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 superfamily 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 neovascularization. (Blood. 2010;116(18):3677-3680)

Introduction

The bone morphogenic protein (BMP) antagonist gremlin1 induces angiogenesis in a BMP-independent manner by binding to as-yet-identified endothelial cell (EC) membrane receptors and activating multiple tyrosine kinase–dependent intracellular signaling pathways in ECs.2,3 Gremlin is produced by human tumors4,5 and is expressed by fibroblast growth factor-2 (FGF2)–activated ECs and tumor endothelium.6 Thus, gremlin may play paracrine/autocrine roles in tumor neovascularization. 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.8 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.

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After photoactivation of EC-bound sulfo-gremlin, VEGFR2 immunocomplexes were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions to transfer the biotin moiety to the interacting receptor, followed by detection with streptavidin–horseradish peroxidase to visualize the biotin–VEGFR2 complex. As shown in Figure 1D, sulfo-gremlin forms a 250 kDa biotin–VEGFR2 complex when cross-linked to the HUVEC surface, VEGFR2 biotinylation being inhibited by an excess of unlabeled gremlin or VEGF-A. Taken together, these data demonstrate the capacity of gremlin to bind VEGFR2 and to compete with VEGF-A for receptor interaction.

We next investigated whether gremlin induces VEGFR2 auto-phosphorylation in ECs.6 In HUVECs, gremlin induced a dose-dependent phosphorylation of tyrosine phosphorylation sites Y175 and Y951 of VEGFR2 (supplemental Figure 1a) with kinetics similar to VEGF-A (Figure 2A-B). VEGFR2 activation is also abrogated by the VEGFR2 kinase inhibitor SU5416, both in gremlin and VEGF-A–activated ECs (Figure 2C). Accordingly, VEGFR2 phosphorylation, abrogated by SU5416, was observed in fetal bovine aortic VEGFR2-GM7373 ECs stimulated by gremlin or VEGF-A after immunostaining with anti–phospho-VEGFR2 (pY175) antibodies (supplemental Figure 2). VEGFR2 engagement leads to a rapid internalization of the activated receptor.9 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 VEGF-R2 highlighted the internalization of VEGF-R2 dimers in the early endosomal compartment2 after stimulation with gremlin or VEGF-A but not with FGFR2 (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,2,3 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 antibody10 or the cyclic peptide cyclo-VEGCI1 (Calbiochem).11

To further assess the role of VEGF-R2 in mediating the proangiogenic activity of gremlin, we transfected murine aortic ECs (MAECs) expressing low levels of VEGF-R2 with a murine VEGFR2 complementary DNA giving rise to VEGF-R2-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-VEGCI (supplemental Figure 5).

Figure 1. Gremlin binds VEGFR2. (A) sVEGFR2D1–7 (Calbiochem) was immobilized at approximately 0.83 pmol/mm² to a CMS sensorchip (BIAcore) that was previously activated with a mixture of 0.2M N-ethyl-N-(3-dimethylaminopropyl)-carbodiimide hydrochloride and 0.05M N-hydroxysuccinimide (35 μL; flow rate: 10 μL/min). Increasing concentrations of gremlin were injected in HBS-EP buffer (BIAcore) for 4 minutes (sample volume: 40 μL; flow rate: 5 μL/min; dissociation time: 4 minutes). The response (in response units) was recorded as a function of time. An overlay plot is shown of all sensorgrams after subtraction of their respective control sensorgrams. Binding parameters, calculated by the nonlinear curve–fitting software package BIAevaluation 3.2 (BIAcore Inc) applied to all sensorgrams simultaneously using a single-site model with drifting baseline, indicate that gremlin/VEGFR2 interaction occurs with Kd = 47 ± 15nM. Under the same experimental conditions, VEGF-A/VEGFR2 interaction occurs with Kd = 3 ± 1nM. (B) Gremlin (25nM) was injected over the sVEGFR2-coated sensor chip in the absence or in the presence of soluble sVEGFR1-Fc, sVEGFR2-Fc, sVEGFR3-Fc, or sFGFR1-Fc (all at 314nM). Binding data were plotted as percentage of maximal bound analyte (recorded at the end of injection) and represent the mean of 2-3 independent experiments. (C) Ninety-six–well plates coated with 100 μL of 250 ng/mL sVEGFR1-Fc or sVEGFR2-Fc were incubated with VEGF-A (20 ng/mL dissolved in phosphate-buffered saline containing 0.1% BSA, 5.0mM (ethylenedinitrilo)tetraacetic acid, 0.004% Tween 20 in presence of different competitors and incubated for 1 hour at 37°C followed by 1-hour incubation at room temperature. Bound VEGF-A was detected with an anti–human VEGF monoclonal antibody (R&D Systems). Gremlin competes with VEGF-A for the binding to immobilized sVEGFR2-Fc (●) in a competitive ELISA for which VEGF-E (○) and FGFR2 (□) were used as positive and negative controls, respectively. At variance, gremlin did not compete with VEGF-A for the binding to immobilized sVEGFR1-Fc (□), whereas placenta growth factor (PGIF) (△) was fully effective. (D) HUVECs were incubated with 5.0nM gremlin conjugated with the bifunctional photoactivatable biotin–label transfer cross-linker Sulfo–SBED Biotin Label transfer reagent (Pierce) (sulfo-gremlin) in the absence or in the presence of a molar excess of unlabeled gremlin or VEGF-A. After ultraviolet irradiation, cell lysates (1.0 mg of protein) were immunoprecipitated with anti-VEGFR2 antibody (Santa Cruz Biotechnology), separated on a sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel under reducing conditions and probed with streptavidin–horseradish peroxidase to visualize the biotin–VEGFR2 complex. Uniform loading of the gel was confirmed by probing the membrane with the anti-VEGFR2 antibody.
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 (VEGFR2KI)\(^\text{12}\), or by competition with an excess of free sVEGFR2 (Figure 2F-G; supplemental Figures 6-7). In all the assays, VEGF-A 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\(^\text{2}\). 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\(^\text{13}\) and in human diseases\(^\text{14}\). However, BMP-independent mechanism(s) may also be involved in gremlin signaling\(^\text{2,15}\). Our data reveal the previously unrecognized capacity of gremlin to specifically bind to and promote activation of the major proangiogenic receptor VEGFR2 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 VEGFR2 ligands to a member of the cystine-knot BMP antagonists distinct from VEGFs\(^\text{16}\). Gremlin is expressed by parenchymal and stromal cells in human tumors\(^\text{2,4,5}\). 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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