A Synthetic Glycosaminoglycan Mimetic Binds Vascular Endothelial Growth Factor and Modulates Angiogenesis*

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In a previous study, we showed that in situ injection of glycosaminoglycan mimetics called RGTA (ReGeneraTing Agents) enhanced neovascularization after skeletal muscular ischemia (Desgranges, P., Barcaud, C., Caruelle, J. P., Barritault, D., and Gautron, J. (1999) FASEB J. 13, 761–766). In the present study, we showed that the RGTA OTR4120 modulated angiogenesis in the chicken embryo chorioallantoic membrane assay, in a dose-dependent manner. We therefore investigated the effect of OTR4120 on one of the most specific angiogenesis-regulating heparin-binding growth factors, vascular endothelial growth factor 165 (VEGF165). OTR4120 showed high affinity binding to VEGF165 (Kd ≥ 2.2 nM), as compared with heparin (Kd = 15 nM), and potentiated the affinity of VEGF165 for VEGF receptor-1 and -2 and for neuropilin-1. In vitro, OTR4120 potentiated VEGF165-induced proliferation and migration of human umbilical vein endothelial cells. In the in vivo Matrigel plug angiogenesis assay, OTR4120 in a concentration as low as 3 ng/ml caused a 6-fold increase in VEGF165-induced angiogenesis. Immunohistochemical staining showed a larger number of well differentiated VEGFR-2-expressing cells in Matrigel sections of OTR4120-treated plug than in control sections. These findings indicate that OTR4120 enhances the VEGF165-induced angiogenesis and therefore may hold promise for treating disorders characterized by deficient angiogenesis.

Angiogenesis, which is essential during wound repair (1), is a dynamic process that is highly regulated by signals from the surrounding extracellular matrix (ECM) (2, 3). Proliferation, migration, and differentiation of endothelial cells during wound repair are influenced by cells, cytokines, and the organization and quality of ECM components (4).

Proteoglycans are a subset of complex polysaccharides bound covalently to a core protein in the ECM. These proteoglycans include glycosaminoglycan (GAG) side chains such as heparan sulfate (HS) or dermatan sulfate (DS). GAG may act as an endothelial cell receptor that recognizes the ECM (5) and regulates cellular events. Furthermore, GAG plays a key role in the bioavailability of heparin-binding growth factors (HBGF) by providing storage and protection sites (6). The exact mechanisms by which GAG participates in this multifactorial process remain unclear but may include modulation of endothelial cells-ECM interactions or signaling of angiogenic or anti-angiogenic factors (7).

Thus, depending on their structure and concentration, GAG molecules may exert a variety of regulatory effects on the local bioavailability of angiogenic growth factors such as VEGF (8).

VEGF induces endothelial cell proliferation, migration, and differentiation. In vivo, VEGF is a homodimeric glycoprotein whose monomer exists as five isoforms produced by alternative splicing of a single gene, which yields polypeptides with 121, 145, 165, 189, and 206 amino acid residues, respectively (9). These VEGF isoforms differ in their affinity for heparin and ECM components. The best characterized VEGF is the heparin-binding 165-amino acid form, VEGF165 (10, 11). Two tyrosine kinase receptors that are VEGF high affinity receptors have been identified in humans, VEGFR-1 (fms-like tyrosine kinase, Flt-1) (12) and VEGFR-2 (kinase domain region, KDR) (13). An additional binding site, neuropilin-1 (NP1), acts as a co-receptor for VEGF165, forming a complex with VEGFR-2 and thereby enhancing VEGF165-induced effects (14).

We have developed synthetic polymers called RGTA for ReGeneraTing Agents (15). These polymers are engineered to mimic the stabilizing and protecting properties of GAG toward HBGF. They are obtained by grafting specific amounts of carboxylate and sulfate groups onto a dextran backbone. These molecules stimulate tissue repair when applied to the site of injury or systemically (16). They expedite healing of skin (17), bone (18, 19), colonic (20), and corneal (21) injuries and have at least 10 time less anticoagulant activity than does heparin. In addition, these polymers enhance in vivo endothelialization (22) of vascular pros-thesis and partly prevent in vivo damage caused by skeletal muscle (23) and myocardial ischemia (24). RGTA interacts with and protects various HBGFs, such as fibroblast growth factor (FGF) and transforming growth factor β, against proteolytic degradation (25, 26).

The in vitro and in vivo effects of RGTA, together with evidence that VEGF165 acts as a mitogen, migratory, and differentiating factor for endothelial cells (as demonstrated for example by its ability to reduce ischemic brain (27) and muscle (8) damage), prompted us to investigate the mechanisms by which RGTA OTR4120 may modulate the biological activities of VEGF165.
EXPERIMENTAL PROCEDURES

Materials—Dextran 40 USP was from Amersham Biosciences (Uppsala, Sweden), and heparin and collagen type I were from Sigma (Saint Quentin Fallavier, France). Purified recombinant human VEGFR-1-Fc, VEGFR-2-Fc, and NP-1-Fc chimera were purchased from R&D Systems Europe (Abingdon, UK). Human recombinant VEGF, was expressed in Sf9 insect cells and purified by cationic-exchange and heparin-affinity chromatography, as reported previously (28). BCA protein assay kit and lodobeads® were purchased from Perbio Science France (Bezons, France). EBM-2 BulletKit® medium, fetal bovine serum (FBS), trypsin-EDTA, and human umbilical vein endothelial cells (HUVECs) were purchased from Biowhittaker Clonetics (Emerainville, France). Matrigel™ was from BD Biosciences (Becton Dickinson, Le Pont de Clai, France), and Boyden chambers were from Costar (Avon, France). Rabbit polyclonal antibodies against VEGFR-1 (H-225) or VEGFR-2 (C-20) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Swiss mice were from Janvier (Le Genest St Isle, France). Housing and anesthesia complied with the guidelines developed by our institution’s Animal Welfare Department in accordance with the European Guide for the Care and Use of Laboratory Animals.

Synthesis of OTR4120—OTR4120 is a carboxymethyl-dextran sulfate with a degree of substitution of 1.13 for sulfate residues and 0.46 for carboxymethyl residues, for a maximal degree of substitution of 3.0 (29, 30). These substitution degrees are similar to those assayed in heparin used as a control. The molecular mass of OTR4120 is 77 kDa. Chick Embryo Chorioallantoic Membrane Assay—The in vivo chicken embryo chorioallantoic membrane (CAM) angiogenesis model was used to investigate the angiogenic potency of OTR4120 (31). Briefly, Leghorn fertilized eggs (Pindos, Greece) were incubated for 4 days at 37 °C. Then, a window was opened on the eggshell, exposing the CAM. The window was covered with sterile tape, and the eggs were returned to the incubator. On day 9 of embryo development, 20 μl of distilled water alone as a control or containing OTR4120 (1.5 or 15 μg) was applied on a 1-cm² area of the CAM inside a silicon ring. After 48 h of incubation at 37 °C, CAMs were fixed in situ with saline-buffered formalin, excised from the eggs, placed on slides, and left to dry in air. Pictures were taken through a stereoscope equipped with a digital camera, and the total length of the vessels was measured using the ImagePC image analysis software (Scion Corp.). Each sample was tested three times using 15–20 eggs for each data point. For the total RNA extraction, instead of in situ fixation, the CAM inside the ring was excised from five eggs for each sample tested, washed three times in phosphate-buffered saline (PBS), pH 7.4, and stored at −80 °C until use.

RNA Isolation—Total RNA isolation was performed using NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany). CAMs from five eggs for each sample tested were disrupted by grinding with a pestle and mortar to a fine powder, in the presence of liquid N2. The powder was immediately mixed with lysis buffer RA1 containing β-mercaptoethanol and passed several times through a 0.9-mm syringe needle. Extraction was conducted as recommended by the manufacturer. The integrity of isolated RNA was assessed by electrophoresis on 1.3% formaldehyde/agarose gels containing 0.5 μg/ml ethidium bromide, and quantification was performed spectrophotometrically.

Reverse Transcriptase-PCR—The RT-PCR reactions for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), VEGFR-1, and VEGFR-2 mRNAs were performed using the OneStep RT-PCR kit (Qiagen, Hilden, Germany) and PTC-200 Peltier Thermal Cycler (M. J. Research), with 200–250 ng of total RNA per reaction. Primers used for VEGFR-1, VEGFR-2, and GAPDH were designed according to the chicken sequences available (GenBank™ accession numbers NM001004368, NM204252, and NM204305, respectively) and were as follows: VEGFR-1 sense, CAC AGC GAG TGA GTA CAA AGC; VEGFR-1 antisense, TCC AGC ATG ATT TGG TAG ATC TC; VEGFR-2 sense, CAG CAC CAT GCA GGA TCA GG; VEGFR-2 antisense, GCC AGC AAG TGG GAG TTT CC; GAPDH sense, ACG GAT TTG GCC GCA GTA TTG GC; GAPDH antisense, GCA GGA TGC GAA ACT GAG C.

The reverse transcriptase reaction was performed using a mix of Omniscript and Senscript reverse transcriptase for 30 min at 50 °C, followed by incubation at 95 °C for 15 min to activate the HotStarTaq DNA polymerase, inactivate the reverse transcriptase, and denature the cDNA template. After the denaturation step, 35 amplification cycles (94 °C for 1 min, 57 °C for 1 min, and 72 °C for 1 min for GAPDH; 94 °C for 45 s, 56 °C for 45 s, and 72 °C for 1 min for VEGFR-1; 94 °C for 45 s, 57 °C for 45 s, and 72 °C for 1.5 min for VEGFR-2) were performed and followed by a final DNA synthesis step at 72 °C for 10 min. PCR reactions were not in the saturating phase, and DNA contamination was ruled by performing PCR without the reverse transcription step (data not shown). The RT-PCR products of all the reactions were subjected to electrophoresis on 1% agarose gels containing 0.5 μg/ml ethidium bromide, photographed using a digital camera, and quantified using the Image PC image-analysis software (Scion Corp.). The ratio of electrophoretic band values of each PCR product over the GAPDH electrophoretic band value, used as loading control, represents the expression of each gene. Each sample was tested three times using five eggs for each data point.

Preparation of OTR4120- and Heparin-Bovine Serum Albumin Complexes—OTR4120 and heparin were covalently coupled to bovine serum albumin (BSA) as previously described by Soulie et al. (32). Briefly, heparin or OTR4120 (912 mg) and BSA (34 mg) were dissolved in 5 ml of potassium phosphate buffer (0.2 M, pH 8.0). Then, 25 mg of sodium cyanoborohydride was added, and the mixture was incubated for 2 days at 37 °C. At the end of incubation, mixtures were dialyzed against water and freeze-dried. The residue was dissolved in 50 mM Tris-HCl, pH 7.4. The OTR4120-BSA and heparin-BSA complex concentrations were determined with the BCA protein assay kit with BSA as the standard.

The complexes were subjected to SDS-10% polyacrylamide gel electrophoresis and visualized by Coomassie Blue staining. The solutions were aliquoted and stored at −80 °C.

Radio-iodination of VEGF165—Radio-iodination was carried out using the lodobeads® method. 125I-VEGF165 was separated from free iodine using a heparin-Sepharose column (0.2 ml bed volume) pre-equilibrated with PBS/0.2% gelatin and eluted with PBS/0.2% gelatin and 2 M NaCl. The specific activity of radio-iodinated VEGF165 was 1–3 × 10⁶ cpm/nl.

Binding of 125I-VEGF165 to OTR4120-BSA and Heparin-BSA Complexes—The surface of 96-well ELISA plates were coated overnight at 4 °C with 100 μl of 0.2 μg/ml OTR4120-BSA or heparin-BSA as a positive control in 50 mM Tris-HCl, pH 7.4, supplemented with 12.7 mM EDTA. After three washes with PBS containing 0.05% Tween 20 (washing buffer), nonspecific interactions were saturated with PBS containing 3% BSA for 1 h at room temperature. Then, 2·10⁴ cpm 125I-VEGF165 (177 pm) and unlabeled VEGF165 in concentrations ranging from 0 to 25 nM were added to a final volume of 100 μl/well in PBS containing 1% BSA. After incubation overnight at 4 °C, the wells were washed five times with washing buffer and, after solubilization with 0.2 M NaOH, the radioactivity in each well was measured in a γ-counter (1275 minigamma counter, LKB Wallac). Each curve was analyzed according to the Scatchard procedure by fitting to a logistic curve using the Ligand software (33). All experiments were carried out in triplicate and were repeated five times.
125I-VEGF165 Binding to Soluble Receptors (VEGFR-1, VEGFR-2, and NP-1)—Purified recombinant human VEGFR-1-Fc, VEGFR-2-Fc, or NP-1-Fc chimeras in a concentration of 1 μg/ml in 100 μl PBS/well were adsorbed on the surface of 96-well ELISA plates overnight at 4 °C. The wells were washed three times with washing buffer, and the nonspecific interactions were saturated with PBS containing 3% BSA, for 1 h at room temperature. Blocking solution was removed, and then 2.10^5 cpm of 125I-VEGF165 was added with or without heparin or OTR4120 in increasing concentrations (0–100 μg/ml). After incubation overnight at 4 °C, free 125I-VEGF165 was removed by five washes with washing buffer. Solubilization was achieved using 0.2M NaOH, and the radioactivity in each well was measured in a γ-counter. All experiments were carried out in triplicate and were repeated five times.

Saturation binding experiments were performed in the presence of VEGF165 at increasing concentrations (0–0.15 nM for VEGFR-1, 0–1.2 nM for VEGFR-2, and 0–0.3 nM for NP-1) and of heparin or OTR4120 at 100 ng/ml, a concentration previously shown to induce maximal VEGF165 binding to its receptors. Each curve was analyzed according to the Scatchard procedure by fitting to a logistic curve using the Ligand software (33). All experiments were carried out in triplicate and were repeated three times with similar results.

Cell Culture—HUVECs were grown in EBM-2 BulletKit medium supplemented with 2% FBS, at 37 °C in a 5% CO₂-humidified atmosphere. The cells were used between passages 2 and 5.

Cell Proliferation Assay—HUVECs were seeded in a density of 2·10^4 cells/well into 12-well tissue culture plates in EBM-2 medium supplemented with 2% FBS. After 24 h, the cells were starved with EBM-2 medium supplemented with 0.5% FBS. After 24 h, VEGF165 (3 ng/ml), OTR4120, or heparin (0.3, 3, or 33 ng/ml) alone or in combination (as specified in

**FIGURE 1.** OTR4120 induces angiogenesis in chicken embryo chorioallantoic membrane. A window was opened on the egg shell and 20 μl of distilled water alone as the control or containing 1.5 or 15 μg of OTR4120 was applied to 1 cm² of the CAM inside a silicon ring. After 48 h, CAMs were fixed and excised from the eggs. A, photographs were taken and total length of the vessels was measured. Each sample was tested three times using 15–20 eggs for each data point. Results are expressed as mean ± S.E. *****, p < 0.001. Alternatively, the CAM inside the ring was excised. Total RNA were extracted and RT-PCRs for GAPDH, VEGFR-2 (B), and VEGFR-1 (C) mRNAs were performed. The RT-PCR products were subjected to electrophoresis, photographed, and quantified. The ratio of electrophoretic band values of each PCR product versus GAPDH electrophoretic band value represents the expression of each gene. Each sample was tested three times using five eggs for each data point. Results are expressed as mean ± S.E. *****, p < 0.001 for both VEGFR-2 and VEGFR-1.

**FIGURE 2.** 125I-VEGF165 binds to OTR4120-BSA and heparin-BSA complexes. The surface of 96-well ELISA plates was coated with OTR4120-BSA (A) or heparin-BSA (B) complexes. 125I-VEGF165 in a concentration of 20 pmol was allowed to bind in the presence or absence of unlabeled VEGF165 overnight at 4 °C. After washing, the radioactivity in each well was measured in a γ-counter. All experiments were carried out in triplicate and were repeated five times.
the results section) were added. After 72 h of incubation, cells were washed with PBS, dissociated with trypsin-EDTA (Clonetics Biowhitaker), and counted using a Coultronics Coulter counter (Beckman Coulter, Roissy, France). The experiment was performed in triplicate and was repeated three times. Results are reported as the mean (±S.E.) percentage of growth in the presence of stimulators compared with the control.

Chemotactic Migration Assay—HUVECs migration in response to VEGF<sub>165</sub> and/or OTR4120 was assessed in a 24-well modified Boyden chamber as described previously (31). A polycarbonate membrane filter with 8-μm pore diameter was coated with collagen type I to promote cell adhesion. The coated filter was placed in the microchamber. HUVECs were loaded into the upper part of chemotaxis chamber at 10<sup>5</sup> cells/well in 0.1 ml EBM-2 medium supplemented with 0.2% FBS. The lower wells of the chamber were filled with 0.6 ml of the same medium containing VEGF<sub>165</sub> (3 ng/ml), OTR4120 (10 ng/ml), or both, as chemoattractants. After 12-h incubation at 37 °C, cells on the upper surface of the filters were completely removed by wiping with a cotton tip. Then, the filters were fixed with methanol, and migrated cells were stained with Crystal Violet. The cells that had migrated through the pores to the lower filter surface were counted per microscopic field magnified ×100. The area of endothelial cells that migrated through the filter was quantified using the NIH Image analysis software. Results are reported as the mean of seven independent high power fields/well provided by three independent experiments. Migration of unstimulated cells served as the control.

Matrigel<sup>TM</sup> Plug Assay—Mice were injected subcutaneously with 0.3 ml of Matrigel<sup>TM</sup> at 4 °C, alone or with VEGF<sub>165</sub> (50 ng/ml), OTR4120 (3 ng/ml), heparin (3 ng/ml), or OTR4120 or heparin combined with VEGF<sub>165</sub> (n = 5). The Matrigel<sup>TM</sup> rapidly formed a single solid gel plug. After 6 days, the mouse skins were pulled back to expose the plug, which remained intact. The plugs were dissected out and frozen in liquid nitrogen. Sections of 8 μm thickness were cut using a cryostat (Leica), fixed with acetone at 4 °C for 20 min, and stained with Gomori-Trichrome. Histological slides were evaluated to determine the Matrigel<sup>TM</sup> plug area infiltrated by endothelial cells; to this end, NIH Image software was used on eight Matrigel<sup>TM</sup>-plug sections/mouse, as described previously (34). Experiment was repeated twice.

Immunohistochemistry Analysis of VEGFR Expression in Matrigel<sup>TM</sup>—Frozen Matrigel<sup>TM</sup> sections (8 μm) were fixed for 20 min in acetone at 4 °C. The sections were re-hydrated and saturated in PBS containing 1% BSA and 2% normal goat serum. Endogenous biotin was blocked using the Vector blocking kit (Vector Laboratories, Burlingame, CA). Then, the sections were incubated for 1 h at room temperature with a rabbit polyclonal antibody against VEGFR-1 or VEGFR-2 (5 μg/ml) in saturation buffer. After two washes in PBS, 0.2% Tween 20, sections were incubated for 1 h at room temperature with biotiny-
RESULTS

OTR4120 Induces Angiogenesis—We first tested the angiogenic potency of OTR4120, using the chicken embryo CAM assay. This assay has been used previously to evaluate the effects of GAGs and heparin on angiogenesis (35). The effect of OTR4120 is dose-dependent. While 1.5 µg/cm² of OTR4120 applied to CAM increased the vascular network by 30% compared with control CAM, the 15 µg/cm² dose inhibited vessels development (Fig. 1A). In keeping with these data, expression of both VEGFR-1 and VEGFR-2 was increased in OTR4120-treated CAM (Fig. 1, B and C). GAGs are known to bind angiogenic growth factors such as VEGF₁₆₅ and to modulate VEGF₁₆₅ bioavailability. Since VEGF₁₆₅ was expressed in CAM (36), we hypothesized that the angiogenic activity of OTR4120 occurred through VEGF₁₆₅ activity.

OTR4120 Binds to VEGF₁₆₅—The interactions between VEGF₁₆₅ and OTR4120 were studied by radio-receptor assays using OTR4120-coated microtiter plate. Specific binding of ¹²⁵I-VEGF₁₆₅ was determined with or without a 1000-fold molar excess of unlabeled VEGF₁₆₅. Specific bindings of ¹²⁵I-VEGF₁₆₅ to OTR4120 (Fig. 2A) or to heparin (Fig. 2B) as the positive control was observed. A molar excess of 1000-fold of unlabeled VEGF₁₆₅ displaced 85% and 60% of ¹²⁵I-VEGF₁₆₅ bound to OTR4120 and heparin, respectively. Neither control nor BSA alone or uncoupled OTR4120 bound ¹²⁵I-VEGF₁₆₅ (data not shown). Saturation ligand binding was demonstrated. Scatchard analysis of ¹²⁵I-VEGF₁₆₅ displacement by unlabeled VEGF₁₆₅ performed using Ligand software (37) showed a Kᵦ of 2.2 ± 2 nM for OTR4120 and 15 ± 4 nM heparin (Fig. 3, A and B).

OTR4120 Potentiates the Binding of ¹²⁵I-VEGF₁₆₅ to Its High Affinity VEGF₁₆₅ Receptors—To determine the effect of OTR4120 on the binding of ¹²⁵I-VEGF₁₆₅ to VEGF receptors, we conducted experiments in which ¹²⁵I-VEGF₁₆₅ bound to the extracellular domain of VEGF receptors in the presence of increasing OTR4120 concentrations. According to the concentration of OTR4120 or heparin, a bell-shaped curve was obtained for the effect on VEGF₁₆₅ binding to its receptors; binding was enhanced with low concentrations and decreased with higher concentrations (Fig. 4). The presence of heparin or OTR4120 had only a very slight effect on ¹²⁵I-VEGF₁₆₅ binding to VEGF₁. Indeed, ¹²⁵I-VEGF₁₆₅ binding was increased less than 2-fold and 1.5-fold, respectively, in the presence of OTR4120 and heparin, respectively. In contrast, OTR4120 or heparin concentrations ranging from 1 to 100 ng/ml markedly and dose-dependently increased ¹²⁵I-VEGF₁₆₅ binding to VEGF₁ and NP-1. When 100 ng/ml OTR4120 or heparin was used, VEGF₁₆₅ binding to VEGF₁ and VEGF₂ was increased 7.6- and 3.4-fold, respectively. Similarly, with the extracellular domain of NP-1, binding increased 3.5- and 1.9-fold with 100 ng/ml OTR4120 or heparin, respectively. Higher concentrations of OTR4120 or heparin dose-dependently inhibited ¹²⁵I-VEGF₁₆₅ binding to both VEGF₁ and NP-1. Similar results were obtained with covalent cross-linking experiments (data not shown). Saturation radio-ligand binding experiments indicated that the Kᵦ of VEGF₁₆₅ binding to the VEGF₁ receptor was decreased 8.7- and 3.3-fold in the presence of OTR4120 and heparin, respectively (TABLE ONE). The Kᵦ values for VEGF₁₆₅ binding to VEGF₁ and NP-1 decreased 6.5- and 8.3-fold, respectively, in the presence of OTR4120 and 5.2- and 38-fold, respectively, in the presence of heparin. In addition, the maximal binding capacity (B₅₀) of VEGF₁₆₅ binding to VEGF₁ was decreased by more than 4.4-fold in the presence of OTR4120, whereas no effect was noted for VEGF₁ or NP-1.

OTR4120 Potentiates the Biological Activity of VEGF₁₆₅—We next used HUVECs to investigate the ability of OTR4120 to modulate the biological effects of VEGF₁₆₅ including proliferation, migration and differentiation of endothelial cells. Increasing concentrations (0.3–33 ng/ml) of OTR4120, or heparin as a control, were added to HUVECs with or without stimulation by 3 ng/ml VEGF₁₆₅. A 2-fold increase in cell number over that induced by VEGF₁₆₅ alone occurred with 3 or 33 ng/ml OTR4120 or heparin alone (Fig. 5A). In contrast, no significant effect was observed when the cells were incubated with OTR4120 or heparin alone. In subsequent studies, we used in vitro and in vivo assays to investigate the effect of OTR4120 on endothelial cell migration induced by low concentration of VEGF₁₆₅. In a Boyden chamber assay, 3 ng/ml VEGF₁₆₅ induced a slight but statistically significant increase in endothelial cell migration compared with non-stimulated cells (Fig. 5B). Endothelial cell migration induced by 3 ng/ml VEGF₁₆₅ was increased...
more than 2-fold in the presence of 3 ng/ml OTR4120. In our assay, OTR4120 alone in a dose of 3 ng/ml induced a slight but significant increase in endothelial cell migration compared with non-stimulated cells. Similar results were obtained using Matrigel™ plug assay. Matrigel™ plugs with or without VEGF165, OTR4120, or VEGF165 plus OTR4120 were injected subcutaneously into mice. Tissues were examined on day 6 (Fig. 6A). Heparin or OTR4120 alone (panels c and e, respectively) or in combination with VEGF165 (panels d and f, respectively) and VEGF165 alone (panel b) induced increased ingrowth of pseudo-capillary structures into the Matrigel™ compared with Matrigel™ alone (panel a). Quantitative analyses showed that a 2-fold increase in endothelial cell infiltration was observed when 50 ng/ml VEGF165 was added to Matrigel™, as compared with the control (Fig. 6B). Addition of 3 ng/ml OTR4120 or heparin induced a 6-fold increase

TABLE ONE

Determination of affinity constants of VEGF165-VEGFR interaction in the presence of OTR4120 and heparin

|          | VEGFR-1 |          | VEGFR-2 |          | NP-1 |
|----------|---------|----------|---------|----------|------|
|          | $K_d$   | $B_{max}$| $K_d$   | $B_{max}$| $K_d$| $B_{max}$|
| Control  | 59.4 ± 19.6| 10.4 ± 2.7| 292.5 ± 163.8 | 0.87 ± 0.38| 246.4 ± 135.1 | 18.8 ± 11.9 |
| OTR4120  | 6.8 ± 2.6 | 10.3 ± 0.9 | 45.2 ± 11.7 | 3.83 ± 0.57 | 29.6 ± 15.4 | 24.9 ± 4.8 |
| Heparin  | 17.7 ± 9  | 10.2 ± 3.2 | 55.9 ± 41.3 | 0.99 ± 0.28 | 6.5 ± 4.5 | 13.2 ± 2.3 |

FIGURE 5. OTR4120 enhanced VEGF165-induced HUVEC proliferation and migration. A, HUVECs were seeded with 2% FBS and starved with 0.5% FBS. After 24 h, 3 ng/ml VEGF165, OTR4120, or heparin (0.3, 3, or 33 ng/ml) alone or in combination were added. After 72 h, cells were dissociated with trypsin-EDTA and counted. The data are reported as the mean percentage of growth in the presence of stimulators compared with the control, obtained in triplicate in one of three independent experiments, with the indicated standard errors. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. B, HUVECs migration was studied using a modified Boyden chamber. Cells were incubated with or without 3 ng/ml VEGF165 or 10 ng/ml OTR4120 alone or in combination. After 12 h at 37 °C, the cells that had migrated through the pore to the lower filter surface were counted per microscopic fields magnified ×100. The data are reported as the mean of seven independent high power fields/well performed in triplicate in two independent experiments. The migration of unstimulated cells served as control and was taken as 100% migration. *, $p < 0.05$; **, $p < 0.01$.

FIGURE 6. OTR4120 enhanced in vivo angiogenesis. A, Matrigel™ was injected subcutaneously alone (panel a) or with 50 ng/ml VEGF165 (panel b), 3 ng/ml heparin (panel c), 50 ng/ml VEGF165 plus 3 ng/ml heparin mixture (panel d), 3 ng/ml OTR4120 (panel e), or 50 ng/ml VEGF165 plus 3 ng/ml OTR4120 mixture (panel f). The animals were killed 6 days later, and the Matrigel™ plugs were sectioned and stained using Gomori-Trichrome for microscopic observation (magnification: ×100). B, quantification of endothelial cell invasion into the Matrigel™ plug was performed as described under “Experimental Procedures.” Values are the mean area of endothelial cells; bars indicate ± S.E. of eight Matrigel™ fields per mouse (n = 5). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. 
in the angiogenic activity of VEGF<sub>165</sub> whereas a slight but significant angiogenic effect, similar to this obtained with VEGF<sub>165</sub> alone, occurred with OTR4120 or heparin alone.

These results led us to study the biochemical properties of the cells invading the Matrigel<sup>TM</sup>. First, as previously shown in numerous studies (38), the cells present in the Matrigel<sup>TM</sup> plugs were immunoreactive to CD31 antibody, confirming their endothelial nature. The number of endothelial cells detected by immunohistochemistry in differentially treated Matrigel<sup>TM</sup> plug was in accordance with previous quantification of Gomori cell staining (data not shown). In addition, the membrane expression of VEGFR-2 and -1 on invading cell surfaces was assessed immunologically, as shown on Fig. 7. Whereas endothelial cells were diffusely distributed in untreated or OTR4120-treated Matrigel<sup>TM</sup> (Fig. 7, A and B), the combination of OTR4120 and VEGF<sub>165</sub> induced endothelial cell alignment characteristic of pseudo-capillary structures (Fig. 7C). These observations were assumed on previous Matrigel<sup>TM</sup> slides staining. In contrast, no VEGFR-1 immunostaining was detected on these cells (Fig. 7, A’–C’).

DISCUSSION

In this study, we demonstrated that OTR4120, a member of a new pharmacological family called RGTA<sup>®</sup> that mimics the function of natural GAGs, promoted angiogenesis via affinity for VEGF<sub>165</sub>. Angiogenic activity of RGTA<sup>®</sup> has been described in several experimental models (18–21). In a model of skeletal rat muscle ischemia, Desgranges et al. (23) showed that these compounds enhanced muscle fiber regeneration and also angiogenesis after muscular ischemia. In these experiments, a higher density of differentiated blood vessels was observed in RGTA<sup>®</sup>-treated muscles compared with untreated ischemic muscles, supporting a role for RGTA<sup>®</sup> in angiogenesis regulation. Although the molecular mechanisms underlying the effects of RGTA<sup>®</sup> remained unclear, these results suggested that the biological effect of RGTA<sup>®</sup> might be ascribable, at least in part, to activation of endothelial growth factors such as VEGF<sub>165</sub>. Indeed, VEGF<sub>165</sub> expression is up-regulated following skeletal muscle ischemia (39). This hypothesis prompted us to undertake this study.

Several experiments have established that GAGs such as HS or DS play a pivotal role in regulating angiogenesis. Thus, Sassi et al. (40) reported that heparinase treatment inhibited neovascularization in the CAM by impairing growth factor binding to their high affinity receptor. More recently, the biological activity of an endothelial-derived proteoglycan called endocan, which regulates the biological activity of hepatocyte growth factor, was shown to be mediated by its GAG moiety and unrelated to the polypeptide (41). DS chains purified from endocan mimicked the biological activity of native endocan. Moreover, HS proteoglycans contributed to preserve an avascular environment in aqueous humor (42). Thus, many of the effects of HS or DS may be related to regulation of the bioavailability of heparin-binding growth factors. According to their molecular structure and/or their local concentration, these GAGs can inhibit or potentiate the binding and presentation of growth factors to their high affinity tyrosine kinase receptors.

Using binding assay, we documented direct VEGF<sub>165</sub> binding to OTR4120, as previously shown with heparin (43) and the vascular-expressed glypicans-1 (44). The interaction between OTR4120 and other heparin-binding growth factors has been investigated. In a previous study, we showed that RGTA<sup>®</sup> modulated collagen-type expression by interacting with FGF-2 (45). Binding analysis with a biosensor indicates that RGTA<sup>®</sup> binds FGF-2 with an affinity about 7-fold lower than that found with VEGF<sub>165</sub> suggesting that the angiogenic activity of OTR4120 may occur via interaction with VEGF<sub>165</sub> rather than FGF-2. Scatchard analysis indicated that the apparent affinity of VEGF<sub>165</sub> to OTR4120 was greater than its affinity to heparin. Studies of heparin structure have identified specific sulfated or acetylated sequences (46). Although the overall O-sulfonic and carboxymethyl content is similar in OTR4120 and heparin, the heparin-specific sequences, which might interact with VEGF<sub>165</sub>, have not been found in OTR4120, a fact that may explain the affinity differences observed.

Several synthetic components that bind heparin-binding growth factors and modulate their biological activities have been described. Recently, a substituted dextran with carboxymethyl benzylamide (CMDB7) and no sulfate group was shown to bind VEGF<sub>165</sub> (47). CMDB7 inhibited the mitogenic effect of VEGF<sub>165</sub> by preventing its binding to VEGFR-2. The chemically sulfated, yeast-derived phosphomannan PI-88 specifically binds to FGF-1, FGF-2, and VEGF<sub>165</sub> (48). Interestingly, compared with heparin, PI-88 has at least 11- and 3-fold greater affinity for FGF-1 and VEGF<sub>165</sub> respectively, but at least 13-fold lower affinity for FGF-2 (49). However, unlike OTR4120, both CMDB7 and PI-88 failed to potentiate cellular proliferation in response to VEGF<sub>165</sub>. Taken together, these results suggest the existence of specific structural domains involved either in potentiating or in inhibiting the biological activities of VEGF<sub>165</sub>. The OTR4120 angiogenic response is dose-dependent as shown on Fig. 1. This is in accordance with previous results showing that heparin stimulates angiogenesis in low concentrations but inhibits it in high concentrations (35, 50).

To investigate the biological consequences of the OTR4120-VEGF<sub>165</sub> interaction, we first determined that VEGF<sub>165</sub> binding to its high affinity receptors is enhanced in the presence of OTR4120. This finding agrees with previous evidence that VEGFR-1 and -2 are both heparin-sensitive.
Indeed, in addition to the presence of a heparin-binding site in both VEGFR-1 and -2 structures, heparin and glypicanc-1 enhanced VEGF₁₆₅ binding to VEGFR-2 (51). However, heparin failed to enhance VEGF₁₆₅ binding to VEGFR-1 expressing cells. This can be ascribed to the presence of natural heparan sulfate on the cell surface (52).

Differential roles for VEGFR-1 and VEGFR-2 have been established from transgenic studies. Mice deficient in VEGFR-2 showed impairments in vasculogenesis and hematopoiesis (53). Similar experiments suggested a role for VEGFR-1 in angiogenesis (54). Nevertheless, very recent work demonstrated that VEGFR-1 but not VEGFR-2 was involved in the recruitment of hematopoietic stem cells (55). In addition, whereas VEGFR-1 seems to be expressed in quiescent endothelium (56), VEGFR-2 is up-regulated in outgrowth vessels (57).

In agreement with previous studies (58), VEGFR-2 is up-regulated in outgrowth vessels (57). Similarly, VEGFR-2 is modulated specifically by a single concentration of OTR4120. In contrast, VEGFR-1 preferentially activate pathways downstream of VEGFR-2, given that VEGFR-1 expression in contrast to the CAM assay where VEGFR-1 is up-regulated in outgrowth vessels (57).

OTR4120 enhanced VEGF₁₆₅ affinity for VEGFR-1 and -2 and NP-1. Of these three compounds, only VEGFR-2 showed an increase in maximal binding (Bₘₐₓ) to VEGF₁₆₅. VEGF₁₆₅ expression is enhanced after hurt events such as ischemia. Considering the greater affinity of VEGF₁₆₅ for VEGFR-1, we suggest that low VEGF₁₆₅ levels may increase hematopoietic cells recruitment or enhance endothelium preservation and survival via VEGFR-1 activation. OTR4120 may potentiate these biological activities of VEGF₁₆₅. Higher VEGF₁₆₅ concentrations may activate angiogenesis via VEGFR-2. In this situation, OTR4120 may preferentially activate pathways downstream of VEGFR-2, given that VEGFR-1 seems to be up-regulated in outgrowth vessels (57).

The biological activities of VEGF₁₆₅ may be modulated specifically by a single concentration of OTR4120. VEGF₁₆₅ is a cytokine that potentiates angiogenesis via VEGFR-2, given that VEGFR-1 expression in contrast to the CAM assay where VEGFR-1 is up-regulated in outgrowth vessels (57).

In conclusion, numerous studies have established tissue regeneration and angiogenesis stimulation by RGTA®. These effects are seen with the sulfated and carboxymethylated derived dextran OTR4120, which belongs to the RGTA® family. Our study sheds light on the mechanisms underlying the neo-vessel outgrowth-stimulating effect of OTR4120, which is a well characterized and reproducible HS mimetic (30).

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