Endostatin Inhibits Endothelial and Tumor Cellular Invasion by Blocking the Activation and Catalytic Activity of Matrix Metalloproteinase 2

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Endostatin Inhibits Endothelial and Tumor Cellular Invasion by Blocking the Activation and Catalytic Activity of Matrix Metalloproteinase 2[1]

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ABSTRACT

Here we report the inhibition of cellular invasion by a recombinant mouse endostatin and the possible mechanism of the inhibition. Endostatin significantly reduced endothelial as well as tumor cellular invasion into the reconstituted basement membrane *in vitro*. Gelatin zymographic analysis revealed that the activation of promatrix metalloproteinase-2 (proMMP-2) that was secreted from endothelial cells was blocked upon endostatin treatment. Studies with recombinant MMPs confirmed that endostatin inhibited proMMP-2 activation, mediated by both membrane-type 1 MMP and 4-aminophenylmercuric acetate. Furthermore, enzymatic assays using a peptide substrate demonstrated that endostatin inhibited the catalytic activities of both MMP-2 and membrane-type 1 MMP. Finally, coimmunoprecipitation experiments revealed that endostatin formed a stable complex with proMMP-2. These novel findings would, at least in part, explain the mechanism of the potent antiangiogenic and antitumor activities of endostatin.

INTRODUCTION

Angiogenesis, the formation of new blood vessels from preexisting endothelium, is a fundamental step in tumor progression and metastasis (1, 2). Endostatin, a M_r 20,000 proteolytic fragment of collagen XVIII, was discovered as a potent inhibitor of angiogenesis (3). Systematic administration of recombinant endostatin resulted in regression of various tumors in a xenograft model (3). Furthermore, Boehm *et al.* (4) 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 (3) and migration (5), and to induce endothelial cell apoptosis (6). Despite its potent antiangiogenic activity *in vivo* and *in vitro*, the molecular targets of endostatin are not clearly understood.

MMPs,[3] a family of zinc-containing endopeptidases, were largely implicated in angiogenesis and tumor metastasis (7, 8). MMPs mediate selective proteolytic degradation of the extracellular matrix that is required for migration and invasion of endothelial cells at the onset of angiogenesis. Indeed, MMP-1, MMP-2, MMP-9, and MT1-MMP are reported to be produced by endothelial cells (7), and it has been recently shown that tumor angiogenesis is reduced in MMP-2-deficient mice (9).

We have found that endostatin significantly reduces invasion of endothelial as well as tumor cells into the reconstituted basement membrane. Importantly, we provide convincing evidence that endostatin inhibits the proteolytic activation of proMMP-2 and the catalytic activities of MT1-MMP and MMP-2. Therefore, we suggest that these novel functions of endostatin may be, at least in part, responsible for its potent antiangiogenic and antitumor action.

MATERIALS AND METHODS

Cell Culture. HUVECs were isolated from human umbilical cord veins by collagenase treatment as described previously (10) and used in passages 2–7. The cells were grown in M199 medium (Life Technologies, Grand Island, NY) supplemented with 20% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 3 ng/ml basic fibroblast growth factor (Upstate Biotechnology, Lake Placid, NY), and 5 units/ml heparin at 37°C under a humidified 95%–5% (v/v) mixture of air and CO_2. Human fibrosarcoma HT1080 cells and HEK 293 cells were maintained in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Preparation of Recombinant Mouse Endostatin. The mouse endostatin cDNA encoding the COOH-terminal 183 amino acids of mouse collagen XVIII (GenBank accession no. A1326391) was obtained by PCR. The 5′ primer was GGG*AAGCTT*CATACTCATCAGGACTTTCAGC and the 3′ primer was GGG*GGATCC*CTATTTGGAGAAAGAGGTCATG. The PCR fragment was digested with *Hin*dIII and *Bam*HI, and cloned into the pFLAG-CMV-1 vector (KODAK, New Haven, CT) encoding the NH_2-terminal FLAG (DYKDDDDK) epitope and a linker amino acid (L). The resulting construct (pFLAG-CMV-1-endostatin) was cotransfected with pcDNA3.1 (CLONTECH, Palo Alto, CA) into HEK293 cells, and the G418 (0.6 μg/ml)-resistant cells were used for the collection of serum-free conditioned medium. The recombinant mouse endostatin was purified serially by heparin-Sepharose CL-6B column (Amersham-Pharmacia, Seoul, Korea) and Superose 12 column (Amersham-Pharmacia) chromatography as described previously (11). The endostatin fraction was extensively dialyzed in PBS and stored at −20°C.

***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) as described previously (12). Six hundred μl of M199 medium with or without 50 nM PMA (Alexis, Laufelfingen, Switzerland) or 5 ng/ml VEGF was placed in the lower wells. HUVECs or HT1080 cells (1×10^6 cells/ml) were treated with the indicated concentration of endostatin or TIMP-2 for 30 min, and 100 μl of cell suspension was loaded into each of the upper wells. The chambers were incubated for 20 h at 37°C. Cells were fixed and stained with H&E. Nonmigrating cells on the upper surface of the filter were removed by wiping with a cotton swab, and invasive activity was quantified by counting the cells that migrated to the lower side of the filter with optical microscopy at ×200.

Gelatin Zymography. Gelatin zymography was performed in 9% SDS-PAGE that had been cast in the presence of 0.1% gelatin. Samples were prepared in nonreducing loading buffer. After electrophoresis, SDS was removed by 2.5% Triton X-100 to renature gelatinases. Gels were then incubated at 37°C for 24 h in an incubation buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 10 mM $CaCl_2$], and then were stained with 0.25% Coomassie Blue R 250.

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[3] The abbreviations used are: MMP, matrix metalloproteinase; APMA, 4-aminophenylmercuric acetate; HUVEC, human umbilical vein endothelial cell; VEGF, vascular endothelial cell growth factor; PMA, phorbol 12-myristate 13-acetate; MT1-MMP, membrane-type 1 MMP; sMT1-MMP, soluble MT1-MMP; HEK, human embryo kidney; TIMP, tissue inhibitor of metalloproteinase.

Inhibition of MT1-MMP-mediated and APMA-mediated ProMMP-2 Activation by Endostatin. Recombinant human proMMP-2 and transmembrane-deleted sMT1-MMP were expressed in baculovirus/insect *Sf*9 cell system and were purified, as described previously (13, 14). To examine the inhibitory effect of endostatin on the sMT1-MMP-mediated proMMP-2 activation, 10 ng of proMMP-2 was activated by 24 ng of sMT1-MMP in the presence of various concentrations of recombinant endostatin in 40 μl of a MMP assay buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM $CaCl_2$, 100 μM $ZnCl_2$, and 0.025% Brij 35) at 37°C for 3 h. Inhibition of the APMA-mediated proMMP-2 activation by endostatin was examined by the same procedure as was inhibition of the sMT1-MMP-mediated proMMP-2 activation, except incubation was for 30 min and 1 mM APMA was used instead of sMT1-MMP. Processed products from the proMMP-2 were detected by gelatin zymography.

Inhibition of Catalytic Activity of MMPs by Endostatin. The catalytic activity of sMT1-MMP and MMP-2 was analyzed by peptide cleavage assay using a quenched fluorescent peptide, Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (Bachem, Torrance, CA) as a substrate (15). proMMP-2 (20 ng) was activated in the presence of a final concentration of 1 mM APMA at 37°C for 30 min. The activated MMP-2 or sMT1-MMP (18 ng) was incubated in 40 μl of the MMP assay buffer containing 1 μM of the peptide in the presence of various amounts of endostatin at 37°C for 30 min. The reaction was stopped by the addition of 0.1 M sodium acetate (pH 4.0) at final concentration. The fluorescence was measured by a KONTRON SFM 25 fluorometer at excitation wavelength 328 nm and emission 393 nm.

RESULTS AND DISCUSSION

Endostatin Inhibits Endothelial Cell Invasion. The recombinant mouse endostatin was purified from HEK293 cells stably transfected with the pFLAG-CMV-1-endostatin. The purified protein showed a single M_r 20,000 band in SDS gel electrophoresis under reducing condition. The protein was found to inhibit VEGF-induced proliferation and the migration of HUVECs but had no effect on the proliferation of HT1080 cells (data not shown), which indicated that our endostatin had intrinsic properties as described in other studies (3, 5).

To examine whether endostatin regulates endothelial cellular invasion, the ability of HUVECs to penetrate the reconstituted basement membrane Matrigel was assessed. In the absence of VEGF or PMA, endostatin slightly reduced basal invasion of HUVECs into the Matrigel (Fig. 1). Treatment of HUVECs with 5 ng/ml VEGF or 50 nM PMA resulted in ~1.6- and 1.4-fold increase in invasive activity, respectively. Interestingly, endostatin effectively blocked both VEGF- and PMA-enhanced cellular invasion in a dose-dependent manner, with near maximal inhibition at 5 μg/ml (Fig. 1*A*). The FLAG peptide DYKDDDDK (up to 10 μM) had no effect on HUVEC invasion. The inhibitory effect of endostatin was also observed in basic fibroblast growth factor-induced HUVEC invasion (data not shown). Furthermore, TIMP-2 (0.3 μg/ml), a specific inhibitor of MMPs, suppressed VEGF-induced HUVEC invasion by about 50% (Fig. 1*B*). These results indicate that endostatin inhibits invasion of endothelial cells in an agonist-independent fashion and raise the possibility that endostatin may block endothelial invasion, presumably by the inhibition of MMP activity.

Endostatin Inhibits the Activation of ProMMP-2 from the Endothelial Cell Cultures. MMPs are known to be crucial for degrading extracellular matrix components and for promoting both endothelial and tumor cellular invasion *in vitro* and *in vivo* (7, 16, 17). To examine whether the anti-invasive activity of endostatin is correlated with the inhibition of activities of gelatinolytic MMPs, we analyzed the effect of endostatin on the levels and processing of gelatinolytic MMPs in HUVECs. Gelatin zymography of the culture medium of HUVECs revealed the release of M_r 68,000 proMMP-2 and M_r 88,000 proMMP-9, although the proMMP-9 band was barely detectable (Fig. 2, *Lane 1*). PMA treatment of HUVECs resulted in marked generation

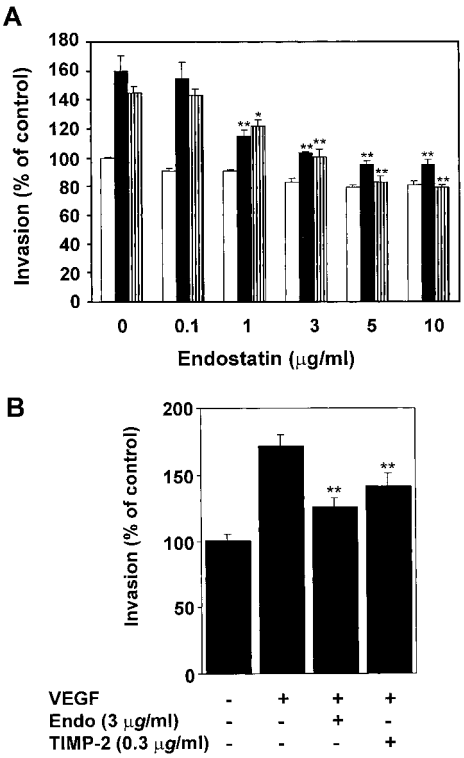

Fig. 1. Effect of endostatin on endothelial cellular invasion. In *A*, HUVECs (1×10^6 cells/ml) were added to Transwell chamber coated with Matrigel and treated with various concentrations of endostatin (0.1, 1, 3, 5, and 10 μg/ml) in the absence (*white bar*) or presence of either 5 ng/ml VEGF (*black bar*) or 50 nM PMA (*striped bar*). After 20 h, the number of invaded cells was counted, and results are expressed as percentage of control (basal invasion with no treatment). *B*, inhibition of VEGF-induced HUVEC invasion by TIMP-2 (0.3 μg/ml). Each value is the mean ± SD of three independent determinations of duplicate experiments. Using a paired Student *t* test: *, $P < 0.05$ (VEGF against the control); **, $P < 0.01$ (PMA alone against the control).

of gelatinolytic bands of M_r 62,000 accompanied by a relative decrease in M_r 68,000 proMMP-2, which is consistent with the activation of proMMP-2 (Fig. 2, *Lane 2*). When HUVECs were treated with PMA and various concentrations of endostatin, the processing of M_r 68,000 proMMP-2 to the M_r 62,000 mature MMP-2 was apparently blocked in a dose-dependent manner, with almost complete inhibition at 3 μg/ml concentration (Fig. 2*A*). In contrast, the FLAG peptide had no effect on the PMA-induced proMMP-2 activation in HUVEC up to 10 μg/ml These results indicate that activation of proMMP-2 secreted from HUVECs can be inhibited by endostatin.

To further confirm whether the inhibition of proMMP-2 activation resulted from the presence of endostatin, endostatin was removed from the purified endostatin solution using anti-FLAG antibody bead (Fig. 2*B*); we then examined activation of gelatinolytic MMPs. Apparently, the endostatin-depleted sample completely lost its inhibitory activity for proMMP-2 activation as shown by gelatin zymography and Western blot analysis (Fig. 2*C*). In addition, it was unable to block endothelial cell invasion (data not shown). We thus confirmed that endostatin inhibited proMMP-2 activation in HUVECs.

Endostatin Inhibits Tumor Cellular Invasion. It has been shown that metastatic tumor cell lines express higher levels of gelatinolytic MMPs than do nonmetastatic counterparts. MMP-2 and MMP-9 are implicated to be most closely correlated with metastatic potential (16, 18–19). Thus, we studied the effect of endostatin on tumor cellular invasion. When the increasing concentrations of endostatin were added into the Matrigel culture of highly metastatic HT1080 cells, endostatin markedly inhibited the invasion of HT1080 cells in a dose-dependent manner, up to 30% at 10 μg/ml of endostatin (Fig. 3).

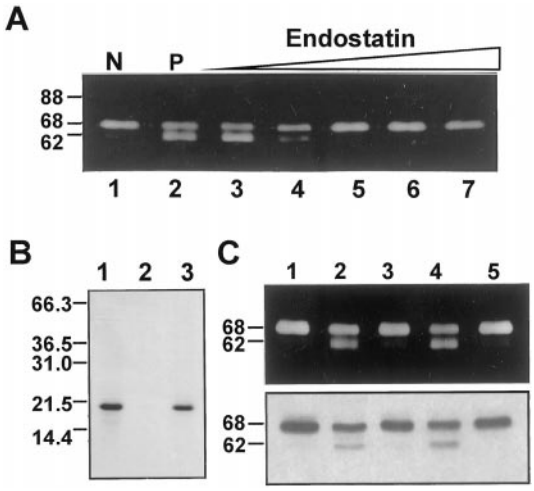

Fig. 2. Inhibition of proMMP-2 activation by endostatin in the culture medium of HUVECs. In *A*, HUVECs were incubated in serum-free conditioned medium for 24 h in the absence or presence of 50 nM PMA and/or various concentrations of endostatin. Then, the culture medium was collected and analyzed by gelatin zymography. *Lane 1*, no treatment (*N*); *Lane 2*, treatment with PMA alone (*P*); *Lanes 3–7*, treatment with PMA with endostatin (0.1, 1, 3, 5, and 10 μg/ml, respectively). In *B*, 200 μl of purified endostatin solution (0.1 μg/ml) were incubated with 100 μl of anti-FLAG antibody-conjugated beads (1 mg/ml) or protein A-Sepharose (3 mg/ml) for 24 h at 4°C on a rocking platform. Ten μl of each supernatant were applied to 15% SDS-PAGE, and the gel was stained with Coomassie Blue. *Lane 1*, the purified endostatin solution; *Lane 2*, the endostatin-depleted solution; *Lane 3*, protein A-Sepharose-treated solution. In *C*, HUVECs were incubated for 24 h in the absence (*Lane 1*), or presence (*Lane 2*), of 50 nM PMA alone, and PMA (50 nM) plus 30 μl of each sample described in *B* [*Lanes 3–5*, (*Lanes 1–3* of *B*)]. The culture medium was analyzed by gelatin zymography (*upper panel*) and by Western blot using anti-MMP-2 antibody (*lower panel*). *On the left*, M_r in thousands.

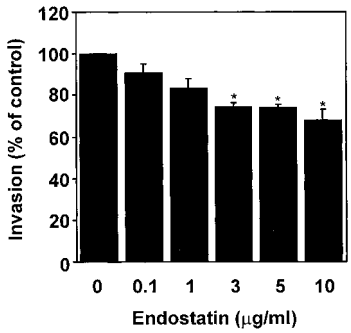

Fig. 3. Inhibition of tumor cellular invasion by endostatin. HT1080 cells (1×10^6 cells/ml) were added to Transwell chamber coated with Matrigel and treated with various concentrations of endostatin (0.1, 1, 3, 5, and 10 μg/ml). The number of invaded cells was counted, and results are expressed as percentage of control (basal invasion in the absence of endostatin). Each value is the mean ± SD of three independent determinations of duplicate experiments. *, $P < 0.05$, when tested against the control using a paired Student *t* test.

Consistently, inhibition of proMMP-2 activation in the culture medium of HT1080 cells was observed by gelatin zymography (data not shown). These results suggest that endostatin could reduce invasion of tumor cells as well as of HUVECs, at least in part, by reducing amounts of active MMP-2 species.

Endostatin Inhibits the Catalytic Activities of MT1-MMP and MMP-2. Because MT1-MMP is known to be an activator of proMMP-2 *in vivo* (20), we examined the effect of endostatin on activation of proMMP-2 by transmembrane-deleted sMT1-MMP *in vitro*. When proMMP-2 was incubated with sMT1-MMP in the absence of endostatin, the proMMP-2 was activated into the M_r 64,000 intermediate form (Fig. 4*A*, *Lane 2*). In the presence of endostatin, activation of proMMP-2 by sMT1-MMP was inhibited by endostatin in a dose-dependent manner (Fig. 4*A*). To examine whether endostatin also inhibits activation of proMMP-2 independent of MT1-MMP, the APMA-mediated proMMP-2 activation was analyzed in the absence or presence of endostatin. Endostatin also inhibited the APMA-mediated proMMP-2 activation in a dose-dependent manner (Fig. 4*B*). Therefore, we conclude that endostatin inhibits the activation of proMMP-2 that is induced by both sMT1-MMP and APMA.

To examine how endostatin inhibits proMMP-2 activation that is mediated by sMT1-MMP and APMA, we have analyzed whether endostatin inhibits catalytic activities of these MMPs. The catalytic activities of both sMT1-MMP and MMP-2 were inhibited by endostatin (Fig. 4*C*). The IC_{50}s of the sMT1-MMP and MMP-2 by endostatin were 2.0 and 0.82 μg/ml, respectively, in a condition that the IC_{50}s by TIMP-2 were 86 and 56 ng/ml, respectively. However, the FLAG peptide had no effect on the catalytic activities of MMP-2 and sMT1-MMP. In addition, the catalytic activity of MMP-9 was also inhibited by endostatin, although the IC_{50} of MMP-9 (4.71 μg/ml) was higher than the IC_{50}s of MMP-2 and sMT1-MMP (data not shown). These results demonstrate that endostatin is an inhibitor of both sMT1-MMP and MMP-2, although the inhibitory activities of endostatin for sMT1-MMP and MMP-2 are approximately 23- and 15-fold weaker than TIMP-2, respectively. Inhibition of the catalytic activities of MT1-MMP and MMP-2 by endostatin explains the inhibition of proMMP-2 activation induced by MT1-MMP and APMA, respectively.

TIMP-2 is known as an endogenous inhibitor of MMP-2 and MT1-MMP (21), and it also inhibits proMMP-2 activation induced by APMA (13) and MT1-MMP (21). In addition, it was reported that

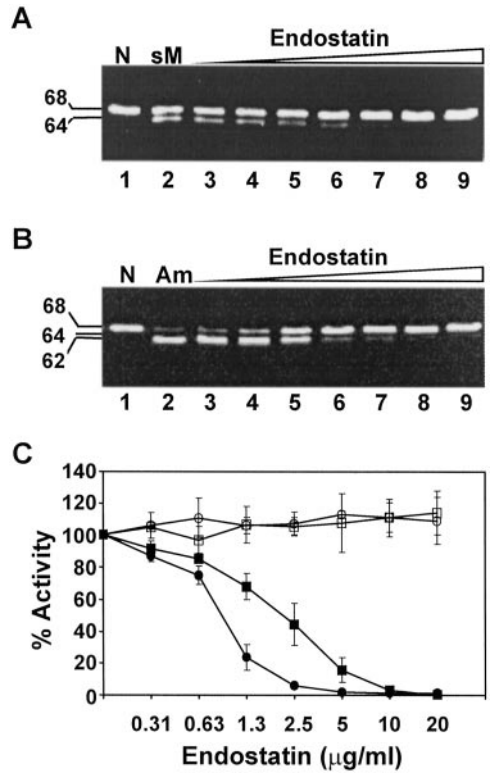

Fig. 4. Inhibition of proMMP-2 activation and the catalytic activities of sMT1-MMP and MMP-2 by endostatin. The recombinant proMMP-2 with various amounts of endostatin (*Lanes 2–9*; 0, 0.31, 0.63, 1.3, 2.5, 5.0, 10, and 20 μg/ml, respectively) was incubated in the presence of either sMT1-MMP (*A*) or APMA (*B*). The reaction products were analyzed by gelatin zymography. *Lane 1* of *A* and *B*, the recombinant proMMP-2 as a control (*N*); *Lane 2* of *A*, sMT1-MMP only (*sM*); *Lane 2* of *B*, APMA only (*Am*). *Numbers on the left*, M_r in thousands. In *C*, the catalytic activity of either sMT1-MMP (□, ■) or the activated MMP-2 (○, ●) was measured by fluorogenic peptide cleavage assay, in the presence of the indicated amounts of endostatin (■, ●) or FLAG peptide (○, □). % *activity*, the catalytic activity of each MMP in the presence of endostatin divided by the catalytic activity in the absence of endostatin. The % activity of each MMP *versus* the amount of endostatin or FLAG peptide was plotted (*C*). Each value is the mean ± SD of three independent determinations of duplicate experiments.

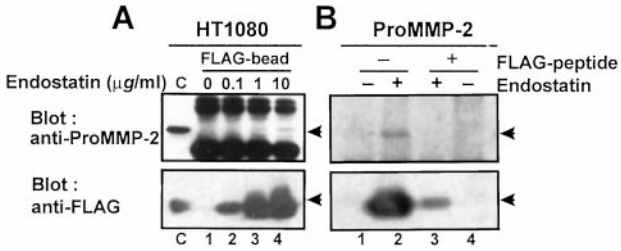

Fig. 5. Interaction of endostatin with proMMP-2. In *A*, the culture medium of HT1080 cells containing ~1 μg/ml proMMP-2 was incubated with various amounts of endostatin as indicated (*Lanes 1–4*; 0, 0.1, 1, and 10 μg, respectively) for 2 h at 4°C. The resulting solution was incubated with anti-FLAG antibody-conjugated beads (25 μl) for an additional 1 h and was precipitated. The precipitates were analyzed by Western blotting using anti-MMP-2 antibody (*upper panel*) and anti-FLAG antibody (*lower panel*), respectively. *Lane C*, 30 μl of HT1080 culture medium (*upper panel*) and 10 ng of FLAG-tagged endostatin (*lower panel*). In *B*, the purified proMMP-2 (250 ng) was incubated with PBS, 1 μg of FLAG-tagged endostatin, 1 μg of FLAG-tagged endostatin plus 10 μg of FLAG peptide, or 10 μg of FLAG peptide alone, in a total volume of 400 μl for 2 h at 4°C. The endostatin was incubated with anti-FLAG antibody-conjugated beads (25 μl) for an additional 1 h. The precipitates were analyzed as described in *A*. *A* and *B*, *arrowheads*, the positions of proMMP-2 (*upper panels*) and endostatin (*lower panels*).

TIMP-2 can inhibit tumor growth, invasion, and metastasis in experimental models (21). In this regard, the effects of endostatin on the inhibition of cellular invasion and MMP inhibitory activities seem very similar to the effects of TIMP-2. However, TIMP-2 was not detectable in the purified endostatin by Western blot analysis using anti-TIMP-2 antibody. We, thus, believe that these novel characteristics of endostatin are not a result of TIMP-2 contamination in the purified endostatin. In addition, because the FLAG peptide did not affect HUVEC invasion and the catalytic activities of MMP-2 and sMT1-MMP, the effects of endostatin are also not a result of the FLAG epitope present in the NH_2 terminus of the recombinant endostatin.

Endostatin Binds to ProMMP-2. Because endostatin inhibited the activation of proMMP-2 by both MT1-MMP and APMA, it is suggested that endostatin may form a stable complex with proMMP-2. To confirm this possibility, we attempted to examine whether exogenously added endostatin could pull down proMMP-2 from the serum-free culture medium of HT1080 cells, which contains approximately 1 μg/ml proMMP-2. As shown in Fig. 5*A* (*upper panel*), proMMP-2 was coprecipitated with exogenously added endostatin in a dose-dependent manner. The levels of endostatin in the precipitates were correlated with those of proMMP-2 (Fig. 5*A*, *lower panel*). To further confirm the direct interaction between these two proteins, purified FLAG-endostatin and proMMP-2 were incubated and immunoprecipitated with anti-FLAG antibody. ProMMP-2 was coprecipitated only in the presence of the FLAG-tagged endostatin as shown in Fig. 5B (*upper panel*, *Lane 2*). This coprecipitation disappeared with competition from the FLAG peptide (Fig. 5*B*, *Lane 3*), which demonstrates the specificity of the coprecipitation assay. These results, thus, indicate that endostatin directly binds proMMP-2, which suggests that such interaction would be important for the inhibition of proMMP-2 activation by MT1-MMP or autocatalytic mechanism. Our results suggest two molecular mechanisms of endostatin action on the activity of MMP-2: (*a*) endostatin inhibits the activation of proMMP-2 by the direct interaction with proMMP-2; and (*b*) endostatin directly inhibits the catalytic activities of the active form of MMP-2 and sMT1-MMP. The latter mechanism is not yet fully understood in the present study and is under investigation.

In conclusion, the data presented here demonstrate that endostatin potently inhibits both the extracellular activation of proMMP-2 by inhibition of MT1-MMP and the catalytic activity of MMP-2 and, thereby, can block the invasiveness of endothelial cells and tumor cells. These findings provide, in part, the mechanism of action of exogenous endostatin associated with its antiangiogenic and antitumor properties.

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