protein, which resulted in the formation of a six-histidine tag. The six-histidine tag could be chelated with metal ions such as Ni^{2+} with a relatively high affinity. These changes simplified the purification and improved the stability of protein. The antiangiogenic efficacy of endostar had been identified.

In the present study, we investigated the influence of endostar on the adhesion, invasion, and migration of MDA-MB-435 cells. We also used gelatin zymography to elucidate the potential of endostar in inhibiting the activities of MMP-2 and MMP-9. We examined the effect of endostar on the related MMPs signal transduction in cancer cell invasion.

Materials and Methods

Materials. Endostar, expressed and purified in *E. coli*, was provided by Simcere Pharmaceutical Research Co., Ltd. (Nanjing, China). Primary antibodies for p38, p-p38, Akt, p-ERK1/2, p-Akt, MMP-2, MMP-9, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibody for ERK1/2 was from Chemicon (Boston, MA). IRDyeTM800 conjugated secondary antibodies were obtained from Rockland Inc. (Philadelphia, PA). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was from Sigma (St. Louis, MO).

Cell Culture. Human breast cancer cell lines MDA-MB-435 were purchased from the Cell Bank of Shanghai Institute of Cell Biology (Shanghai, China). Cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY) containing 10% fetal calf serum (Sijiqing, Hangzhou, China), 100 U/ml penicillin, and 100 mg/L streptomycin, and cells were grown in humidified atmosphere of 5% CO_2 at 37°C.

Wound Healing Assay. Cells were seeded into a six-well tissue culture dish and allowed to grow to 90% confluency in complete medium. Cell monolayers were wounded by a plastic tip (1 mm) that touched the plate as described (13). Wounded monolayers were then washed four times with medium to remove cell debris and incubated in medium in the absence or presence of endostar for various periods of time up to 24 h. Cell migration into the wound surface was monitored by microscopy at various times. Quantification was done by measuring the distance of the wound edge of the migrating cells from the start point to the migrated point.

***In Vitro* Invasion Assay.** The *in vitro* invasion assays were carried out using Transwell chamber with 6.5-mm diameter polycarbonate filters (8-μm pore size, Corning Costar, Cambridge, MA) coated with matrigel (Becton Dickinson, Bedford, MA) as described previously (14). Cells were trypsinized and suspended at a final concentration of 5×10^5 cells/ml in serum-free RPMI-1640 medium. Various concentrations of endostar (125, 250, and 500 μg/ml) were given to the cells for 24 h before seeding. Cell suspension was loaded into each of the upper wells. Fetal calf serum (5%) was used as a chemoattractant in the lower chambers. After incubation for 24 h at 37°C under 5%CO_2/95% air atmosphere, all of the noninvaded cells were removed the upper face of the transwell membrane with a cotton swab; the invaded cells were fixed with 100% methanol and then stained with hematoxylin and eosin (Nanjing Sunshine Biotechnology Ltd., Nanjing, China). The invaded cells were counted microscopically. Ten fields were counted for each assay.

Cell Attachment Assay. Cell attachment was assayed as described previously with slight modifications (15). Microtiter wells were coated with fibronectin (Sigma, St. Louis, MO) overnight. The wells were blocked for 30 min with 0.5% BSA in PBS. Cells were trypsinized and suspended at a final concentration of 5×10^5 cells/ml in serum-free RPMI-1640 medium. Various concentrations of endostar (125, 250, and 500 μg/ml) were given to the cells for 24 h before seeding. One hundred microliters of cell suspension was added to the wells, and the plates were incubated at 37°C for 40 min. The medium was then carefully suctioned out of each well. Each well was washed three times with PBS. The colorimetric MTT-assay was

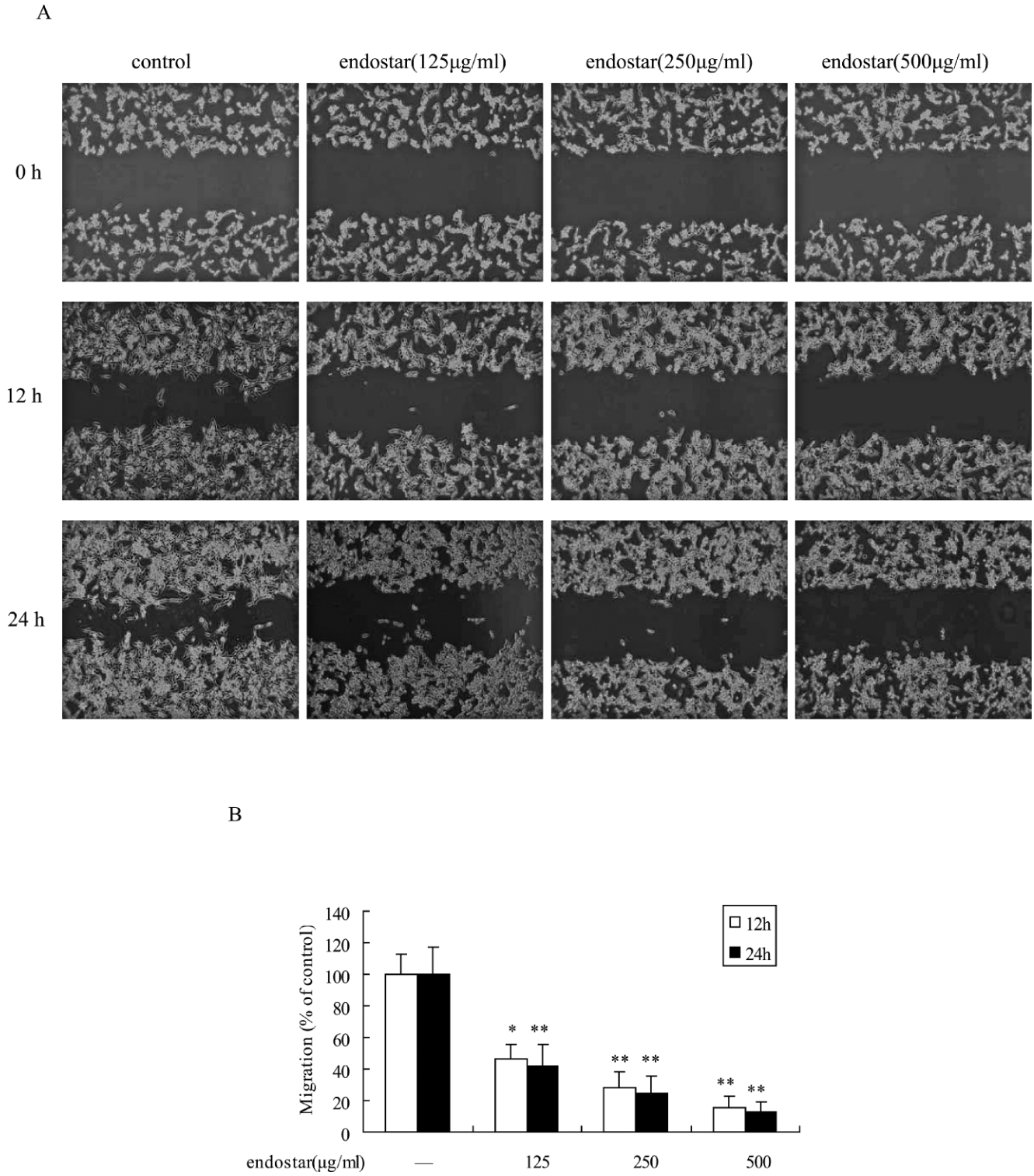

Figure 2. Effect of endostar on MDA-MB-435 cell migration activity *in vitro*. (A) Cell monolayers were wounded by a plastic tip. Wounded monolayers were then washed four times with medium to remove cell debris and incubated in medium in the absence or presence of endostar for various periods of time up to 24 h. Cells were monitored under a microscope equipped with a camera. (B) Quantification of the wound-healing assay. $^{*}P < 0.05$ compared with control; $^{**}P < 0.01$ compared with control.

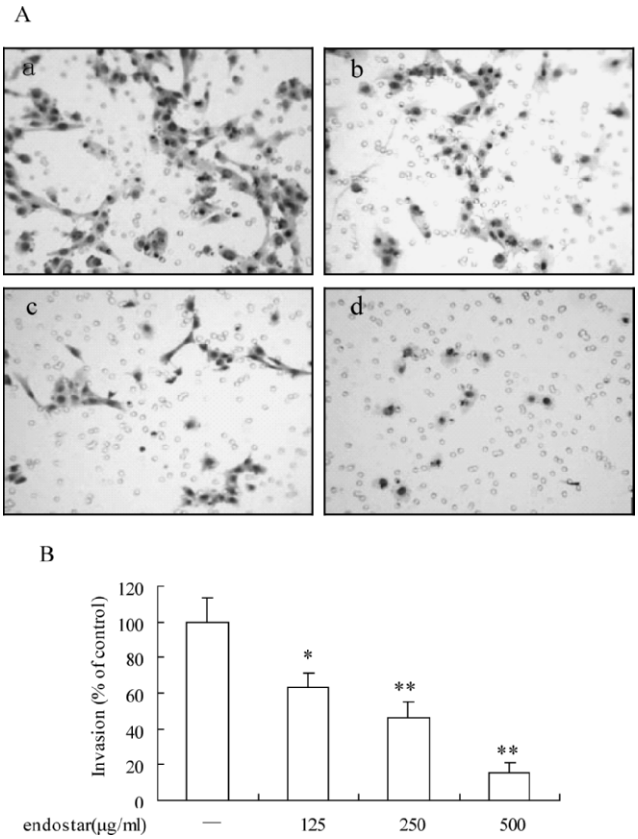

Figure 3. Effect of endostar on MDA-MB-435 cell invasion *in vitro*. Invasion of MDA-MB-435 cells toward conditioned medium was measured using Transwell chambers with 6.5-mm diameter polycarbonate filters (8 μm pore size) coated with matrigel. (A) a, control; b, 125 μg/ml endostar; c, 250 μg/ml endostar; d, 500 μg/ml endostar. Magnification: ×100. (B) Inhibition of the invasion by endostar. Photographs are representative pictures from three independent experiments. $*P < 0.05$ compared with control; $**P < 0.01$ compared with control.

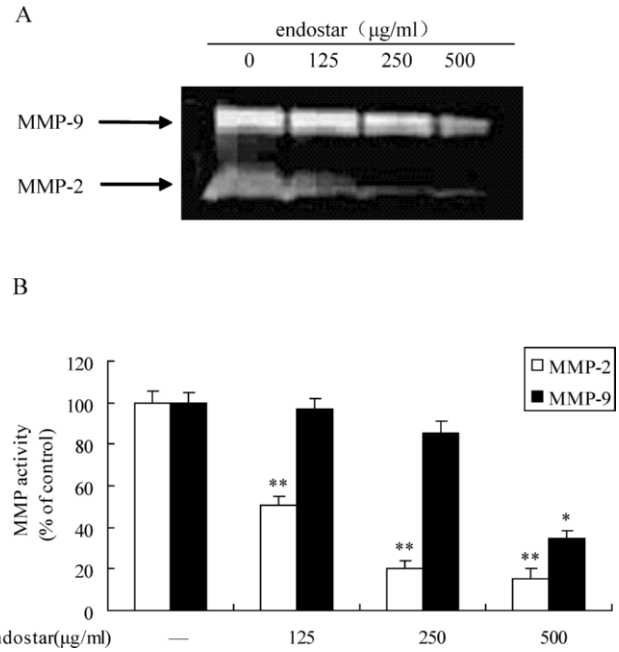

Figure 4. Effect of endostar on the secretion of MMP-2/9 by gelatin zymography. (A) Gelatin zymography analysis of serum-free media conditioned by MDB-MA-435 cells treated with endostar for 24 hrs. (B) The relative enzyme activities of MMP-2 and -9 are expressed as percentages relative to control samples. $*P < 0.05$ compared with control; $**P < 0.01$ compared with control.

used to determine the number of remaining cells (adherent cells).

Gelatin Zymography. Gelatin zymography was performed in 10% SDS-PAGE that had been cast in the presence of 0.1% gelatin (16). After a treatment with endostar (125, 250, and 500 μg/ml) for 24 h, the supernatants were collected. Samples were prepared in nonreducing loading buffer. After electrophoresis, the gels were washed twice with 50 mM Tris-HCl, pH 7.6, containing 5 mM, $CaCl_2$, 1 μM $ZnCl_2$, and 2.5% Triton X-100 (v/v) for 15 min, followed by a brief rinsing in washing buffer without Triton X-100. The gels were then incubated at 37°C for 24 h in 50 mM Tris-HCl buffer containing 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 1% Triton X-100, and 0.02% NaN_3, pH 7.6. The digestion was terminated, and the gels were stained with 0.1% Coomassie Brilliant Blue R250, followed by destaining with 10% acetic acid, 10% methanol. Then the gels were observed by Gel-Pro analyzer.

Western Blot. MDA-MB-435 cells were treated for 24 h with various concentrations of endostar (125, 250, and 500 μg/ml) and cells were collected. Then the cells were lysed in lysis buffer (50 mM Tris-Cl, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% (m/v)NP-40, 0.2 mM PMSF, 0.1 mM NaF, and 1.0 mM DTT), and 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 BCA assay with a Varioskan multimode microplate spectrophotometer (Thermo, Waltham, MA). Then, equal amounts of proteins were separated by SDS-PAGE and transferred onto the PVDF membranes (Millipore, Billerica, MA). The blots were incubated with specific antibodies against indicated primary antibodies overnight at 4°C followed by IRDyeTM800 conjugated secondary antibody for 1 h at 37°C. Detection was performed by the Odyssey Infrared Imaging System (LI-COR Inc., Lincoln, NE). All blots were stripped and reprobed with polyclonal anti-β-actin antibody to ascertain equal loading of proteins.

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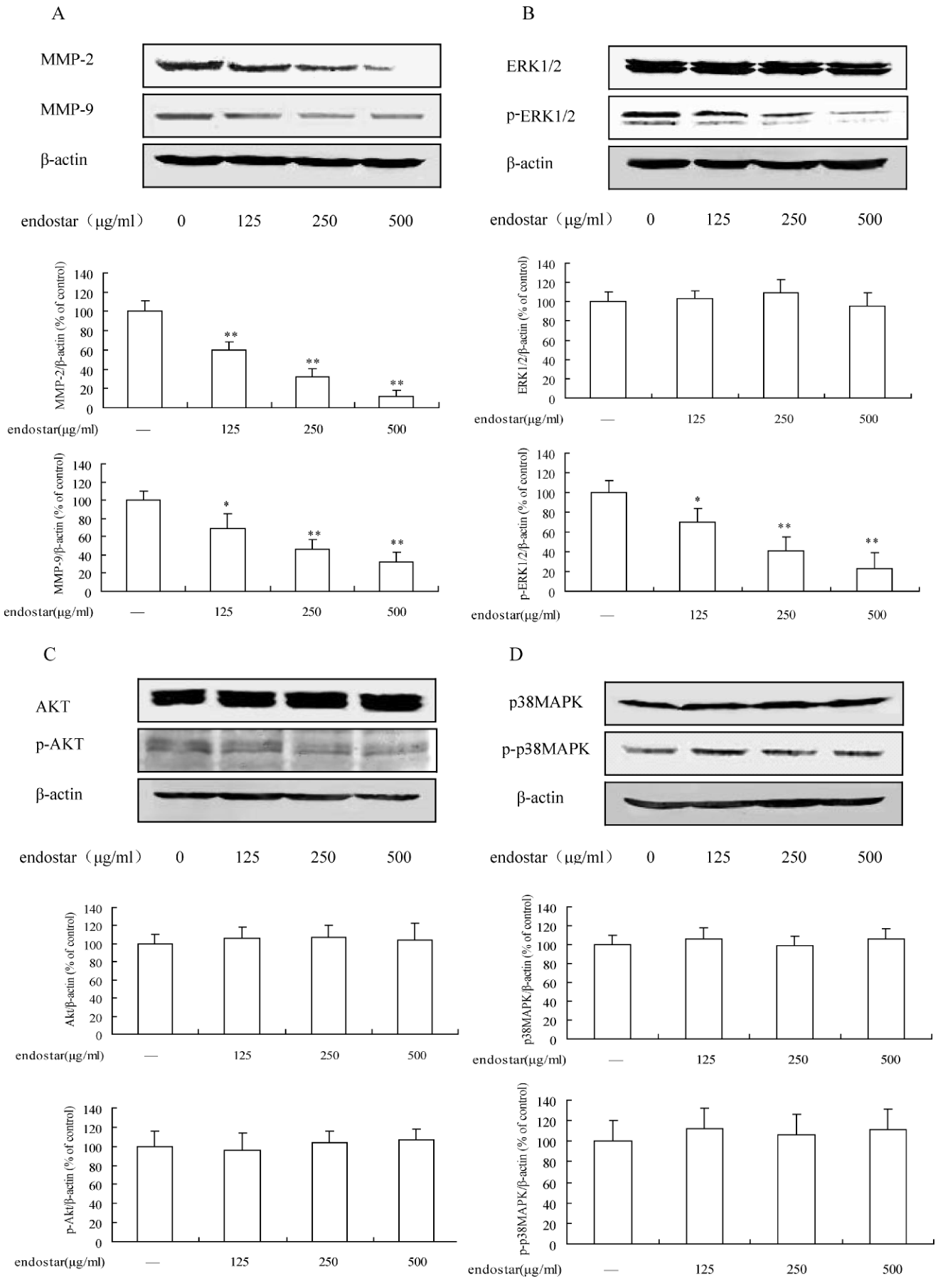
Figure 5. Effects of endostar on the expression of MMP-2/9 and their signaling pathways. Western blot assays were used to examine MMP-2, MMP-9, (A) total and phosphorylation of ERK1/2 (B), Akt (C), and p38 MAPK (D). $*P < 0.05$ compared with control; $**P < 0.01$ compared with control.



Statistical Analysis. All results shown represented the mean ± SEM from triplicate experiments performed in a parallel manner unless otherwise indicated. Statistical analyses were performed using an unpaired, two-tailed Student's *t* test. All comparisons were made relative to untreated controls and significance of difference was indicated as $*P < 0.05$ and $**P < 0.01$.

Results

Effect of Endostar on MDA-MB-435 Cell Adhesion to Fibronectin. Results of MTT assay showed that a 24-h treatment of endostar at various concentrations (0–500 μg/ml) exhibited no cytotoxicity in MDA-MB-435 cells (data not shown). This concentration range was then applied to all subsequent experiments.

Tumor cell adhesion to extracellular matrices and basement membranes is considered to be an initial step in the invasive process for metastatic tumor cells (17). We examined the influence of endostar on the adhesion of MDA-MB-435 cells to the substrates precoated with fibronectin, which is a major basement membrane component. After treatment with endostar (125, 250, and 500 μg/ml) for 24 h, cells were trypsinized and suspended at a final concentration of 5×10^5 cells/ml in serum-free RPMI-1640 medium. Cell suspension was added to the wells, and the plates were incubated at 37°C for 40 min. Medium was then carefully suctioned out of each well. Each well was washed three times with PBS. Then the colorimetric MTT-assay was used to determine the number of remaining cells (adherent cells). Incubation of 250 and 500 μg/ml endostar significantly inhibited cell adhesion to fibronectin-coated substrate (Fig. 1); the inhibition was about 49% and 79% with 250 and 500 μg/ml endostar, respectively.

Effect of Endostar on MDA-MB-435 Cell Migration Activity *In Vitro*. In addition to cell adhesion ability, cell motility was a measure of metastatic potential of cancer cells. To examine the effect of endostar on cancer cell migration, we performed wound healing assays using MDA-MB-435 cells. Confluent monolayers of cells were scratched to form wound and cultured in the absence or presence of various concentrations of endostar (125, 250, and 500 μg/ml) for 24 h. Phase contrast images were photographed. Endostar affected wound healing migration of MDA-MB-435 cells. After 24 h approximately, cells in the control group efficiently spread into the wound area to such an extent that the wound boundary was almost indistinguishable, whereas very a few cells in endostar-treated group spread forward (Fig. 2A). The migration distances between the leading edge and the wound line were compared between endostar-treated and control cells. As shown in Figure 2B, cellular migration was inhibited by up to 58%, 76%, and 84% at 24 h with 125, 250, and 500 μg/ml endostar, respectively.

Effect of Endostar on MDA-MB-435 Cell Invasion *In Vitro*. To explore whether the invasion activity was inhibited within highly invasive tumor cell (MDA-MB-435) in response to endostar, we examined the invasion activity using a matrigel-coated membrane. After a treatment with endostar (0, 125, 250, and 500 μg/ml) for 24 hrs, cells were harvested and seeded in a Transwell chamber and then incubated at 37°C. After stimulating MDA-MB-435 cells with fetal calf serum for 24 h, a large number of cells invaded to the lower side of the filter in the Transwell Chamber. The invaded cells were counted microscopically. Figure 3 indicated that endostar dramatically reduced the number of invading cells, this inhibition occurred in a concentration-dependent manner (125 to 500 μg/ml). The inhibition percentage of 125, 250, and 500 μg/ml endostar was about 37%, 54%, and 85%, respectively.

Effect of Endostar on MMP-2 and -9 Enzyme Activity. MMPs are known to be crucial for degrading extracellular matrix components and for promoting tumor cellular invasion *in vitro* and *in vivo*. To examine whether the anti-invasive activity of endostar was correlated with the inhibition of activities of gelatinolytic MMPs, we analyzed the effect of endostar on the levels of gelatinolytic MMP-2 and MMP-9 in MDA-MB-435 cells. When cells were treated with various concentrations of endostar for 24 h, the supernatants were collected. Samples were prepared in nonreducing loading buffer. After electrophoresis, the gels were incubated with buffer and stained. Then the gels were observed by Gel-Pro analyzer. It was shown that the presence of increased concentrations of endostar reduced MMP-9 and -2 activities. The level of MMP-2 was apparently inhibited with almost complete inhibition at 250 μg/ml concentration (Fig. 4). And the inhibition was about 49%, 80%, and 85% with 125, 250, and 500 μg/ml endostar, respectively. Endostar could also inhibit the level of MMP-9, but the apparent suppression was only seen at 500 μg/ml concentration with inhibition of 65%. These results indicated that secretion of MMP-2 and MMP-9 could be inhibited by endostar.

Effects of Endostar on the Expression of MMP-2/9 and Their Signaling Pathway. To explore whether the increased inhibition of invasiveness potential of MDA-BA-435 cells induced by endostar was associated with the induction of MMP-2 and MMP-9 and their signal pathways, we examined the expression of MMP-2 and MMP-9 in MDA-MB-435 cells. Endostar treatment significantly decreased MMP-2 (72 kDa) expression, but it had only a little effect on MMP-9 (92 kDa) expression. To determine which upstream signaling pathway was influenced by endostar, we examined the expression and phosphorylation of ERK1/2, Akt, and p38 MAPK. Treatment with endostar inhibited phosphorylation of ERK1/2 in a concentration-dependent manner. But endostar didn't inhibit the activation of Akt and p38 MAPK. In all cases, the total steady state of protein levels remained unchanged (Fig. 5).

Discussion

There are several steps in tumor progression that could be regulated by endostar. First, endostar has been shown to inhibit neoangiogenesis of cancer cells (18). Second, endostar can induce the apoptosis of human umbilical vein endothelial cells (HUVECs) (data not published). This study provided evidence that endostar inhibited the adhesion, invasion, migration, and MMP expression in MDA-MB-435 human breast cells. These results proved the inhibitory effect of endostar on breast cancer invasiveness and that endostar could be a potential effector for the prevention of cancer metastasis.

Tumor growth, invasion, and metastasis are a multistep and complex process that includes cell division and proliferation, proteolytic digestion of the extracellular matrix, cell migration through basement membranes to reach the circulatory system, and remigration and growth of tumors at the metastatic sites (19–21). In this study, we have determined adhesion and invasion to show that endostar effectively inhibited the migration of MDA-MB-435 human breast cells. The migration of cancer cells through a coated membrane involves not only ECM degradation, but also requires the ability of adhesive interactions between cells and the matrix. In our study, decreased migration was correlated with decreased adhesion and invasion.

The initial step of tumor cell invasion is characterized by the breakdown of the basement membrane, a process known to be dependent on type IV collagen-degrading enzymes, mainly MMP-2 and MMP-9. Liotta et al. obtained results where type IV gelatinase activity correlated with cancer metastasis. MMPs degrade the basement membrane and extracellular matrix, thus facilitating the invasion of malignant cells through connective tissues and blood vessel walls and resulting in the establishment of metastases (22). MMP expression, although low or undetectable in most normal tissues, is substantially increased in the majority of malignant tumors. Numerous studies (23–28) in a variety of tumor types, including lung, colon, breast, and pancreatic carcinomas, demonstrate overexpression of MMPs in malignant tissues in comparison with adjacent normal tissues. In general, the gelatinases (MMP-2 and MMP-9) have been most consistently detected in malignant tissues and associated with tumor aggressiveness, metastatic potential, and a poor prognosis. Most published papers determined the downregulation of MMPs by possible antimetastatic agents, not by measuring MMPs activities directly. In this study, we observed downregulation of the secretion and expression of MMP-2 and MMP-9 activities by endostar compared with controls. Although this was not a direct inhibition of their enzymatic activities, this inhibition of MMP-2 and MMP-9 would probably be responsible for the decrease of invasion of MDA-MB-435 cells.

Activation of ERK1/2 has been shown in other systems to be the mechanism for promoting the production of MMPs (29), which are important for cell proliferation, invasion, and neovascularization. It has been shown that inhibition of p-ERK1/2 may lead to a reduction in the expression of MMP-2, as well as in the invasion of tumor cells (30, 31). Thus, it is possible that endostar may decrease breast cancer cell invasion by inhibiting the production of MMPs via inactivation of ERK1/2. As evidenced from the above results, endostar may be a powerful candidate in the development of preventive agents for cancer metastases.

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