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Brief report

Gremlin is a novel agonist of the major proangiogenic receptor VEGFR2

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The bone morphogenic protein antagonist gremlin is expressed during embryonic development and under different pathologic conditions, including cancer. Gremlin is a proangiogenic protein belonging to the cystine-knot superfam-ily that includes transforming growth factor-β proteins and the angiogenic vascular endothelial growth factors (VEGFs). Here, we demonstrate that gremlin binds VEGF receptor-2 (VEGFR2), the main transducer of VEGF-mediated angiogenic signals, in a bone morphogenic protein-independent manner. Similar to VEGF-A, gremlin activates VEGFR2 in endothelial cells, leading to VEGFR2-dependent angiogenic responses in vitro and in vivo. Gremlin thus represents a novel proangiogenic VEGFR2 agonist distinct from the VEGF family ligands with implications in vascular development, angiogenesis-dependent diseases, and tumor neoangi-valorization. (Blood. 2010;116(18):3677-3680)

Introduction

The bone morphogenic protein (BMP) antagonist gremlin induces angiogenesis in a BMP-independent manner by binding to as-yet-unidentified endothelial cell (EC) membrane receptors and activating multiple tyrosine kinase–dependent intracellular signaling pathways in ECs.3,4 Gremlin is produced by human tumors5,6 and is expressed by fibroblast growth factor-2 (FGF2)–activated ECs and tumor endothelium.2 Thus, gremlin may play paracrine/autocrine roles in tumor neoangiogenesis. The identification of the EC receptors activated by gremlin has so far been unsuccessful.

Vascular endothelial growth factor receptor-2 (VEGFR2) is the major proangiogenic tyrosine kinase receptor expressed by ECs and is activated by different members of the vascular endothelial growth factor (VEGF) family.2,5 Both gremlin and VEGFs belong to the cystine-knot protein superfamily,7 suggesting possible structural and/or functional similarities among these proangiogenic factors. On this basis, we investigated the capacity of gremlin to interact with and activate VEGFR2. The results demonstrate that gremlin binds and activates VEGFR2, leading to VEGFR2-dependent angiogenic responses in vitro and in vivo.

Methods

Ligand-receptor interaction assays

Interaction of VEGF-A and gremlin (R&D Systems) with the immobilized extracellular domain of VEGFR2 (sVEGFR2; Calbiochem) was analyzed by surface plasmon resonance (BIAcore Inc) and by competitive enzyme-linked immunosorbent assay (ELISA). VEGFR2 interaction on the EC surface was characterized by cross-linking experiments, whereas VEGFR2 dimerization was assessed by fluorescence resonance energy transfer analysis.

In vitro angiogenic assays

Motility and 3-dimensional gel invasion assays were performed on human, murine, and bovine ECs.1 When indicated, ECs were stably transfected with a pcDNA3.1 expression vector harboring the mouse VEGFR2 complementary DNA.

In vivo angiogenic assays

Alginate beads (5 μL) containing gremlin (100 ng per embryo) were grafted on the chorioallantoic membrane (CAM) of fertilized chicken eggs at day 11. After 72 hours, new blood vessels converging toward the implant were counted.

Details of experimental procedures and analysis are provided in supplemental Methods (available on the Blood Web site; see the Supplemental Materials link at the top of the online article).

Results and discussion

Surface plasmon resonance analysis was used to assess the ability of gremlin to bind the extracellular domain of VEGFR2. As shown in Figure 1A, gremlin binds sVEGFR2 immobilized on a BIAcore sensor chip (Kd = 47 ± 15 nM). The interaction is inhibited by an excess of soluble sVEGFR2-Fc but not by sVEGFR1-Fc, sVEGFR3-Fc, or sFGFR1-Fc (Figure 1B). Similar to VEGF-E, gremlin inhibits the binding of VEGF-A to immobilized sVEGFR2-Fc in competitive ELISA but, unlike the VEGFR1-specific agonist placenta growth factor, it does not affect VEGF-A interaction with sVEGFR1-Fc (Figure 1C). To assess whether gremlin/VEGFR2 interaction occurs also under physiologic conditions, we incubated human umbilical vein ECs (HUVECs) with gremlin conjugated with a bifunctional photoactivatable biotin-label transfer cross-linker...
abrogated by the VEGFR2 tyrosine kinase inhibitor SU5416. Both VEGFR2 activation is also and Y951 of VEGFR2 (supplemental Figure 1a) with kinetics dependent phosphorylation of tyrosine phosphorylation sites Y1175 (pY1175) antibodies (supplemental Figure 2). VEGFR2 engages or VEGF-A after immunostaining with anti–phospho-VEGFR2 fetal bovine aortic VEGFR2-GM7373 ECs stimulated by gremlin and VEGF-A–activated ECs (Figure 2C). Accordingly, VEGFR2 phosphorylation, abrogated by SU5416, was observed in gremlin or VEGF-A but not with FGF2 (supplemental Figure 3). Thus, in keeping with its ability to induce focal adhesion kinase, mitogen-activated protein kinase extracellular signal-regulated kinase1/2, and transcription factor nuclear factor κB activation, gremlin activates VEGFR2 similar to VEGF-A. Tyrosine phosphorylation of cellular proteins (supplemental Figure 1a), chemotactic migratory response and small GTPase Rac activation (supplemental Figure 4), as well as the formation of 3-dimensional EC sprouts (Figure 2E) are similar in HUVECs stimulated by either gremlin or VEGF-A. These activities are blocked by SU5416 or by receptor-binding competitors such as neutralizing anti-VEGFR2 antibody or the cyclic peptide cyclo-VEGI (Calbiochem). To further assess the role of VEGFR2 in mediating the proangiogenic activity of gremlin, we transfected murine aortic ECs cotransfected with enhanced yellow fluorescent protein–tagged and enhanced cyan fluorescent protein–tagged VEGFR2 highlighted the internalization of VEGFR2 dimers in the early endosomal compartment after stimulation with gremlin or VEGF-A but not with FGF2 (supplemental Figure 3). Indeed, confocal analysis shows that both gremlin and VEGF-A induce VEGFR2 phosphorylation and internalization of the activated receptor in HUVECs (Figure 2D). In addition, fluorescence resonance energy transfer analysis of bovine aortic ECs cotransfected with enhanced yellow fluorescent protein–tagged and enhanced cyan fluorescent protein–tagged VEGFR2 highlighted the internalization of VEGFR2 dimers in the early endosomal compartment after stimulation with gremlin or VEGF-A but not with FGF2 (supplemental Figure 3). Thus, in keeping with its ability to induce focal adhesion kinase, mitogen-activated protein kinase extracellular signal-regulated kinase-2, and transcription factor nuclear factor κB activation, gremlin activates VEGFR2 similar to VEGF-A. Tyrosine phosphorylation of cellular proteins (supplemental Figure 1a), chemotactic migratory response and small GTPase Rac activation (supplemental Figure 4), as well as the formation of 3-dimensional EC sprouts (Figure 2E) are similar in HUVECs stimulated by either gremlin or VEGF-A. These activities are blocked by SU5416 or by receptor-binding competitors such as neutralizing anti-VEGFR2 antibody or the cyclic peptide cyclo-VEGI (Calbiochem). To further assess the role of VEGFR2 in mediating the proangiogenic activity of gremlin, we transfected murine aortic ECs (MAECs) expressing low levels of VEGFR2 with a murine VEGFR2 complementary DNA giving rise to VEGFR2-MAECs. The motogenic activity exerted by gremlin or VEGF-A is dramatically up-regulated in these cells compared with parental MAECs. Again, the activity of both motogens is inhibited by SU5416 and cyclo-VEGI (supplemental Figure 5).
In keeping with these in vitro observations, the angiogenic activity exerted by gremlin and VEGF-A ex vivo in the human umbilical artery ring EC sprouting assay and in vivo in the chick embryo CAM assay is significantly inhibited by SU5416, the VEGFR2 inhibitor I (VEGFR2-KI), or by competition with an excess of free sVEGFR2 (Figure 2F-G; supplemental Figures 6-7).

In all the assays, VEGFR2 inhibitors do not affect the activity of FGF2 (data not shown).

Here, we demonstrate that the angiogenic activity of gremlin is mediated by VEGFR2. Most importantly, BMP2 does not prevent gremlin from binding and activating VEGFR2 (supplemental Figure 8). Similarly, BMP4 does not affect the angiogenic activity of gremlin and its interaction with ECs. We therefore propose that distinct domains of gremlin may mediate the interaction with VEGFR2 or BMPs, respectively.

The capacity of gremlin to bind BMPs and to inhibit their interaction with the cognate transforming growth factor-β family receptors is thought to play a role in embryonic development and in human diseases. However, BMP-independent mechanism(s) may also be involved in gremlin signaling. Our data reveal the previously unrecognized capacity of gremlin to specifically bind to and promote activation of the major proangiogenic receptor VEGF2 in a BMP-independent manner. Thus, gremlin may exert both BMP-dependent and BMP-independent functions in different physio-pathologic conditions by inhibiting BMP-mediated transforming growth factor-β receptor activation or by a direct activation of VEGF receptor signaling, respectively. These findings extend the number of proangiogenic VEGF2 ligands to a member of the cystine-knot BMP antagonists distinct from VEGFs.

Gremlin is expressed by parenchymal and stromal cells in human tumors. Our observations may provide novel insights for the understanding of the biology of vascular development and of angiogenesis-dependent diseases, including cancer.

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Authorship

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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