Endostatin Blocks Vascular Endothelial Growth Factor-mediated Signaling via Direct Interaction with KDR/Flk-1*

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Endostatin, a fragment of collagen XVIII, is a potent anti-angiogenic protein, but the molecular mechanism of its action is not yet clear. We examined the effects of endostatin on the biological and biochemical activities of vascular endothelial growth factor (VEGF). Endostatin blocked VEGF-induced tyrosine phosphorylation of KDR/Flk-1 and activation of ERK, p38 MAPK, and p125 FAK in human umbilical vein endothelial cells. Endostatin also inhibited the binding of VEGF165 to both endothelial cells and purified extracellular domain of KDR/Flk-1. Moreover, the binding of VEGF121 to KDR/Flk-1 and VEGF121-stimulated ERK activation were blocked by endostatin. The direct interaction between endostatin and KDR/Flk-1 was confirmed by affinity chromatography. However, endostatin did not bind to VEGF. Our findings suggest that a direct interaction of endostatin with KDR/Flk-1 may be involved in the inhibitory function of endostatin toward VEGF actions and responsible for its potent anti-angiogenic and anti-tumor activities in vivo.

Angiogenesis, the formation of new capillaries from the pre-existing blood vessels, is critical for tumor growth and metastasis (1–5). Endostatin, a 20-kDa proteolytic fragment of collagen XVIII, was discovered as a potent inhibitor of angiogenesis originally from murine hemangioendothelioma cell medium (4). Subsequently, the recombinant endostatin was shown to inhibit tumor growth and metastasis in various animal models (4). Furthermore, repeated cycles of endostatin therapy resulted in prolonged tumor dormancy without resistance to endostatin (5). On the cellular level, endostatin has been shown specifically to block proliferation and migration of endothelial cells and to induce endothelial cell apoptosis (4, 6, 7). 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 and is involved in the differentiation of endothelial cells and the development of the vascular system (8–10). In particular, it is thought that VEGF is the most important angiogenic factor closely associated with induction and maintenance of the neovascularization 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 (10, 11). 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 related mainly to the mitogenic and chemotactic responses, whereas Flt-1 is required for endothelial cell morphogenesis (12, 13). Recent studies (14–16) 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 FAK. In addition, endothelial nitric-oxide synthase is considered an essential mediator of VEGF-induced angiogenesis (17, 18).

Previous studies have reported that endostatin inhibits endothelial cell proliferation, migration, and angiogenesis in response to VEGF (6, 19), but its mechanism of action is not clearly delineated. Here we report that endostatin blocks VEGF-induced tyrosine phosphorylation of KDR/Flk-1 and activation of ERK, p38 MAPK, and p125 FAK, which are downstream events of KDR/Flk-1 signaling and are involved in the mitogenic and motogenic activities of VEGF in endothelial cells. Furthermore, we demonstrated that endostatin inhibits the binding of VEGF to endothelial cells and to its cell surface receptor, KDR/Flk-1. The binding partner for endostatin is KDR/Flk-1 but not VEGF. Our results suggest that direct interaction of endostatin with KDR/Flk-1 blocks the binding of VEGF to endothelial cells and the VEGF-induced signaling of KDR/Flk-1 itself and its downstream signaling events, resulting in the inhibition of VEGF-induced endothelial cell proliferation and migration.

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1 The abbreviations used are: VEGF, vascular endothelial growth factor; KDR, kinase insert domain-containing receptor; Flk-1, fetal liver kinase 1; Flt-1, fms-like tyrosine kinase 1; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; FAK, focal adhesion kinase; bFGF, basic fibroblast growth factor; HUVECs, human umbilical vein endothelial cells; PBS, fetal bovine serum; HRP, horseradish peroxidase; CMV, cytomegalovirus; PBS, phosphate-buffered saline; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; MMP, matrix metalloproteinase.
Experimental Procedures

Materials—VEGF165, VEGF121, basic fibroblast growth factor (bFGF), protein A-agarose, and antibody to p125FAK were from Upstate Biotechnology (Lake Placid, NY). Tissue culture dishes and plasticware were obtained from Falcon Products. M199 and heparin were purchased from Invitrogen. KDR/Flik-1-Fc, Flt-1-Fc, and antibody for VEGF were from R&D Systems (Minneapolis, MN). Anti-KDR/Flik-1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine antibody was from Transduction Laboratories (Lexington, KY). Antibodies for phospho-specific ERK (Thr-202/Tyr-204) and ERK2 were obtained from New England Biolabs (Beverly, MA). Antibodies for human IgG-HRP and mouse IgG-HRP were from Pierce. (3H)Thymidine, 125I-VEGF165, NHS-activated Sepharose, and the chemiluminescent substrate for horseradish peroxidase were from Amersham Biosciences. All other reagents were purchased from Sigma unless indicated otherwise.

Cell Culture—Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord veins by collagenase treatment as described previously (20), and only passages 2–6 were used. The cells were grown in M199 supplemented with 20% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, 3 ng/ml bFGF, and 5 μg/ml heparin at 37 °C under a humidified 5% CO 2 atmosphere before seeding. One hundred cells/well onto gelatin-coated 24-well plates. After 24 h, the medium was replaced with M199 containing 1% FBS with or without various amounts of VEGF. After 6 h, the medium was replaced with M199 containing 1% FBS and cells were stimulated by the addition of VEGF (10 ng/ml). After stimulation, cells were lysed in 1 ml of lysis buffer (20 mM Tris/HCl, pH 8.0, 2 mM EDTA, 137 mM NaCl, 1 mM Na 2 VO 3, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, and 1% Triton X-100). Lysates were clarified by centrifugation at 15,000 × g for 10 min, and the resulting supernatants were immunoprecipitated with either rabbit anti-Flik-1 or anti-FAK antibody at 4 °C for 3 h followed by the addition of protein A-agarose beads at 4 °C for 1 h. Immunoprecipitates were washed three times with lysis buffer, solubilized in SDS-PAGE sample buffer containing β-mercaptoethanol, and further analyzed by Western blotting.

Western Blotting—Cell lysates or immunoprecipitates from HUVECs were run in SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The blocked membranes were then incubated with the indicated antibodies, and the immunoreactive bands were visualized using a chemiluminescent substrate.

Endothelial Cell Migration Assay—The counting assay was performed as described previously (19). Briefly, HUVECs were seeded at a density of 2 × 10 5 cells/well in gelatin-coated 24-well plates. After 24 h, cells were washed twice with M199 and incubated for 6 h in M199 containing 1% FBS. Cells were stimulated by the addition of 10 ng/ml VEGF for 30 h, followed by the addition of 1 μCi/ml of (3H)Thymidine for 6 h. High molecular weight DNAs were precipitated using 5% trichloroacetic acid at 4 °C for 30 min. After two washes with ice-cold H 2 O, 3H radioactivity was solubilized in 0.2 n NaOH, 0.1% SDS and determined by scintillation counter. Each sample was assayed in duplicate, and statistical analysis was performed using Student’s t test based on comparisons with control samples tested at the same time.

Results

Endostatin Inhibits VEGF 165-induced Tyrosine Phosphorylation of KDR/Flik-1 in HUVECs—According to the previous reports (6, 19), endostatin inhibits VEGF-induced endothelial cell proliferation, migration, and angiogenesis, but its mechanism is not clear. To investigate the molecular mechanisms of endostatin associated with its anti-angiogenic activities, the recombinant mouse endostatin was expressed and purified from HEK293 cells stably transfected with the pFLAG-CMV-1-endostatin as described previously (21). After dialysis against PBS, the purity of recombinant mouse endostatin as checked by SDS-PAGE was as high as 97%. The binding of 125I-VEGF to HUVECs was performed as described previously (20), and assay was repeated twice. (3H)Thymidine incorporation assay was conducted in a 96-well plate. The cells were washed and blocked with 3% BSA in M199 for 2 h. After 10 min of preincubation of KDR/Flik-1-Fc (25 ng/ml) in M199 containing 25 μM HEPES/NaOH, pH 7.4, and 0.1% BSA with or without various amount of endostatin, the (3H)Thymidine incorporation assay was repeated twice. The counting assay was performed on ELISA plates overnight at 4 °C. The wells were washed and blocked with M199 containing 3% BSA at room temperature for 2 h. 125I-VEGF 165 (0.125 nM) was added to each well. The binding was allowed to proceed at room temperature for 2 h. The wells were washed three times with 400 μl PBS. Bound materials were eluted by boiling with 50 μl of reducing SDS-loading dye. Samples (25 μl) were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane, and KDR/Flik-1-Fc was detected using anti-human IgG-HRP.

Binding of 125I-VEGF to Immobilized Endostatin—Various amounts of endostatin in 100 μl of PBS were coated on ELISA plates overnight at 4 °C. The wells were washed and blocked with M199 containing 3% BSA at room temperature for 2 h. 125I-VEGF 165 (0.125 nM) was added to each well. The binding was allowed to proceed at room temperature for 2 h. The wells were washed three times with PBST. Bound 125I-VEGF 165 were removed by the addition of 150 μl of 0.1 n NaOH at room temperature for 30 min, and the radioactivity was determined in a γ-counter. Each sample was assayed in triplicate, and the assays were repeated twice.

Statistical Analysis—The p values were calculated from Student’s t test based on comparisons with control samples tested at the same time.

Results

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Binding of KDR/Flik-1-Fc to the Immobilized VEGF—VEGF 165, and VEGF 121 (80 ng/well) in 100 μl of PBS were immobilized to 96-well plates. The wells were washed and blocked with 3% BSA in M199 for 2 h. After 10 min of preincubation of KDR/Flik-1-Fc (25 ng/ml) in M199 containing 25 μM HEPES/NaOH, pH 7.4, and 0.1% BSA with or without various amount of endostatin, the (3H)Thymidine incorporation assay was repeated twice. The counting assay was performed on ELISA plates overnight at 4 °C. The wells were washed and blocked with M199 containing 3% BSA at room temperature for 2 h. 125I-VEGF 165 (0.125 nM) was added to each well. The binding was allowed to proceed at room temperature for 2 h. The wells were washed three times with PBST. Bound 125I-VEGF 165 were removed by the addition of 150 μl of 0.1 n NaOH at room temperature for 30 min, and the radioactivity was determined in a γ-counter. Each sample was assayed in triplicate, and the assays were repeated twice.

Statistical Analysis—The p values were calculated from Student’s t test based on comparisons with control samples tested at the same time.
that endostatin used in this study had the intrinsic properties described in other studies (6, 24).

Because previous studies have shown that VEGF induces proliferation and migration through activation of its cell surface receptor, KDR/Flk-1 (10, 17), we investigated the effects of endostatin on VEGF-induced KDR/Flk-1 phosphorylation. When HUVECs were stimulated with 10 ng/ml VEGF165 for 5 min, a protein migrating at 205 kDa was strongly tyrosine-phosphorylated (Fig. 2A). Preincubation of HUVECs with 10 μg/ml endostatin (30 min) prior to VEGF165 stimulation dramatically reduced VEGF-induced tyrosine phosphorylation of this protein. To determine whether the VEGF-induced tyrosine-phosphorylated protein is KDR/Flk-1, tyrosine phosphorylation of KDR/Flk-1 was analyzed by immunoprecipitation with anti-KDR/Flk-1 and then immunoblotting with anti-phos-phototyrosine antibody. As shown Fig. 2B, VEGF165 induced tyrosine phosphorylation of KDR/Flk-1; this phosphorylation was blocked by endostatin pretreatment. These results indicate that endostatin blocks VEGF-induced KDR/Flk-1 activation in endothelial cells.

**Endostatin Inhibits VEGF165-stimulated ERK, p38 MAPK, and p125FAK Activation**—To further determine the inhibitory effect of endostatin on VEGF-induced KDR/Flk-1 activation, we investigated the effect of endostatin on the intracellular signaling pathways induced by VEGF. Because previous studies have demonstrated that VEGF induces a rapid activation of ERK and p38 MAPK, which are downstream signaling molecules of KDR/Flk-1 (14, 15), the effects of endostatin on VEGF-induced ERK and p38 MAPK activation were assessed. Subconfluent HUVECs were preincubated with various concentrations of endostatin for 30 min and then stimulated with 10 ng/ml of VEGF165 for 10 min. Fig. 3A shows that VEGF stimulated the phosphorylation of ERK and p38 MAPK, and endostatin inhibited VEGF-induced ERK and p38 MAPK activation in a dose-dependent manner. Pretreatment of endothelial cells with endostatin (10 μg/ml) for various times prior to the addition of VEGF165 (10 ng/ml) showed that endostatin almost completely blocked VEGF-induced ERK and p38 MAPK phosphorylation after 30 min of preincubation (Fig. 3B). When HUVECs were treated simultaneously with endostatin and VEGF165, the inhibitory activity was greatly reduced.

To confirm the inhibitory effect of endostatin on KDR/Flk-1 signaling, we investigated whether endostatin could block p125FAK tyrosine phosphorylation, which lies downstream of the KDR/Flk-1 receptor (15). Preincubation of HUVECs with endostatin (30 min) caused a marked decrease in p125FAK tyrosine phosphorylation induced by VEGF165 (Fig. 3C). These results suggest that endostatin can block multiple signaling events of VEGF in endothelial cells, presumably via VEGF receptor inactivation.

**Endostatin Blocks the Binding of VEGF165 to Its Receptors**—To determine the mechanism by which endostatin blocks VEGF-mediated KDR/Flk-1 activation, we investigated the effect of endostatin on the binding of VEGF165 to HUVECs. Fig. 4A shows that pretreatment of endostatin (30 min) prior to the addition of 125I-VEGF165 blocked the binding of VEGF to HUVECs in a dose-dependent manner (IC50 ~ 1.25 μg/ml). When endothelial cells were preincubated with endostatin for 30 min, the specific binding of 125I-VEGF165 (0.125 nM) was reduced to 3.7% by the presence of 10 μg/ml of endostatin (Fig. 4B). The inhibitory activity of endostatin (10 μg/ml) was markedly reduced without preincubation (52.0%). These results suggest that preincubation of endostatin prior to VEGF treatment is necessary to block the VEGF binding to endothelial cells.

To further confirm the inhibitory activity of endostatin on the interaction between VEGF and its receptors, using an ELISA binding assay we examined the effect of endostatin on the binding of VEGF with KDR/Flk-1-Fc, a soluble fusion protein containing the extracellular domain of KDR/Flk-1. As shown in Fig. 4C, preincubation of endostatin (10 μg/ml) with KDR/Flk-1-Fc blocked the interaction between VEGF and KDR/Flk-1-Fc in a dose-dependent manner (IC50 ~ 2 μg/ml). The binding of Plt-1-Fc to immobilized VEGF165 was also blocked by endostatin. We next investigated the effect of preincubation time of endostatin with KDR/Flk-1-Fc on the binding of VEGF to KDR/Flk-1-Fc. The longer preincubation showed less binding of KDR/Flk-1-Fc to the immobilized VEGF (Fig. 4D). We also examined whether endostatin can affect the
dissociation rate of KDR/Flk-1-Fc from the preformed VEGF and FKDR/Flk-1-Fc complex. Endostatin even at a high concentration (10 μg/ml) had no significant effect on the dissociation rate of KDR/Flk-1-Fc from VEGF and its receptor complex (data not shown). These results suggest that preincubation of endostatin prior to VEGF treatment is necessary to block VEGF binding to endothelial cells and that endostatin inhibits VEGF-induced signaling via the direct blockage of VEGF and its receptor KDR/Flk-1 interaction.

**Endostatin Blocks the Binding of VEGF₁₂₁ to KDR/Flk-1 and VEGF₁₂₁-stimulated ERK Activation**—VEGF₁₂₁ and VEGF₁₀₅ are two major isoforms of VEGF generated via an alternative splicing mechanism from a unique gene (10, 17). VEGF₁₀₅ but not VEGF₁₂₁ binds to heparin-like molecules, and the binding is related to the biological function of VEGF₁₀₅. To clarify the involvement of the heparin binding domain of VEGF in the inhibition of VEGF-induced endothelial cell responses by endostatin, we investigated the effect of endostatin on the binding of VEGF₁₂₁ to KDR/Flk-1 and VEGF₁₂₁-stimulated ERK activation in endothelial cells. Preincubation of endosta-
tin (10 min) with KDR/Flk-1-Fc blocked the binding of KDR/Flk-1-Fc to the immobilized VEGF121 in a dose-dependent manner (Fig. 5A). The IC50 value was 3.5 μg/ml, similar to that of VEGF165. The longer preincubation of endostatin with KDR/Flk-1-Fc showed a lower level of binding of KDR/Flk-1-Fc to VEGF121 in the presence of the same amount of endostatin (Fig. 5B). As shown in Fig. 5C, both VEGF121 and VEGF165 stimulated phosphorylation of ERK in endothelial cells, and these effects were blocked by the treatment of endostatin. These results suggest that endostatin can inhibit the interaction between VEGF121 and its receptor KDR/Flk-1 and block VEGF121-induced ERK activation in endothelial cells.

Endostatin BINDs to KDR/Flk-1 but Not to VEGF—Because endostatin blocks the interaction between VEGF and its receptor KDR/Flk-1, we investigated whether endostatin directly binds to either KDR/Flk-1 or VEGF. Endostatin affinity chromatography showed that KDR/Flk-1 bound to the endostatin-immobilized beads but not to control beads (Fig. 6A). Flt-1-Fc also bound to the endostatin-immobilized beads (data not shown). In an ELISA assay, 125I-VEGF165 did not bind to immobilized endostatin (Fig. 6B). Affinity chromatography also showed that there was no interaction between endostatin and VEGF (data not shown). These results suggest that direct interaction of endostatin with KDR/Flk-1 but not with VEGF may be involved in the inhibitory activity of endostatin on VEGF-induced signaling, proliferation, and migration of endothelial cells.

**DISCUSSION**

Several reports demonstrate that endostatin, a potent inhibitor of angiogenesis, specifically blocks the proliferation and migration of endothelial cells induced by angiogenic factors including VEGF (6, 19) and inhibits tumor growth and metastasis in various animal models (4). However, the molecular mechanisms of endostatin-mediated anti-angiogenesis and tumor regression are not yet clear. To delineate the mechanisms involved in the anti-angiogenic activity of endostatin, we investigated the effect of endostatin on VEGF action by cell biological and biochemical experiments. VEGF is the most important angiogenic molecule associated with tumor-induced neovascu-
larization. In this paper, 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 p125FAK, which 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. Finally, endostatin directly binds to KDR/Flk-1 but not to VEGF. 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 signaling such as ERK, p38 MAPK, and p125FAK, resulting in the inhibition of VEGF-induced proliferation and migration of endothelial cells.

Endostatin Suppresses VEGF-induced Endothelial Cell Signaling—Previous reports showed that endostatin inhibits VEGF-induced proliferation and migration of endothelial cells (6, 19). Because recent studies suggest that the VEGF receptor
KDR/Flk-1 and not Flt-1 is involved in proliferation and migration of endothelial cells in response to VEGF (12, 13). We first revealed that endostatin could interfere with VEGF-induced activation of its receptor KDR/Flk-1. Endostatin blocked VEGF165-induced phosphorylation of KDR/Flk-1 in human endothelial cells. This finding was further supported by our data that endostatin clearly suppressed the downstream events of VEGF165-induced KDR/Flk-1 signaling, such as activation of ERK and p38 MAPK and p125FAK tyrosine phosphorylation, which are requisite for the mitogenic and motogenic activities of VEGF in endothelial cells (14–16). Endostatin also inhibited VEGF121-induced activation of ERK in HUVECs. These results supposed that the anti-angiogenic action of endostatin against VEGF is associated with its ability to block VEGF receptor KDR/Flk-1 activation.

Endostatin Blocks the Interaction between VEGF and Its Receptor, KDR/Flk-1, and Binds to KDR/Flk-1 but Not to VEGF—How does endostatin block the VEGF-mediated endothelial cell signaling events that result in the blocking of VEGF-induced proliferation and migration of endothelial cells? There are at least three different possible mechanisms: (i) endostatin directly interferes in the interaction between VEGF and its receptor, KDR/Flk-1, via its direct binding either to VEGF or to KDR/Flk-1; (ii) binding of endostatin to its receptor-like molecules present on the surface of endothelial cells transfers intracellular signals to block VEGF-mediated signaling; and (iii) internalized endostatin blocks the VEGF-mediated signaling. Our results showed that endostatin blocks the binding of VEGF to its receptors present on the surface of HUVECs and also interferes in the interaction between VEGF and KDR/Flk-1-Fc in a dose-dependent manner. It is worthwhile to note that endostatin blocked VEGF-induced tyrosine phosphorylation of KDR/Flk-1, proliferation, and migration of endothelial cells and activation of ERK, p38 MAPK, and p125FAK as well as the binding of VEGF to endothelial cells and to KDR/Flk-1 with an IC50 in the μg/ml range. This working concentration was at a level similar to that shown in other reports (7, 25). However, some groups have demonstrated that endostatin blocks VEGF-induced endothelial cell migration with an IC50 in the ng/ml range (6, 26). Thus, the working concentration of endostatin is still controversial.

What is the binding partner for endostatin? Our experiments, including endostatin affinity chromatography, showed that the binding partner for endostatin is KDR/Flk-1 but not VEGF. Preincubation of endothelial cells with KDR/Flk-1-Fc was necessary for the efficient blockage of interaction between VEGF and its cell surface receptors or KDR/Flk-1-Fc. These results may be the reason why preincubation of HUVECs with endostatin prior to VEGF treatment is required for the efficient inhibition of VEGF-induced phosphorylation of KDR/Flk-1 and intracellular signaling pathways. Previously reported results also show that preincubation of endothelial cells is necessary for the blockage of VEGF-induced migration (6, 19). The requirement of preincubation for the efficient activity of endostatin also supports the finding that the binding partner for endostatin is KDR/Flk-1 not VEGF. Although most of the VEGF-induced responses in endothelial cells are mediated through KDR/Flk-1, a recent study suggested that Flt-1 but not KDR/Flk-1 may be involved in VEGF-induced formation of capillary networks via the stimulation of nitric oxide release in HUVECs (27). Endostatin blocks the tube formation of endothelial cells (28). Our results showed that endostatin also bound to Flt-1 and blocked the interaction between VEGF and Flt-1. It is worthwhile to note that endostatin neither competes with the binding of bFGF to human tissues nor affects FGF receptor signaling (29, 30), although endostatin was originally identified based on its inhibitory activity of bFGF-induced endothelial cell proliferation (4). Although we could not completely rule out the other mechanisms, our observations suggest that direct interaction of endostatin with KDR/Flk-1 can block the binding of VEGF to its receptor present on the surface of endothelial cells and may be involved in the inhibitory activity of endostatin on VEGF-induced angiogenesis.

Model for Anti-angiogenic Activity of Endostatin—Although several groups have found endostatin-binding proteins and suggested models to delineate the mechanism of endostatin action, the molecular mechanisms of endostatin-mediated anti-angiogenesis and tumor regression are not fully understood. Endostatin induces tyrosine kinase signaling through the Shb adaptor protein and enhanced apoptosis in bFGF-treated endothelial cells (30). Endostatin inhibits pro-MMP-2 activation mediated by membrane type 1 MMP and the catalytic activities of both MMP-2 and membrane type 1 MMP resulting in the inhibition of endothelial and tumor cell invasion (21). Endostatin also binds with α5- and α6-integrins on the surface of endothelial cells and inhibits integrin-dependent endothelial cell migration (25). The interaction of endostatin with tropomyosin causes disruption of microfilament integrity leading to inhibition of endothelial cell migration, induction of endothelial cell apoptosis, and ultimately inhibition of tumor growth (31).

A recent study has shown that endostatin interacts with endothelial cell surface glycans via its glycosaminoglycan chain and that glycans are necessary for the inhibitory activity of endostatin on VEGF165 but not VEGF121-induced endothelial cell migration; endostatin, however, efficiently blocks both VEGF165 and VEGF121-induced endothelial cell migration (26). Another study shows that heparin binding is not involved in the function of endostatin (29). An in situ-binding assay showed that endostatin binds predominantly to its functional target (blood vessels) and co-localizes largely with bFGF in human breast carcinoma tissues (29). However, in situ endostatin binding to blood vessels is resistant to treatment with heparinase and is not affected by bFGF and heparin. These results indicate that binding of endostatin to blood vessels is not mediated by heparan sulfate proteoglycans and that heparin binding domain of endostatin on VEGF-induced endothelial cell migration, induction of endothelial cell apoptosis, and ultimately inhibition of tumor growth (31).

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REFERENCES
1. Folkman, J. (1995) Nat. Med. 1, 27–31
2. Jackson, J. R., Seed, M. P., Kircher, C. H., Willoughby, D. A., and Winkler, J. D. (1997) FASEB J. 11, 457–465
3. Risau, W. (1997) Nature 386, 671–674
4. O’Reilly, M. S., Boehm, T., Shing, Y., Fukui, N., Vastano, G., Lane, W. S., Flynn, E., Birkhead, J. R., Olsen, B. R., and Folkman, J. (1997) Cell 88, 277–285
5. Boehm, T., Folkman, J., Browder, T., and O’Reilly, M. S. (1997) Nature 386, 404–407
6. Yamaguchi, N., Anand-Apte, B., Lee, M., Sasaki, T., Fukui, N., Shapiro, R., Que, I., Lowik, C., Timpl, R., and Olsen, B. R. (1999) EMBO J. 18, 4414–4423
7. Dhanabal, M., Ramchandran, R., Waterman, M. J., Lu, H., Knebelman, B., Segal, M., and Sukhatme, V. P. (1999) J. Biol. Chem. 274, 11721–11726
8. Ferrara, N. (1996) Eur. J. Cancer 32, 2413–2422
9. Ferrara, N., and Davis-Smyth, T. (1997) Endocr. Rev. 18, 4–25
10. Veikkola, T., and Aaltio, R. (1999) Semin. Cancer Biol. 9, 211–220
11. McMahon, G. (2000) Oncologist 5, 3–10
12. Bernatchez, P. N., Soker, S., and Siros, M. G. (1999) J. Biol. Chem. 274, 31047–31054
13. Davis-Smyth, T., Chen, H., Park, J., Presta, L. G., and Ferrara, N. (1996) *EMBO J.* **15**, 4919–4927
14. Pedram, A., Razandi, M., and Levin, E. R. (1998) *J. Biol. Chem.* **273**, 26722–26728
15. Rousseau, S., Huile, F., Landry, J., and Huet, J. (1997) *Oncogene* **18**, 2169–2177
16. Abedi, H., and Zachary, I. (1997) *J. Biol. Chem.* **272**, 15442–15451
17. Zachary, I. (2001) *Am. J. Physiol.* **280**, C1375–C1386
18. Ziche, M., Morbidelli, L., Choudhuri, R., Zhang, H. T., Donnini, S., Granger, H. J., and Bicknell, R. (1997) *J. Clin. Invest.* **99**, 2625–2634
19. Taddei, L., Chiarugi, P., Brogelli, L., Cirri, P., Magnelli, L., Raeger, G., Ziche, M., Granger, H. J., Chiarugi, V., and Ramponi G. (1999) *Biochim. Biophys. Res. Commun.* **263**, 340–345
20. Jaffe, E. A., Nachman, R. L., Becker, C. G., and Minick, C. R. (1973) *J. Clin. Invest.* **52**, 2745–2756
21. Kim, Y. M., Jang, J. W., Lee, O. H., Yeon, J., Choi, E. Y., Kim, K. W., Lee, S. T., and Kwon, Y. G. (2000) *Cancer Res.* **60**, 5410–5413
22. Lee, O. H., Kim, Y. M., Lee, Y. M., Moon, E. J., Lee, D. J., Kim, J. H., Kim, K. W., and Kwon, Y. G. (1999) *Biochem. Biophys. Res. Commun.* **264**, 743–750
23. Bae, D. G., Gho, Y. S., Yoon, W. H., and Chae, C. B. (2000) *J. Biol. Chem.* **275**, 13588–13596
24. Dhanabal, M., Ramchandran, R., Volk, R., Stillman, I. E., Lombardo, M., Irues-Arispe, M. L., Simons, M., Sukhatme, V. P. (1999) *Cancer Res.* **59**, 189–197
25. Rehn, M., Veikkola, T., Kukk-Valdre, E., Nakamura, H., Ilmonen, M., Lombardo, C., Pihlajaniemi, T., Alitalo, K., and Vuori, K. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 1024–1029
26. Karumanchi, S. A., Jha, V., Ramchandran, R., Karihaloo, A., Tsaikas, L., Chan, B., Dhanabal, M., Hanai, J. I., Venkataraman, G., Shriver, Z., Keiser, N., Kalluri, R., Zeng, H., Mukhopadhyay, D., Chen, R. L., Lander, A. D., Hagiwara, K., Yamaguchi, Y., Sassekharan, R., Cantery, L., and Sukhatme, V. P. (2001) *J. Biol. Chem.* **275**, 13588–13596
27. Bussolati, B., Dunk, C., Grohman, M., Kontos, C. D., Mason, J., and Ahmed, A. (2001) *Am. J. Pathol.* **159**, 993–1008
28. Joki, T., Machluf, M., Atala, A., Zhu, J., Seyfried, N. T., Dunn, I. F., Abe, T., Carroll, R. S., and Black, P. M. (2001) *Nat. Biotechnol.* **19**, 35–39
29. Chang, Z., Chou, A., and Friedl, A. (1999) *Am. J. Pathol.* **155**, 71–76
30. Dixelius, J., Larsson, H., Sasaki, T., Holmqvist, K., Lu, L., Engstrom, A., Timpl, R., Welsh, M., and Claesson-Welsh, L. (2000) *Blood* **95**, 3403–3411
31. MacDonald, N. J., Shivers, W. Y., Narum, D. L., Plum, S. M., Wingard, J. N., Fuhrmann, S. R., Liang, H., Holland-Linn, J., Chen, D. H., and Sim, B. K. (2001) *J. Biol. Chem.* **276**, 25190–25196