

Inhibition of vascular endothelial growth factor signaling by endostatin

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Angiogenesis, the formation of new capillaries from the preexisting blood vessels, is critical for tumor growth and metastasis. Endostatin, a 20-kDa proteolytic fragment of collagen XVIII, was discovered as a potent inhibitor of angiogenesis originally from murine hemangioendothelioma cell medium. Subsequently, the recombinant endostatin was shown to inhibit tumor growth and metastasis in various animal models. Furthermore, repeated cycles of endostatin therapy resulted in prolonged tumor dormancy without resistance to endostatin. On the cellular level, endostatin has been shown to specifically block proliferation and migration of endothelial cells, and induce endothelial cell apoptosis. However, the molecular mechanisms of endostatin-mediated anti-angiogenesis and tumor regression are not yet clear.

Vascular endothelial growth factor (VEGF), a potent mitogen for endothelial cells, is an important mediator of angiogenesis, that is involved in the differentiation of endothelial cells and the development of the vascular system. In particular, it is thought that VEGF is the most important angiogenic factor closely associated with induction and maintenance of the neovasculature in human tumors. The increased expression of VEGF mRNA has been detected in a variety of tumors and recently tumor VEGF level was recognized as an important prognostic marker of tumor angiogenesis. VEGF exerts its effects through binding to its two receptor tyrosine kinases, KDR/Flk-1 and Flt-1, expressed on endothelial cells. KDR/Flk-1 is mainly related to the mitogenic and chemotactic responses, whereas Flt-1 is required for endothelial cell morphogenesis. Recent studies have identified the VEGF-induced signaling events in endothelial cells including extracellular signal-regulated kinases (ERKs), p38 mitogen-activated protein kinase (p38 MAPK), and p125 focal adhesion kinase (p125^{FAK}). In addition, endothelial nitric oxide synthase is considered to be an essential mediator of VEGF-induced angiogenesis.

Previous studies have reported that endostatin inhibits endothelial cell proliferation, migration, and angiogenesis in response to VEGF, but its mechanism of action is not clearly delineated. In this study, we

present several novel observations. First, endostatin blocks the VEGF-induced tyrosine phosphorylation of KDR/Flk-1 in endothelial cells. Second, endostatin suppresses the VEGF-induced activation of ERK, p38 MAPK, and p125^{FAK} that are downstream events of the KDR/Flk-1 signaling and are involved in the mitogenic and motogenic activities of VEGF in endothelial cells. Third, endostatin inhibits the binding of VEGF to endothelial cells and to its cell surface receptor, KDR/Flk-1. Fourth, endostatin directly binds to KDR/Flk-1 but not to VEGF. Fifth, endostatin internalized into endothelial cells by its binding to the VEGF receptors. Sixth, anti-endostatin monoclonal antibody neutralizing interaction between endostatin and KDR/Flk-1 was able to block internalization of endostatin into endothelial cells. The epitope of endostatin-neutralizing antibody is located in the central region (residues 70-100) of endostatin. Our findings clearly indicate that direct interaction of endostatin with the VEGF receptor KDR/Flk-1 blocks the binding of VEGF to its receptor, the VEGF-induced signaling of KDR/Flk-1 itself and its downstream signalings such as ERK, p38 MAPK, and p125^{FAK}, resulting in the inhibition of VEGF-induced proliferation and migration of endothelial cells.

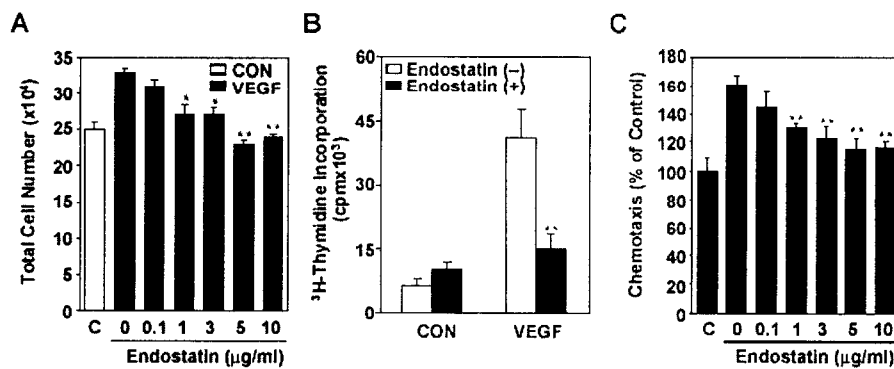


Fig.1. Endostatin inhibits VEGF-induced proliferation and migration of HUVECs. (A) HUVECs were pretreated for 30 min with various concentrations of endostatin before exposure to VEGF₁₆₅ (10 ng/ml). After incubation for 48 h, total cell number was counted under a microscope. (B) Cultured HUVECs were stimulated with 10 ng/ml VEGF₁₆₅ in the absence or presence of 10 µg/ml endostatin and allowed to proliferate for 36 h. ³H-thymidine incorporation was measured during the last 6 h of incubation. (C) Various concentrations of endostatin (0.1, 1, 3, 5, and 10 µg/ml) were pretreated for 30 min prior to treatment of 10 ng/ml VEGF. After a 4 h incubation, chemotaxis was quantified by counting the cells that migrated to the lower side of the filter with optical microscopy at x200 magnification. The basal migration in the absence of VEGF was 65 ± 3 cells/field. Each sample was assayed in duplicate, and

the assays were repeated twice. *, $P < 0.05$; **, $P < 0.01$ vs. VEGF.

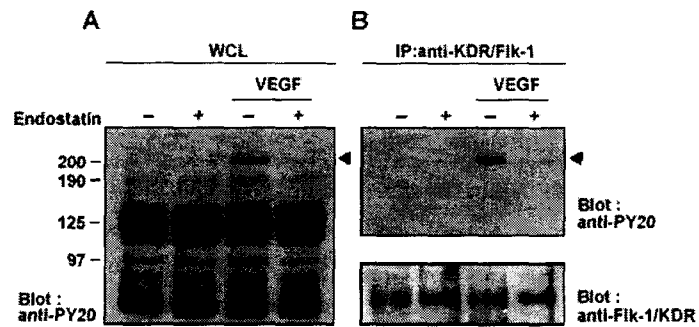


Fig.2. Endostatin inhibits VEGF-stimulated KDR/Flk-1 phosphorylation in HUVECs. HUVECs were pretreated with 10 $\mu\text{g/ml}$ of endostatin for 30 min, and stimulated with 10 ng/ml of VEGF₁₆₅ for 10 min. Whole cell lysates (A) or anti-KDR/Flk-1 immunoprecipitates (B) were analyzed by SDS-PAGE. Immunoblot analysis was performed with anti-phosphotyrosine antibody (A and upper panel of B). Blot was reprobated with anti-KDR/Flk-1 antibody (lower panel of B). Arrow indicates tyrosine-phosphorylated protein with molecular weight of 205 kDa.

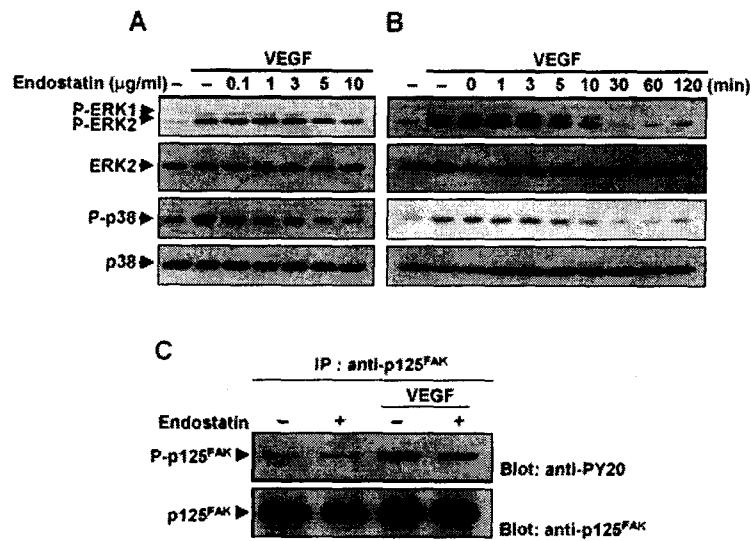


Fig.3. Endostatin blocks VEGF₁₆₅-induced MAP kinase and p125^{FAK} activation. (A) Endostatin inhibits VEGF₁₆₅-induced ERK and p38 MAPK activation in a dose-dependent manner. HUVECs were pretreated with various concentrations of endostatin for 30 min, and then stimulated with 10 ng/ml of VEGF₁₆₅ for 10 min. (B) The preincubation effect of endostatin on the inhibition of VEGF-induced ERK and p38 MAPK activation. HUVECs were pretreated with 10 µg/ml of endostatin for the indicated time periods and then stimulated with 10 ng/ml of VEGF₁₆₅ for 10 min. In A and B, activation of ERK and p38 MAPK by VEGF was determined by Western blotting using antibodies against phosphorylated forms of ERKs (P-ERK1 and P-ERK2) and p38 MAPK (P-p38). The membranes were stripped and re probed with antibody against p38 MAPK. (C) Endostatin inhibits VEGF-induced tyrosine phosphorylation of p125^{FAK}. HUVECs were pretreated with 10 µg/ml of endostatin for 30 min, and then stimulated with 10 ng/ml of VEGF for 10 min. Cell lysates were immunoprecipitated with anti-p125^{FAK} antibody. The precipitates were analyzed by immunoblotting with anti-phosphotyrosine antibody to assay for p125^{FAK} tyrosine phosphorylation (P-p125^{FAK}) or anti-p125^{FAK} antibody for p125^{FAK} protein levels (p125^{FAK}).

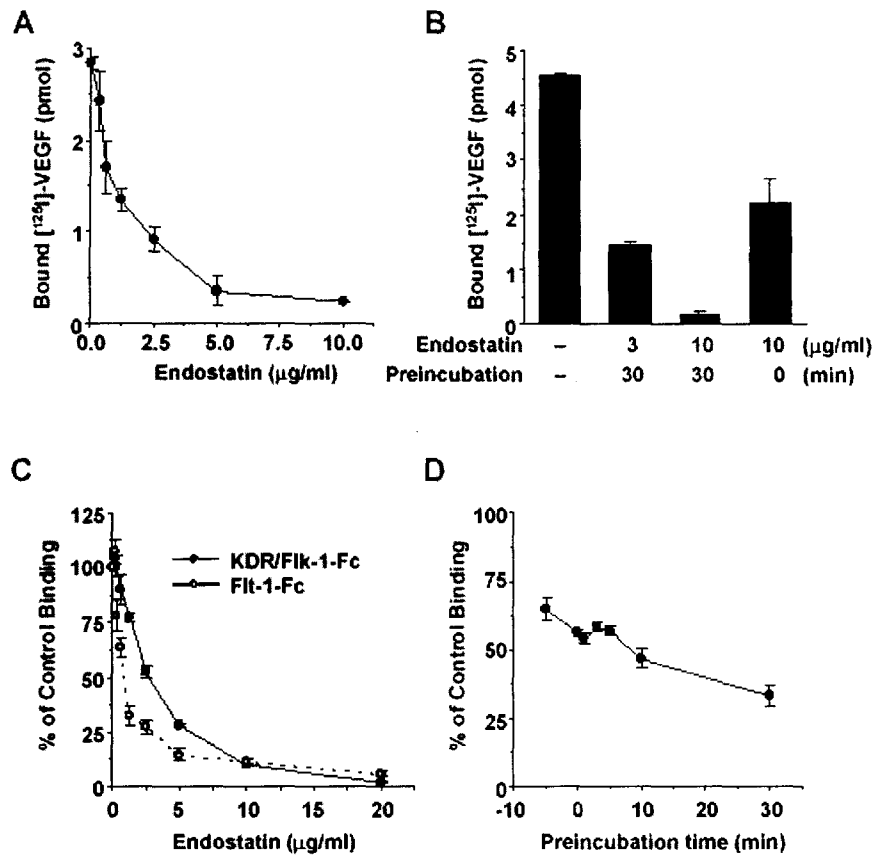


Fig.4. Endostatin blocks the binding of VEGF₁₆₅ to its receptors. (A) Endostatin inhibits the binding of KDR/Flk-1-Fc and Flt-1-Fc to immobilized VEGF₁₆₅ in a dose-dependent manner. Various concentrations of endostatin were preincubated with 25 ng/ml of KDR/Flk-1-Fc or Flt-1-Fc for 10 min, and then the mixtures were added to the VEGF₁₆₅-coated wells for 2 h. The amount of bound KDR/Flk-1-Fc or Flt-1-Fc was determined with anti-human IgG-HRP using a chemiluminescent substrate. (B) Inhibitory activity of endostatin on the VEGF and KDR/Flk-1 interaction depends on the preincubation time of endostatin with KDR/Flk-1. Endostatin (5 $\mu\text{g/ml}$) and KDR/Flk-1-Fc (25 ng/ml) were preincubated for various time periods and the mixtures were added to the VEGF₁₆₅-coated wells. The amount of bound KDR/Flk-1-Fc was determined as described in (A).

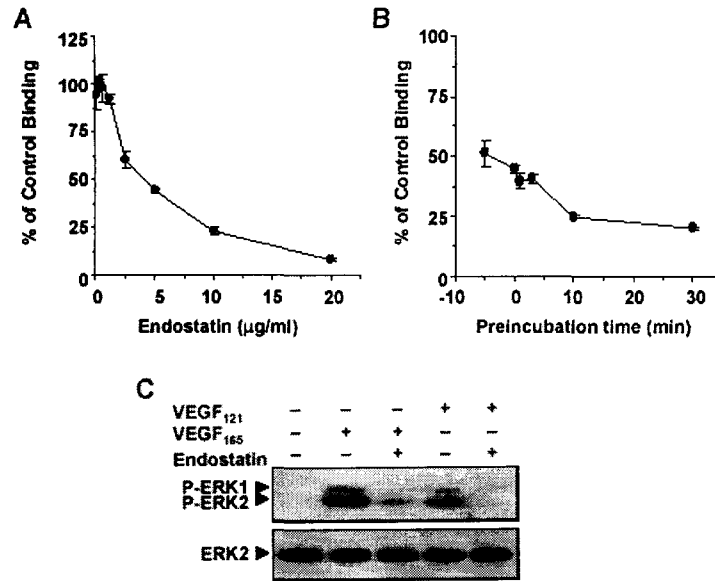


Fig.5. Endostatin blocks the binding of VEGF₁₂₁ to KDR/Fik-1 and VEGF₁₂₁-stimulated ERK activation. (A) Endostatin inhibits the binding of KDR/Fik-1-Fc to immobilized VEGF₁₂₁ in a dose-dependent manner. (B) Inhibitory activity of endostatin on the VEGF₁₂₁ and KDR/Fik-1 interaction depends on the preincubation time of endostatin with KDR/Fik-1. (C) Endostatin blocks VEGF₁₂₁-induced ERK activation. Experiments were carried out as described in Fig. 3 and Fig. 2.

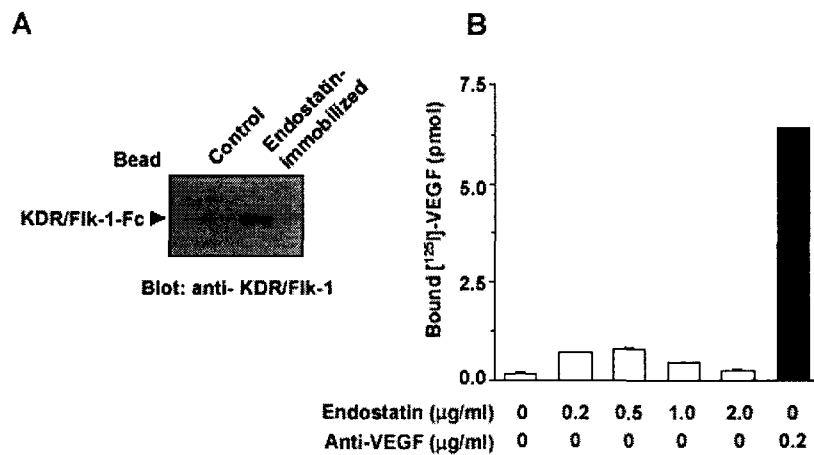


Fig.6. Endostatin binds to KDR/Fik-1 but not to VEGF. (A) Endostatin binds to KDR/Fik-1.

KDR/Flk-1-Fc was incubated with control or endostatin-immobilized beads. After bound materials were eluted, samples were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes and KDR/Flk-1-Fc was detected using anti-human IgG-HRP. (B) Endostatin does not bind to VEGF. ¹²⁵I-VEGF was added to the wells of ELISA plate coated with various amounts of endostatin. After 2 h incubation, the bound radioactivity was determined. The wells coated with anti-VEGF antibody were used as a positive control.

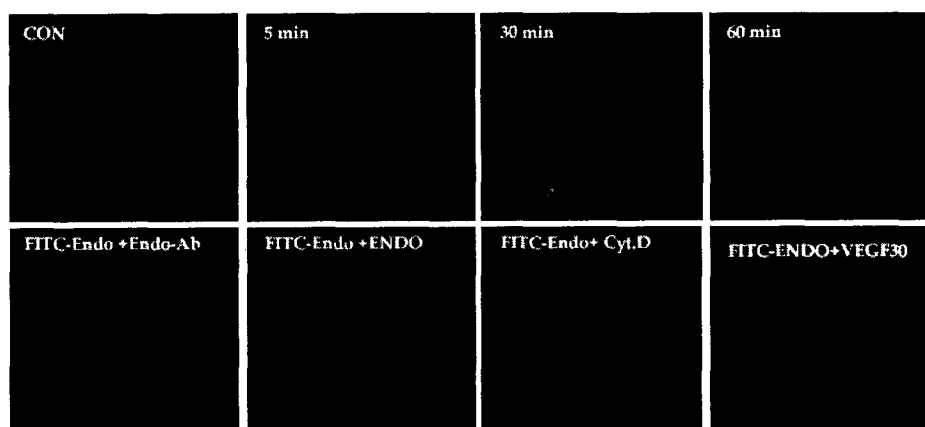


Fig.7. Internalization of endostatin into endothelial cells. HUVECs were incubated in the absence (CON) or presence of 10 μ g/ml FITC-conjugated endostatin for the indicated periods (upper panel). HUVECs were pretreated with endostatin neutralizing antibody, 50 μ g/ml unlabeled endostatin, cytochalasin D, and VEGF prior to addition of 10 μ g/ml FITC-conjugated endostatin (lower panel).

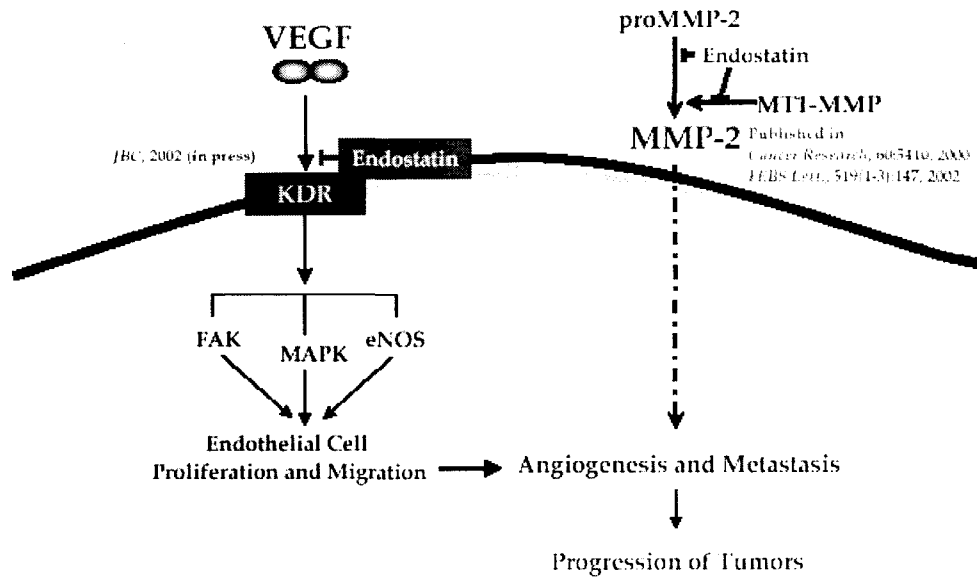


Fig.8. Proposed mechanism of anti-angiogenic and anti-tumor activities of endostatin. We previously reported that endostatin inhibits endothelial and tumor cellular invasion by blocking the activation and catalytic activity of matrix metalloproteinase-2 (MMP-2) (Can Res, 2000).

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