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Endostar, a modified endostatin inhibits non small cell lung cancer cell in vitro invasion through osteopontin-related mechanism

Qinggui Ni [a,b], Hui Ji [a,*], Zhihui Zhao [c], Xin Fan [b], Chengyun Xu [a]

[a] Department of Pharmacology, China Pharmaceutical University, Nanjing 210009, China
[b] Simcere Pharmaceutical Research Institute, Nanjing 210042, China
[c] Life Science College, Nanjing Normal University, Nanjing 210046, China

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ABSTRACT

In this study, we studied the inhibition of non small cell lung cancer (NSCLC) cells invasion by a recombinant human Endostar, a modified endostatin and the possible osteopontin-related mechanism. The results showed that Endostar significantly inhibited highly metastatic NSCLC (NCI-H460) cells in vitro invasion. ELISA demonstrated that reduction of osteopontin level in the medium by Endostar may be responsible for the inhibition of invasion. RT-PCR assay and western blot analysis revealed that the reduction of osteopontin was due to under-regulation of osteopontin expression. Furthermore, Endostar also inhibited osteopontin-induced less metastatic NSCLC (A549) cells invasion, indicating that Endostar may have other different osteopontin-related mechanism. In an adhesion assay, we found that Endostar reduced NCI-H460 cells binding to osteopontin. Flow cytometric analysis suggested that the reduction of adhesion may be related to under-regulation of its receptors (CD44v6 and $\alpha_v\beta_3$ integrin) expression. Additionally, we found, via gelatin zymographic analysis, that osteopontin-induced the expression and activation of pro-matrix metalloproteinase (MMP)-2 and pro MMP-9 secreted from A549 cells were blocked upon Endostar treatment, indicating that Endostar may block osteopontin-mediated signal transduction pathways through MMP families. The above results indicate that Endostar may have an intrinsic non-angiogenesis-related antitumor activity through osteopontin-related mechanism against NSCLC, including osteopontin change and osteopontin signal transduction blockade. Tumor cell invasion is important for tumor metastasis, our findings suggest that it is probably a good strategy to put Endostar into treatment of NSCLC metastasis.

1. Introduction

Endostatin, a carboxyl-terminal proteolytic fragment of collagen XVIII, was discovered as a potent inhibitor of angiogenesis (O'Reilly et al., 1997).Tumor progression and metastasis of many animal and human tumors in mice were inhibited by endostatin, for example, Human laryngeal squamous cell carcinoma in nude mice (Yao et al., 2004), spontaneous mammary carcinoma in transgenic mice (Calvo et al., 2002), liver metastases from murine colorectal cancer cells injected into the spleen (Te Velde et al., 2005), murine lung adenocarcinoma (LA795) growing subcutaneously in mice (Xia et al., 2003), etc. A combination of endostatin plus thrombospondin-1, or a combination of endostatin plus carboplatin prevented all metastases, significantly inhibited primary tumors (Abraham et al., 2003). Furthermore, Boehm et al. (1997) reported that repeated cycles of endostatin therapy prolonged tumor dormancy without resistance to endostatin. On the cellular level, endostatin was shown to inhibit endothelial cell proliferation (O'Reilly et al., 1997) and migration (Yamaguchi et al., 1999), and to induce endothelial cell apoptosis (Dhanabal et al., 1999). Researchers attributed these activities to anti-angiogenic mechanism of endostatin.

However, in other studies, a non-angiogenesis-related mechanism of endostatin was observed. Te Velde et al. (2005) reported that 2 h pretreatment in vivo with endostatin inhibited intrahepatic tumour growth by early inhibition of tumour cell seeding in the liver and induced a two-fold decrease of tumor cell adhesion to endothelial cells under flow conditions. Kim et al. (2000) demonstrated that endostatin blocked the invasiveness of tumor cells through inhibiting both the extracellular activation of pro-matrix metalloproteinase (MMP)-2 and the catalytic activity of pro MMP-2. These may indicate that Endostar had its intrinsic properties, especially in tumor metastasis.

The roles of osteopontin in tumor have been discussed in a number of papers. The findings that osteopontin expression correlates with tumor progression in breast cancers (Tuck et al., 1998), stomach (Ue et al., 1998), lung (Chambers et al., 1996), prostate (Khodavirdi et al., 2006), liver (Pan et al., 2003), and colon (Agrawal et al., 2002), and that osteopontin concentration in the plasma of patients with metastatic disease is significantly higher than that in normal sera (Senger et al., 1988; Fedarko et al., 2001) implicate its role in the

* Corresponding author. Department of Pharmacology, China Pharmaceutical University, 24TongJiaXiang, 210009, Nanjing, China. Tel.: +86 25 86021369.
E-mail address: huijicpu@163.com (H. Ji).

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regulation of tumor cell migration and metastasis. In fact, numerous studies in cultured cells have shown that osteopontin expression renders cells more tumorigenic and/or metastatic (Khodavirdi et al., 2006). In contrast, down-regulation of osteopontin expression by antisense approach reduced cell growth in soft agar and in mice as primary tumors or experimental metastasized foci (Rangaswami et al., 2006).

Endostar is a modified endostatin. It was approved for a treatment of clinical non-small cell lung cancer (NSCLC) with combination of cis-platinum or other chemotherapy drugs in China. In this study, we attempted to dissect the non-angiogenesis-related antitumor activity of Endostar on NSCLC cells invasion and its possible osteopontin-related mechanism.

2. Materials and methods

2.1. Tumor cell culture

NSCLC cell line NCI-H460(with high-lung -metastatic activity) and A549 (ATCC,U.S.A.) were routinely cultured in DMEM medium (Gibco, U.S.A) supplemented with 10% fetal bovine serum. 100 units/ml penicillin, 100 μg/ml streptomycin, at 37 °C under a humidified 95%–5% (v/v) mixture of air and CO_2. Cell viability was determined by Trypan blue staining. Confluent cultures were harvested by brief trypsinisation (0.05% trypsin in 0.02% EDTA), and resuspended in the medium to a final concentration of 1×10^6cells/ml.

2.2. In vitro invasion assay

The in vitro invasion assay was carried out using transwell chamber with 6.5-mm diameter polycarbonate filters (8 μm pore size, Greiner Bio-one, Germany) as described in the instructions. The NCI-H460 or A549 cells suspension (1×10^6cells/well) was added to the upper chamber of transwell cell culture chambers coated with or without 35 μg Matrigel (BD Biosciences, U.S.A.). DMEM supplemented with 10% fetal bovine serum was used as a chemoattractant in the lower wells, while DMEM /0.1% bovine serum albumin was added to the control wells. When required, human recombinant osteopontin (Sigma, U.-S.A) was added at the concentration of 100 ng/ml. Different concentrations of Endostar (0.1, 0.5, 2.5, 12.5, and 62.5 μg/ml. Simcere, China) or equal concentrations of control buffer were added. The inserts were incubated for 24 h at 37 °C. The cells were labelled with 8 μM Calcein-AM (Sigma, U.S.A) in DMEM for 45 min at 37 °C. Thereafter, the migrated cells were harvested by brief trypsinisation (0.05% trypsin in 0.02% ethylenediaminetetraacetic acid (EDTA)). The number of migrated cells was measured with a fluorescence plate reader (TECAN infinite M200, TECAN, Switzerland) at an excitation wave length of 485 nm and an emission wave length of 520 nm. The invasion index (*I*%) was calculated according to the following formula: $(I\%) = (RFU_1 - RFU_0) / (RFU_2 - RFU_0) \times 100\%$ (RFU_1 = the relative fluorescence units obtained from cells that migrated through an ECM coated membrane towards 10% fetal bovine serum, RFU_2 = the relative fluorescence units obtained from cells that migrated through an uncoated membrane towards 10% fetal bovine serum, and RFU_0 = the relative fluorescence units obtained from cells that passively passed through an uncoated membrane in the absence of fetal bovine serum).

2.3. Enzyme-linked immunosorbent assay (ELISA)

After tumor cells (1×10^6/well) were treated with the different concentration of Endostar (0.1, 0.5, 2.5, 12.5, and 62.5 μg/ml) for 24 h under cell culture condition, the culture medium was collected and osteopontin level was determined by ELISA as described in the instructions (R&D System, U.S.A). A polyclonal antibody was used to osteopontin immobilized on a microtitre plate to bind osteopontin in the sample. Further detection was done with a second monoclonal antibody labelled with horseradish peroxidase (HRP). Tetramethyl benzidine was used as a substrate for a coloring reaction, which were read spectrophotometrically on a plate reader (TECAN infinite M200) at OD 492.and the strength of coloring was proportional to the quantity of osteopontin.

2.4. Real-time PCR (RT-PCR)

For the determination of mRNA levels in the cultured cells, Total cells RNA from ELISA were extracted and RT-PCR assay was designed: forward: 5'-TGAG-TCTGGAAATAACTAATGTGTTTGA-3',reverse:5'-GAACATAGACATAACCCTGAAGCTTTT-3'. RT-PCR assay was performed as indicated in the instruction (QIAGEN, Germany). All samples were run for 28 cycles in triplicate and three control samples with no template were included for each run. The bands of beta-actin were used as the reference.

2.5. Western blot analysis

Total cell extracts from ELISA were prepared by adding 500 μl lysis buffer (0.1 M Tris–HCl/0.4% Triton X-100), followed by a centrifugation at 10,000 g at 4 °C for 30 min. Proteins (20–50 μg/cell sample), assessed by the BCA protein assay (Pierce, U.S.A) were run in a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) before transferring onto a Polyvinylidene Fluoride (PVDF) membrane (Whatman, Germany). Membranes were first blocked overnight at 4 °C with phosphate buffered saline (PBS, PH7.4) containing 5% powdered milk and 0.1% Tween 20 and were then incubated for 2 h with the primary antibodies (Chemicon, U.S.A), Membranes were then incubated for 1 h with the adequate horse-radish peroxidase conjugated secondary antibodies (Donkey anti-rat, Proteintech, U.S.A). Peroxidase was revealed by the enhanced chemiluminescence assay (Pierce, U.S.A).To normalize sample loading, glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Biolegend, U.S.A) blot was done.

2.6. Adhesion assay

Adhesion assay was assessed as described (Standal et al., 2004). 96-well plates were coated overnight at 4 °C with 100 ng/ml human recombinant osteopontin in PBS (PH7.4), 50 μl/well or bovine serum albumin (100 ng/ml in PBS, 50 μl/well). The plates were then washed and blocked with bovine serum albumin (10 mg/ml, 100 μl/well) for 1 h at room temperature. Before use the plates were washed 3 times and dried completely. The cells were washed twice and resuspended in DMEM/0.1% bovine serum albumin at a concentration of 1×10^6cells/ml and labeled for 1 h at room temperature with 8 μM Calcein-AM. Subsequently, the cells were washed 4 times and 80,000 cells per well were seeded and incubated with the different concentration of Endostar (0.1, 2.5, 62.5 μg/ml) for 1 h at 37 °C in 5% CO_2. non-adherent cells were carefully removed by washing. The cells were thereafter solubilized by adding 1% Triton X-100, 50 μl/well. Fluorescence intensity at 520 nm was measured with a TECAN infinite 200 fluorescence reader. The excitation wavelength was 485 nm.

2.7. Flow cytometric analysis

NCI-H460 cells were incubated in the culture medium with the different concentration of Endostar(0.1, 2.5, 62.5 μg/ml) for 12 h at 37 °C in 5% CO_2. The cells were then collected and stained with fluorescence-labeling anti-CD44v6 antibody (Chemicon, U.S.A) or anti-$\alpha_V\beta_3$ integrin (Chemicon, U.S.A). Flow cytometric analysis of cell surface CD44v6 and $\alpha_V\beta_3$ integrin were performed using a FACS Calibur analyzer (Beckon Dickinson Immunocytometry Systems, U.S.A).

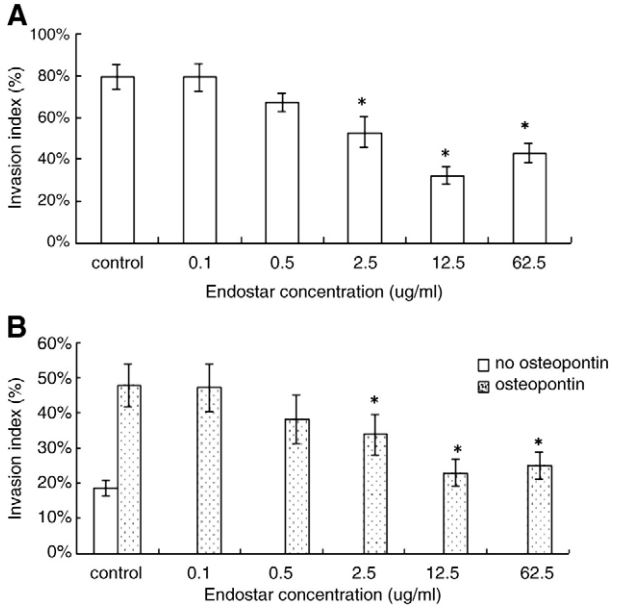

Fig. 1. Inhibition of Endostar on tumor cells invasion. Tumor cells (1×10^6cells/well) were added to transwell chamber coated with Matrigel and were incubated with various concentrations of Endostar (0.1, 0.5, 2.5, 12.5, and 62.5 μg/ml) for 24 h at 37 °C. The control was added equal concentrations of control buffer. DMEM supplemented with 10% fetal bovine serum was used as a chemoattractant. A. Invasion index of NCI-H460 cells and effect of Endostar on NCI-H460 cells invasion. B. Invasion index of A549 cells and effect of Endostar on A549 cells invasion in the presence or the absence of 100 ng/ml of osteopontin. Each value is the mean ± S.D. of three independent determinations of duplicate experiments. *$P<0.05$, when tested against the control using a paired student's *t* test.

2.8. Zymography experiments

The gelatinolytic activity was measured as described (Philip et al., 2001). The A549 cells were pretreated with osteopontin (100 ng/ml) in serum-free medium for 45 min and then incubated with Endostar (0.5, 12.5 μg/ml) for 12 h at 37 °C. The conditioned medium was collected by centrifugation, concentrated, and dialyzed. Protein concentrations were measured using BCA protein assay. The samples containing equal amount of total proteins were mixed with sample buffer in absence of reducing agent, incubated at room temperature for 30 min, and loaded onto zymography SDS-PAGE containing gelatin (0.5 mg/ml). The gels were washed and incubated in the buffer (50 mM Tris–HCl (pH 7.5) containing 100 mM $CaCl_2$, 1 M $ZnCl_2$, 1% (v/v) Triton-X100, and 0.02% (w/v) NaN3) for 16 h. The gels were stained with Coomassie Blue and destained. Negative staining showed the zones of gelatinolytic activity.

2.9. Statistical analysis

Statistical analysis of data was done by student' *t* test using SPSS software. Difference was considered to be statistically significant at $P<0.05$.

3. Results

3.1. Inhibition of Endostar on in vitro NSCLC cells invasion

Two NSCLC lines, NCI-H460 and A549, were chosen for this investigation. NCI-H460 cells showed high invasion index. When the increasing concentrations of Endostar (0.1, 0.5, 2.5, 12.5, and 62.5 μg/ml) were added into the matrigel culture, the invasion was markedly inhibited in a dose-dependent manner as compared to the control (buffer without Endostar) with almost maximal inhibition at 12.5 μg/ml (Fig. 1A). Invasion index of A549 cells was lower, but 100 ng/ml of osteopontin induced A549 cells to high invasion activity. Effect of Endostar on osteopontin-induced A549 cells invasion was similar to that of NCI-H460 (Fig. 1B). The results indicated that Endostar can inhibit either NSCLC cells invasion or osteopontin-induced NSCLC cells invasion.

3.2. Reduction of Endostar on osteopontin level in the culture medium of NSCLC cells by the under-regulation of osteopontin expression

To examine whether Endostar inhibits NSCLC cells invasion by osteopontin-related mechanism, osteopontin level in the culture medium of both NCI-H460 and A549 cells were determined. We found that osteopontin level was higher in the culture medium and were decreased by Endostar in a dose-dependent manner, which was consistent with that of the invasion assay (Fig. 2A), indicating that inhibition of Endostar on the invasion may be due to reduction of osteopontin level. RT-PCR assay (Fig. 2B) and western blot analysis (Fig. 2C) showed a close correlation between the inhibition of osteopontin expression (gene and protein level) and the reduction of osteopontin level. However, osteopontin level in the culture medium of A549 cells was lower as indicated in Fig. 2A, and inhibition of Endostar on osteopontin-induced NSCLC cells invasion may refer to other osteopontin-related mechanism without osteopontin change.

3.3. Reduction of Endostar on NSCLC (NCI-H460) cells binding to osteopontin by the under-regulation of osteopontin receptor expression

Osteopontin functions through its interaction with the integrin and CD44 families of cell surface. We performed an adhesion assay

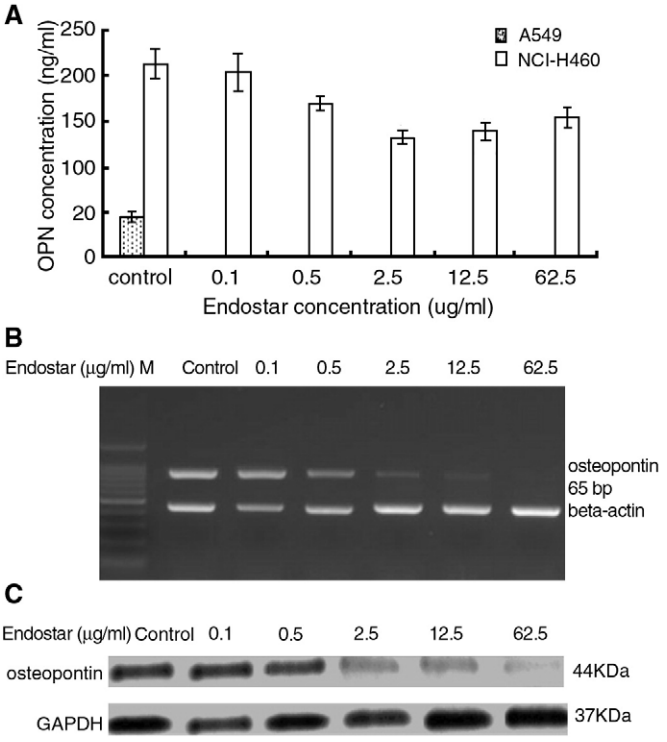

Fig. 2. Reduction of Endostar on osteopontin level in the culture medium of NSCLC cells by the under-regulation of osteopontin expression. Tumor cells (1×10^6cells/well) were incubated with various concentrations of Endostar (0.1, 0.5, 2.5, 12.5, and 62.5 μg/ml) for 24 h at 37 °C. The control was added equal concentrations of buffer. A. Osteopontin level in the culture medium of both NCI-H460 and A549 cells was determined by ELISA, respectively. B. The total NCI-H460 cells RNA from ELISA was extracted. The regulation of osteopontin gene expression was analyzed by RT-PCR. Band of beta-actin is a reference. C. Total NCI-H460 cells protein from ELISA was prepared. The protein levels were determined by western blot analysis. GAPDH protein was used as the reference. Each value is the mean ± S.D. of three independent determinations of duplicate experiments. *$P<0.05$, when tested against the control using a student's *t* test.

with highly metastatic NCI-H460 cells to determine whether Endostar inhibits osteopontin binding to its receptors. The results showed that Endostar blocked the binding in a dose-dependent manner (Fig. 3A). In two other separate experiments, we analyzed osteopontin receptors by flow cytometric and found that the log fluorescence of both CD44v6 (Fig. 3B) and $\alpha_V\beta_3$ integrin (Fig. 3C) was decreased following the treatment of Endostar. The results indicated that Endostar inhibited NSCLC cells adhesion to osteopontin presumably by the under-regulation of expression of osteopontin receptors.

3.4. Blockade of Endostar on osteopontin-induced expression and activation of MMPs of NSCLC (A549) cells

The interactions of osteopontin with its cell surface receptors could induce the activation of various signal transduction pathways. To check whether Endostar suppresses signal transduction pathways of osteopontin downstream, we analyzed the effect of Endostar on the levels and processing of gelatinolytic MMPs in the medium of A549. The results showed that the levels of both pro- and active MMP-2 were higher in osteopontin-treated cells compared with untreated cells (Fig. 4A, B). When cells pretreated with osteopontin followed by treatment with different concentration of Endostar (0.5, 12.5 μg/ml), both pro-MMP-2 expression and activation showed reduction (Fig. 4A). The same results were observed in MMP-9 (Fig. 4B), demonstrating that Endostar blocked the osteopontin-induced osteopontin-related signal transduction pathway.

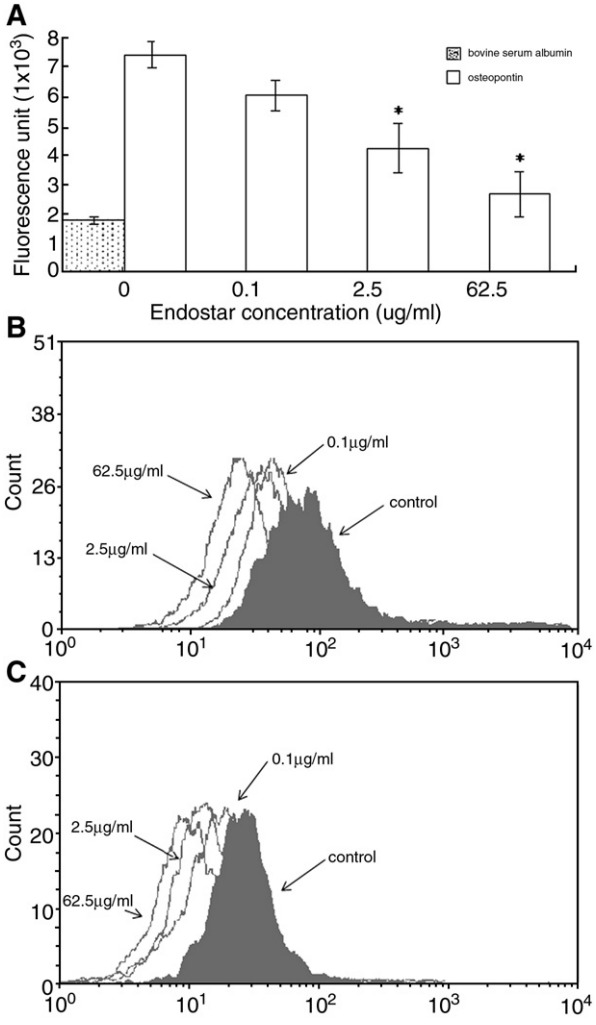

Fig. 3. Blockade of Endostar on NSCLC (NCI-H460) cells binding to osteopontin by the under-regulation of osteopontin receptor expression. A. 96-well plates were coated overnight at 4 °C with 100 ng/ml human recombinant osteopontin in PBS, 50 μl/well or bovine serum albumin (100 ng/ml in PBS, 50 μl/well). NCI-H460 cells (1×10^6cells/well) were added to the plates and incubated with different concentrations of Endostar (0.1, 2.5, 62.5 μg/ml) for 12 h at 37 °C. The control was added equal concentrations of control buffer. Tumor cells binding to osteopontin was determined by an adhesion assay. Each value is the mean ± S.D. of three independent determinations of duplicate experiments. *$P<0.05$, when tested against the control using a Student's t test. B, C. The cells were treated as indicated above and were collected and stained with a fluorescence-labeling anti-CD44v6 antibody or anti-$\alpha_V\beta_3$ integrin. Flow cytometric analysis of cell surface CD44v6 (B) and $\alpha_V\beta_3$ integrin (C) were performed using a FACS Calibur analyzer. Histograms represent the log fluorescence and histograms of the different concentration of Endostar were overlaid. Data shown are representatives of at least three independent experiments.

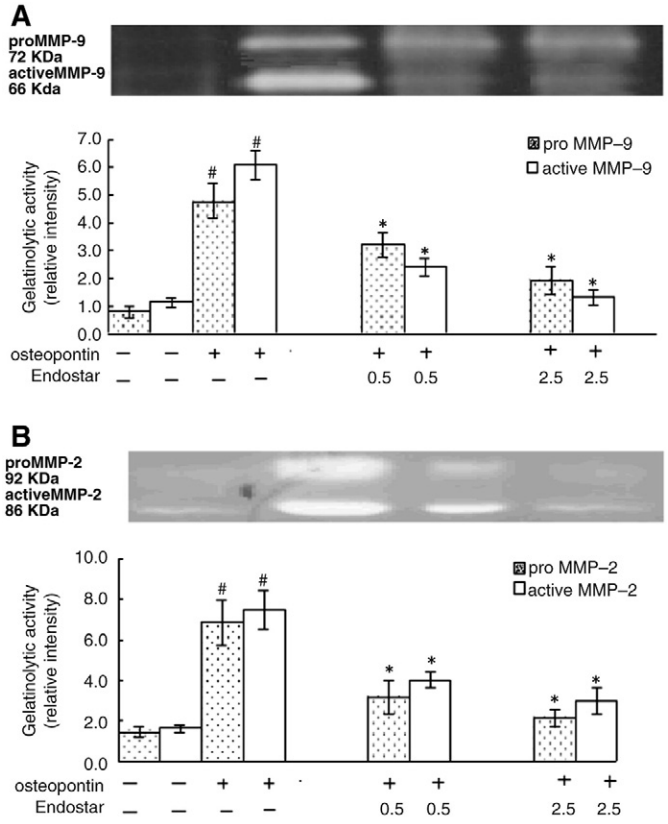

Fig. 4. Effect of Endostar on osteopontin-induced expression and activation of MMPs of NSCLC (A549) cells. A549 cells were treated with or without 100 ng/ml of osteopontin alone or with 100 ng/ml of osteopontin and then with different concentration of Endostar (0.5, 12.5 μg/ml) for 12 h at 37 °C in the culture condition. The medium was collected. MMP-2 (A) and MMP-9 (B) activity was analyzed by gelatin zymography, and the bands were analyzed by densitometry and are represented in the form of a bar graph. The mean values of triplicate experiments are indicated. The relative intensities were analyzed statistically using student's t test (*$P<0.05$) when compared with osteopontin-treated group. #$P<0.05$, when compared osteopontin-untreated group).

4. Discussion

In this study, we found that Endostar inhibited NSCLC cells in vitro invasion, and which was related to lowering of osteopontin expression. Furthermore, Endostar also could inhibit osteopontin-induced NSCLC cells invasion, indicating that Endostar may act also through other different osteopontin-related mechanism.

Osteopontin activates various signaling pathways and functions through the interaction with its receptor on cell surface. It has been shown that osteopontin interacts with a number of different integrins via the RGD sequence, including alpha v beta3, alpha v beta 1and alpha v beta 5 (Sodek et al., 2000; Hu et al., 1995; Hruska et al., 1995). Additional integrins have also been found to interact with osteopontin,

including alpha 4 beta 1(Bayless et al., 1998), alpha 9 beta 1 (Smith et al., 1996), and alpha 8 beta1 (Denda et al., 1998). The best characterized osteopontin receptor is the $\alpha_V\beta_3$ integrin. There is evidence that osteopontin causes cell adhesion, migration, extracellular matrix (ECM) invasion, and cell proliferation by interaction with its receptor $\alpha_V\beta_3$ integrin in various cell types (Panda et al., 1997). CD44 is another important non-integrin receptor of osteopontin and expressed as a standard receptor (CD44 s) and in multiple splice isoforms (CD44v), whose expression is altered during tumor growth and progression. CD44 splice variants are thought to be correlated with invasive growth and metastasis in many tumor types and the v6 variant exon of CD44 (CD44v6) is necessary for osteopontin binding (Ponta et al., 2003). Our results showed that Endostar reduced NCI-H460 cells binding to osteopontin by an under-regulation of both the $\alpha_V\beta_3$ integrin and CD44v6 expression, which indicated that Endostar might block an onset of osteopontin-mediated signaling pathways and inhibit NSCLC cells invasion.

Osteopontin binds its receptors and then activates several signaling pathways that may contribute to tumor progression and metastatic behavior. Two studies suggested that osteopontin bound $\alpha_V\beta_3$ integrin and induced nuclear factor kappa B-mediated pro MMP-2 and pro MMP-9 expression and activation through I kappa B alpha/IKK signaling pathways (Philip and Kundu, 2000; Rangaswami et al., 2006). In this study, we found that Endostar inhibited osteopontin-induced expression and activation of both pro MMP-2 and pro MMP-9. MMPs is a family of zinc-containing endopeptidases and plays a significant role in ECM degradation and facilitate cell motility, tumor growth and metastasis (Philip and Kundu, 2003; Han et al., 2001). MMP-2 and MMP-9 were especially implicated in tumor metastasis (Coussens and Werb, 1996). Our results indicate that Endostar may block downstream osteopontin-mediated signal transduction pathways through MMPs family and inhibit tumor cell invasion.

Take together, Endostar inhibits NSCLC cells invasion through osteopontin-related mechanism including osteopontin change and osteopontin signal transduction blockade, which can explain no-angiogenesis activity of endostatin in tumor treatment, and also provide a basis for the treatment of metastasis by Endostar. Angiogenesis is necessary for sustained growth and metastasis of tumor cells. In some studies, an implicated osteopontin have been reported in angiogenesis. The expression of vascular endothelial growth factor (VEGF), osteopontin, and integrin $\alpha_V\beta_3$ has been demonstrated to be related with angiogenesis (Senger et al., 1996). Endothelial cell migration is stimulated by the osteopontin and there were cooperation between the VEGF and osteopontin. (Shijubo et al., 1999). And also osteopontin contributes to angiogenesis through effects on the expression of VEGF (Takano et al., 2000). And our primary study (data not shown) showed that Endostar can inhibit osteopontin-induced vascular endothelial cell migration through influencing VEGF and its receptor-VEGFR2,which may be a novel anti-angiogenesis mechanism of endostatin. Further study is still in progress. So we consider that it may probably be a good strategy to put Endostar into treatment of NSCLC or other tumor metastasis.

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