Undersulfated, low-molecular-weight glycol-split heparin as an antiangiogenic VEGF antagonist

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Vascular endothelial growth factor (VEGF) represents a target for antiangiogenic therapies in a wide spectrum of diseases, including cancer. As a novel strategy to generate nonanticoagulant antiangiogenic substances exploiting binding to VEGF while preventing receptor engagement, we assessed the VEGF-antagonist activity of a low-molecular-weight (LMW) compound (ST2184, Mw = 5800) generated by depolymerization of an undersulfated glycol-split heparin derivative. The parental compound was obtained by introducing regular sulfation gaps along the prevalently \(N\)-sulfated heparin regions, followed by glycol-splitting of all nonsulfated uronic acid residues (~50% of total uronic acid residues). ST2184 was endowed with a negligible anticoagulant activity after S.C. injection in mice. ST2184 binds VEGF165 as evaluated by its capacity to retard \(^{125}\)I-VEGF165 electrophoretic migration in a gel mobility shift assay and to prevent VEGF165 interaction with heparin immobilized onto a BIAcore sensor chip. Unlike heparin, ST2184 was unable to present \(^{125}\)I-VEGF165 to its high-affinity receptors in endothelial cells and inhibited VEGF165-induced neovascularization in the chick embryo chorioallantoic membrane. Undersulfated, LMW glycol-split heparins may therefore provide the basis for the design of novel nonanticoagulant angiostatic compounds.

Key words: angiogenesis/endothelium/growth factor receptor/heparin/VEGF

Introduction

Uncontrolled endothelial cell proliferation occurs in tumor neovascularization and in angioproliferative and angiogenesis-dependent diseases (Carmeliet and Jain, 2000). Thus, antiangiogenic agents may have broad applicability for the therapy for a wide spectrum of diseases, including cancer.

Vascular endothelial growth factor (VEGF) plays a major role in angiogenesis by acting via its tyrosine kinase receptors (VEGFRs) (Ferrara et al., 2003). VEGF/VEGFR antagonists affect tumor growth and vascularization (Margolin, 2002), and the VEGF-specific antibody bevacizumab exerts antivascular effects in cancer patients (Willett et al., 2004).

Among the five isoforms encoded by the VEGF gene (Ferrara and Davis-Smyth, 1997), VEGF165 plays a major role (Grunstein et al., 2000; Yu et al., 2002). Similar to several angiogenic growth factors, including fibroblast growth factors (FGFs) (Rusnati and Presta, 1996), VEGF165 binds heparin and heparan sulfate (HS) proteinpolylcans (HSPGs) (Gitay-Goren et al., 1992; Tessler et al., 1994). Heparin/HS interaction modulates the activity of VEGF and its interaction with VEGFRs (Cohen et al., 1995; Gitay-Goren et al., 1992; Tessler et al., 1994).

Heparin/HS derivatives and polyanionic heparin-like substances are endowed with antiangiogenic activity (Presta et al., 2003), a major mechanism of action being related to their capacity to bind heparin-binding growth factors and to prevent their productive interaction with signaling receptors (Presta et al., 2003). Interestingly, low molecular weight (LMW) heparin inhibits the angiogenic activity exerted by VEGF165 (Norrb by, 2000; Norrb by and Ostergaard, 1997)

Thus, the design of LMW heparin derivatives as VEGF antagonists can be envisaged.

Recently both thrombotic and hemorrhagic complications have been reported in cancer patients undergoing antiangiogenic therapy targeting the VEGF/VEGFR system (Daly et al., 2003). For instance, pulmonary hemorrhage has been observed in patients undergoing antiangiogenic therapy with the anti-VEGF bevacizumab. Even though the etiology of these complications remain unclear, these findings suggest that the use of antiangiogenic VEGF antagonists endowed with anticoagulant activity, for example, LMW heparin, should be approached with caution.

Recently we generated a novel nonanticoagulant antiangiogenic heparin derivative (ST1514) (Casu et al., 2002, 2004). Sulfation gaps along the regular heparin sequences were generated by selectively removing 2-O-sulfate groups to reach a ratio of about 1:1 between sulfated and nonsulfated uronic acid residues. Next, the C(2)–C(3) bonds of all nonsulfated uronic acid residues were split, generating flexible joints along the heparin chain while minimizing


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cleavage of glycosidic bonds. Because the splitting reaction also occurs at the level of the essential glucuronic acid residue of the active site for antithrombin, ST1514 was no longer anticoagulant, but it showed a potent FGF2 antagonist and angiostatic activity (Casu et al., 2002, 2004).

Here, we assess the capacity of ST2184, a LMW derivative of ST1514 (Casu et al., 2004), to interact with VEGF165 and act as a nonanticoagulant angiogenesis inhibitor.

Results

Structural characterization and anticoagulant activity of ST2184

Figure 1A shows the prevalent structures of heparin, ST1514, and ST2184, as established by $^1$H- and $^{13}$C-nuclear magnetic resonance (NMR) spectra (Casu et al., 2004). The glycol-split compounds consist of heparin tetrasaccharide sequences where one out of two L-iduronic acid residues were glycol-split by controlled periodate oxidation and reduced with sodium borohydride. Being generated by nitrous acid depolymerization, ST2184 bears 2,5-anhydromannose residues at the reducing terminal. $^{13}$C-NMR spectra and 2D experiments (not shown) indicate that ST2184 differs from ST1514 mainly for additional signals associated with terminal 2,5-anhydromannitol (aMan.ol) residues. The intensity of signals of these residues, as referred to the "internal" residues, is compatible with the average molecular weight determined by gel permeation chromatography (GPC) ($M_w = 5800$). The close similarity with the monodimensional and 2D NMR spectra of its precursor points to a similar molecular conformation for ST2184 and ST1514.

As anticipated, the splitting reaction causes a dramatic decrease in the anticoagulant activity of ST2184. Indeed, S.C. injection of ST2184 in BALB/c mice exerted only a limited and transient increase of the activated partial thromboplastin time (APTT) in the plasma of treated animals when compared to the potent and long-lasting anticoagulant activity of heparin (Figure 1B). Similar results were obtained after ST1514 administration (data not shown).

ST2184/VEGF165 interaction

Two experimental approaches were used to assess ST2184/VEGF165 interaction. First, $^{125}$I-VEGF165 was incubated with ST2184 or heparin and subjected to native polyacrylamide gel electrophoresis (Figure 2A). The mobility shift assay demonstrates that slow migrating $^{125}$I-VEGF165 complexes are formed starting from 60 ng or 600 ng/sample of heparin or ST2184, respectively. The slower migration rate of the heparin complex compared to the ST2184 complex is likely due to the smaller size and lower charge density of ST2184 compared to heparin.

Next, we assessed the ability of free VEGF165 to bind to biotinylated heparin immobilized onto a streptavidin-activated BIAcore sensor chip. Increasing concentrations of VEGF165 were injected over the heparin surface (Figure 2B, bottom). A $k_{on}$ of $2.6 \times 10^7$ M$^{-1}$ s$^{-1}$ and a slow $k_{off}$ of $2.7 \times 10^{-3}$ s$^{-1}$ characterized the interaction ($K_d = 100$ nM), whereas VEGF165 did not bind the streptavidin surface in the absence of immobilized heparin (Figure 2B, top).

On this basis, we evaluated the capacity of ST2184 to compete with immobilized heparin for the binding to VEGF165. ST2184 or heparin were preincubated with VEGF165 and injected onto the heparin-coated sensor chip. Both ST2184 and heparin caused a dose-dependent inhibition of immobilized heparin/VEGF165 interaction with ID$_{50}$ values equal to 100 nM and 10 nM, respectively (Figure 2C). A LMWH preparation inhibited VEGF165/heparin interaction with an ID$_{50}$ value equal to 300 nM (Fig. 2C). Thus ST2184 interacts with VEGF165 with an affinity ~10 times lower than heparin but 3 times higher than LMW heparin.

Antiangiogenic VEGF165-antagonist activity of ST2184

As observed for bovine endothelial cells (Gitay-Goren et al., 1992), Scatchard plot analysis of $^{125}$I-VEGF165 binding to human umbilical vein endothelial (HUVE) cells revealed a
high-affinity class of receptors ($K_d = 20$ pM; $B_{\text{max}} = 4$ fmol/10$^6$ cells) and a low-affinity class ($K_d = 0.25$ nM; $B_{\text{max}} = 22$ fmol/10$^6$ cells) (data not shown). Also, in keeping with previous observations (Gitay-Goren et al., 1992), low doses of heparin (up to 1–10 μg/ml) caused a three- to fourfold increase in $^{125}$I-VEGF$_{165}$ binding (Figure 3A). This effect is related to the capacity of heparin to bind directly to VEGFRs facilitating ligand interaction (Dougher et al., 1997), as already observed for FGF–receptor interaction (Klagsbrun and Baird, 1991). At higher doses, heparin caused a significant inhibition of $^{125}$I-VEGF$_{165}$ binding instead.

Unlike heparin, low doses of ST2184 were unable to increase the binding of $^{125}$I-VEGF$_{165}$ to HUVE cells, whereas similar to heparin, higher doses of the compound inhibited $^{125}$I-VEGF$_{165}$ binding both in the absence or in the presence of 1.0 μg/ml heparin (Figure 3A). When tested in a cell proliferation assay, ST2184 was more effective than heparin in inhibiting HUVE cell proliferation.
Next, the anti-VEGF165 activity of ST2184 was evaluated on the chick embryo chorioallantoic membrane (CAM) (Casu et al., 2004). ST2184 caused a significant inhibition of the angiogenic response triggered by VEGF165, similar to its precursor ST1514 (Figure 4A,B). Histological analysis confirmed the inhibitory effect. In contrast, heparin did not affect the angiogenic activity of VEGF165.

Discussion

Our strategy for designing novel nonanticoagulant antian- giogenic heparin derivatives was based on the generation of regular sulfation gaps along the nonsulfated regions of heparin, followed by glycol-splitting of nonsulfated uronic acid residues (Casu et al., 2002, 2004). Within a given range of 2-O-desulfation/glycol-splitting, these heparin derivatives prevent the formation of FGF receptor/FGF2/HSPG ternary complexes and inhibit FGF2-angiogenic activity (Casu et al., 2002, 2004). Here the undersulfated, glycol-split LMW ST2184 compound binds VEGF165 and exerts a significant VEGF165 antagonist activity and antiangiogenic action in the CAM assay, a model in which unmodified heparin is inactive (see Figure 4 and Casu et al., 2004).

Glycol-split heparin chains are more flexible than unmodified chains and conformationally driven to adopt geometries unfavorable for formation of ternary complexes with FGF2 and its receptor(s) (Casu et al., 2002, 2004). Indeed, unlike heparin, ST2184 was unable to facilitate the interaction of VEGF165 with its receptors. Instead, at very high concentrations both ST2184 and heparin sequester the growth factor and inhibit ligand binding and cell proliferation. However, only ST2184 exerts a significant antiangiogenic activity in vivo. The incapacity to support VEGF–VEGFR interaction may at least partly explain the angiostatic activity of ST2184.

Heparin is used in therapy as an anticoagulant and antithrombotic drug (Fareed et al., 2000). When administered to cancer patients, heparin increases survival times (Lebeau et al., 1994; Zacharski et al., 2000). Beneficial effects of heparin in cancer are though to be associated with the binding to and inhibition of one or more proteins overexpressed by tumor cells (Engelberg, 1999). Heparin and its LMW derivatives are currently being investigated as antitumor agents (Lebeau et al., 1994; Zacharski et al., 2000), and therapeutic regimes have been proposed to exploit their antmetastatic activity (Varki and Varki, 2002). However, the anticoagulant properties of heparin involve hemorrhagic risks, and nonanticoagulant variants of the polysaccharide endowed with potential antitumor properties are warranted (Lapierre et al., 1996). This is of importance when considering that antiangiogenic anti-VEGF interventions may be associated with hemorrhagic complications in cancer patients (Daly et al., 2003). Under- sulfated, glycol-split ST1514 and its LMW derivative ST2184 are endowed with a negligible anticoagulant activity when compared to heparin. These compounds may therefore provide the basis for the design of novel nonanticoagulant LMW angiostatic molecules.

Materials and methods

Materials

125I-VEGF165 was from Amersham Pharmacia Biotech (Little Chalfont, U.K.) and recombinant VEGF165 from Calbiochem Biochemicals (Darmstadt, Germany). Porcine mucosal heparin sodium salt (170 IU/mg, FU IX, average Mw = 13,600) was from Laboratorio Derivati Organici (Trino Vercellese, Italy). The other chemicals were from Sigma Chemical (St. Louis, MO).

Preparation of LMW ST2184

ST2184 was prepared by controlled nitrous acid depolymerization of glycol-split ST1514 (Casu et al., 2004). Briefly, 4.0 g of ST1514 were dissolved in 65 ml H2O at 4°C. NaNO2 (75 mg) was added and the pH adjusted to 2.0 with 0.1 M HCl. The solution was stirred at 4°C for 20 min. The pH
was then brought to 7.0. Solid NaBH₄ (1.0 g) was added in several portions under stirring. After 2–3 h the pH was adjusted to 4.0 with HCl, and the solution was neutralized with NaOH. The product was isolated by ethanolic precipitation. The extent of glycol-splitting was evaluated by integration of ¹³C NMR signals at 106.5 and 102 ppm, corresponding to C₁ of the split uronic residues and 2-O-sulfated iduronic residues, respectively. Yield: 75%. The average Mw, as evaluated by GPC, is 5800 (polydispersion 1.4) (Casu et al., 2004). LMW heparin was prepared from unfractionated heparin as for ST2184, using 100 mg NaNO₂ at 0°C. The relative content of terminal a.Man.ol residues (TR) with respect to total glucosamine residues was evaluated for both LMW compounds by integration of all anumeric ¹³C NMR signals at 98–107 ppm (a) and those at 82, 85, and 87 ppm (b) corresponding to total C₄, C₂, and C₅ of the terminal anhydromannitol unit (see Casu et al., 2004). TR (≈ 2b/3a + 2b) was 0.14 and 0.13 for LMW heparin and ST2184, corresponding to average Mn values (calculated from ratios aMan/total residues, including terminal aMan.ol) of 4200 and 3900 Da, respectively.

**Plasma clotting assay**

ST2184 or heparin were injected S.C. in BALB/c mice at 100 mg/kg. Mice were sacrificed by ether inhalation at different time intervals; blood was taken by intracardiac puncture using a plastic syringe containing 0.126 M sodium citrate (1:10, v/v) and platelet-poor plasma was obtained by centrifugation. APTT was measured using optimised Thrombofax reagent (Ortho Diagnostic Systems, Milan, Italy). Reference mouse platelet-poor plasma was used for comparison.

**Gel mobility shift assay**

¹²⁵I-VEGF₁₆₅ (10⁵ cpm/2 ng) was preincubated at room temperature for 30 min with ST2184 or heparin. Samples were then loaded on a native 7% polyacrylamide gel. The gel buffer was 10 mM Tris (pH 7.4) and 1 mM ethylenediamine tetra-acetic acid (EDTA), and the electrophoresis buffer was 40 mM Tris (pH 8.0), 40 mM acetic acid, 1 mM EDTA (Wu et al., 2002). The gel was run at 6 V/cm for 2 h, transferred to 3MM paper, dried under vacuum, and autoradiographed.

**BIAcore binding assay**

A BIAcore F1 sensorchip was activated with streptavidin. Then, heparin biotinylated on its reducing end was allowed to react with the streptavidin-coated surface. VEGF₁₆₅ (300 nM) alone or in the presence of heparin derivatives was dissolved in binding buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P₂₀, pH 7.4) and injected over the heparin surface for 5 min. Then the sensor chip was washed with the same buffer until dissociation was observed. The surface plasmon resonance signal was expressed in terms of resonance units (RU).

¹²⁵I-VEGF₁₆₅ binding assay

HUVE cells (Clonetics, Cambrex, Milan, Italy) were seeded at 200,000/well in 24-well plates and incubated overnight in M199 medium (Invitrogen, Carlsbad, CA) containing 20% fetal bovine serum, 2 mM glutamine, and 2 ng/ml FGF2. For Scatchard plot analysis, cells were seeded. Then, cells were incubated for 2 h at 4°C with 0–1.26 nM ¹²⁵I-VEGF₁₆₅ and 900 ng/ml unlabeled VEGF₁₆₅ dissolved in 0.2 ml M199 with 0.2% gelatin, 1.25 mM HEPES. Cells were then solubilized with 1% Triton X-100 and radioactivity measured using a γ-counter. Binding data were analyzed by the Scatchard procedure using PRISM software. For competition binding studies, cells were incubated with ¹²⁵I-VEGF₁₆₅ (4.0 ng/ml) in the presence of 10–1000 μg/ml of heparin or of ST2184 both in the absence or in the presence of 1.0 μg/ml heparin. Nonspecific binding in the presence of 900 ng/ml of unlabeled VEGF₁₆₅ was subtracted from all the values.

**HUVE cell proliferation assay**

Cells were seeded at 20,000/cm² and incubated overnight in complete EGM2 medium (BioWhittaker, Verviers, Belgium). Next, cells were treated for 72 h with fresh EGM2 medium in the presence of 0.1–1.0 mg/ml of ST2184 or heparin. Finally, cells were trypsinized and counted.

**CAM assay**

Gelatin sponges (1 mm³) were placed onto the CAM of fertilized chicken eggs on day 8 of development, immediately followed by topical administration (2–3 μl) of VEGF (500 ng/egg) in the presence of vehicle or of 100 μg/egg of heparin derivatives (Casu et al., 2004). Treatments were repeated daily for 4 days. At days 8 and 12, the number of macroscopic vessels converging toward the implant were assessed under a stereomicroscope. Also, angiogenesis was quantified at day 12 by a planimetric method of point counting on paraffin-embedded 8-mm sections (Casu et al., 2004).

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**Abbreviations**

aMan.ol, anhydromannitol 6-sulfate; APTT, activated partial thromboplastin time; CAM, chorioallantoic membrane; EDTA, ethylenediamine tetra-acetic acid; FGF, fibroblast growth factor; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; HUVE, human umbilical vein endothelial; LMW, low molecular weight; NMR, nuclear magnetic resonance; VEGF, vascular endothelial growth factor; VEGFR, tyrosine kinase VEGF receptor.

**References**


