Structural basis and potential role of heparin/heparan sulfate binding to the angiogenesis inhibitor endostatin

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Recombinant mouse endostatin produced by mammalian cells was shown to bind to heparin with a $K_d$ of 0.3 μM, suggesting that this interaction may play a role in its anti-angiogenic activity. Alanine mutagenesis demonstrated that a major site of four clustered arginines (positions 155, 158, 184 and 270) and a second site (R193,R194) are essential for binding. The same epitopes also participate in endostatin binding to heparan sulfate and sulfatides but not in its binding to the extracellular protein ligands fibulin-1 and fibulin-2. Analyses with various heparin fragments demonstrated a minimum size (12mer) for efficient binding to endostatin and a crucial role of 2-O- and 6-O-sulfation. Furthermore, a substantial proportion (10–50%) of heparan sulfate chains obtained from various tissues showed a distinct binding to endostatin, indicating its potential to interact with extracellular and/or membrane-bound proteoglycans. Angiogenesis induced by basic fibroblast growth factor-2 (FGF-2), but not by vascular endothelial growth factor (VEGF), in a chick chorioallantoic membrane assay could be inhibited by vascular endothelial growth factor (VEGF), in a chick chorioallantoic membrane assay could be inhibited by heparin, but their mode of interaction with the physiological substrate heparan sulfate and their structural requirements are less well established. Platelet factor 4 (PF4) was shown to inhibit FGF-2-mediated mitosis and migration of endothelial cells (Sato et al., 1990; Watson et al., 1999). The abundant FGF-2 is one of the best characterized members of a family of structurally related cytokines that have strong mitogenic and angiogenic activity. This activity is mediated through their binding to several tyrosine kinase receptors, thereby inducing intracellular signaling cascades (Klint and Claesson-Welsh, 1999). FGFs bind to membrane-bound and extracellular heparan sulfate proteoglycans and this binding is considered to be important for their extracellular storage, conferring protease resistance and co-receptor action (Galzio et al., 1997). A minimum FGF-2 binding tetra- to hexasaccharide sequence in heparan sulfate contains N-sulfated GlcN units and a single IdoA 2-O-sulfate group (Habuchi et al., 1992; Maccarana et al., 1993; Tyrrell et al., 1993), while the presence of 6-O-sulfate and larger oligosaccharide structures is required to allow receptor activation (Guimond et al., 1993; Ishihara et al., 1993; Pye et al., 1998). These findings along with crystallographic analyses of oligosaccharide binding sites on FGF-1 and -2 (Faham et al., 1996; DiGabriele et al., 1998; Venkataraman et al., 1999) have led to the concepts that heparan sulfate-mediated FGF-2 dimerization in cis-orientation or bridging of FGF-2 with its tyrosine kinase receptors are required for induction of mitogenic activity (Guimond et al., 1993; Herr et al., 1997; Moy et al., 1997). This work and similar concepts also generated questions as to how biodiversity of heparan sulfates is regulated at the cell and tissue level to accommodate specific requirements during development and the need to interact with diverse mitogenic cytokines (Salmivirta et al., 1996; Lindahl et al., 1998; Lyon and Gallagher, 1998).

Introduction

Formation of new blood vessels by sprouting from the existing microvascular system, which is referred to as angiogenesis, plays a critical role not only during embryonic development but also during subsequent growth and tissue regeneration, including the efficient growth of tumors (Risau, 1995, 1997; Hanahan and Folkman, 1996). Angiogenesis is initiated by various cytokines such as basic fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF), and at later stages becomes dependent on platelet-derived growth factor and transforming growth factor β1 (Beck and D’Amore, 1997; Risau, 1997). These processes are controlled by a variety of endogenous angiogenesis inhibitors (Cao, 1998), including chemokines, thrombospondins, hormones and the proteolytic fragments angiotatin and endostatin, derived from plasminogen and collagen XVIII, respectively (O’Reilly et al., 1994, 1997). The latter two have received particular attention because of their ability to reduce tumor angiogenesis and growth in experimental mouse models (O’Reilly et al., 1996, 1997; Boehm et al., 1997; Dhanabal et al., 1999a; Yamaguchi et al., 1999). The molecular mechanisms of how such inhibitors interfere with stimulators of angiogenesis have not previously been elucidated. As discussed for endostatin (Hohenester et al., 1998), a possible mechanism is competition for the heparin/heparan sulfate binding sites, which act as co-receptors for several cytokines.
et al., 1994), possibly by interfering with FGF-2 binding to the two types of FGF receptors involved and with FGF-2 dimerization (Perollet et al., 1998), processes that are in part sensitive to heparin inhibition. Yet a recombinant PF4 mutant that lacks heparin-binding was still active in several of these assays (Maione et al., 1991). Thrombospondin-1 was shown to bind directly to FGF-2 and to block its binding to endothelial cells, interactions that could both be inhibited by heparin (Taraboletti et al., 1997).

We have recently used mammalian cells to prepare recombinant endostatin in a highly soluble form (Sasaki et al., 1998) that was not available from bacterial production (Boehm et al., 1998; Dhanabal et al., 1999a). This has set the stage for identifying its heparin-binding epitope by site-directed mutagenesis and determining structural parameters of heparin/heparan sulfate that are required for binding. Furthermore, the use of a sensitive FGF-2-dependent angiogenesis assay demonstrated a lack of inhibition by endostatin after abolishing its heparin binding. This is the first evidence for a potential biological role of heparin binding of endostatin.

Results

Characterization of the heparin-binding epitope of endostatin by site-directed mutagenesis

Previous studies have demonstrated that recombinant mouse endostatin obtained from transfected human kidney cells is folded into a compact globular structure (Hohenester et al., 1998) and shows a distinct binding to heparin. Furthermore, side chain modifications indicated that several arginines but not the lysines contribute to this interaction (Sasaki et al., 1998). In order to map the binding epitope, we have now mutated to alanine nine solvent-exposed arginines either individually or in double and triple combinations (Table I). Alanine mutagenesis was also applied to three hydrophobic residues (F162, F165, L185), which also showed an unusual surface exposure (Hohenester et al., 1998). Furthermore, since human endostatin has been shown to bind one atom of zinc (Ding et al., 1998), we produced two more mutants, D207A and H134A/D136A, in order to prevent this ligation.

All 16 of these mutants could be readily obtained from serum-free culture medium of transfected human 293-EBNA cells in yields comparable to that of the wild-type protein (Sasaki et al., 1998), indicating that the mutations did not interfere with proper folding. They could easily be purified by heparin chromatography at low ionic strength, followed by molecular sieve chromatography, where they eluted in the monomer position (Sasaki et al., 1998). In SDS–PAGE, all except one showed the same 22 kDa band and a purity of >95% (data not shown). The mobility of mutant H134A/D136A was slightly lower. The purity of a few of these mutants was underscored by Edman degradation, which demonstrated a single APLAHTH N-terminal sequence with APLA being derived from the expression vector signal peptide cleavage region (Pöschl et al., 1996).

Atomic emission spectroscopy demonstrated a zinc content of 1.02 ± 0.06 atoms for endostatin and the same value for the mutant R158/184/270A, indicating full occupation of the single zinc binding site. This content was decreased to 0.07 and 0.02 in the mutants H134A/D136A and D207A, respectively. Mass spectrometry showed a molecular mass of 20 595 Da for endostatin (calculated 20 724 Da) and of 20 342 Da for mutant R158/184/270A (calculated 20 469 Da), consistent with the removal of the C-terminal Lys, as also shown by carboxypeptidase treatment. A polyclonal antiserum and several monoclonal antibodies had ELISA titers within a narrow range for endostatin and all mutants, indicating the same set of immunological epitopes.

In analytical heparin affinity chromatography, all mutants bound completely in 0.05 M Tris–HCl pH 7.4, and the NaCl concentrations required for displacement were determined (Table I). When compared with endostatin (0.35 M NaCl), four single Arg mutations showed a distinct reduction to either 0.25 M (R155A,R184A) or 0.19 M (R158A,R270A) with an even greater reduction in the double and triple mutants. The elution behavior of R158/270A (0.11 M) and R158/184/270A (0.08 M) indicated that they no longer bind heparin at physiological ionic strength. A smaller but significant reduction in binding was observed for mutant R193/194A (0.27 M). Mutations of some other Arg residues (R169, R241, R259), of three hydrophobic residues and of the zinc binding site (0.35 M NaCl) were determined (Table I). When compared with endostatin (calculated 0.25 M), four single Arg mutations showed a distinct reduction to either 0.25 M (R155A,R184A) or 0.19 M (R158A,R270A) with an even greater reduction in the double and triple mutants. The elution behavior of R158/270A (0.11 M) and R158/184/270A (0.08 M) indicated that they no longer bind heparin at physiological ionic strength. A smaller but significant reduction in binding was observed for mutant R193/194A (0.27 M). Mutations of some other Arg residues (R169, R241, R259), of three hydrophobic residues and of the zinc binding site had no apparent effect on heparin binding (Table I).

Endostatin has also been shown to bind to sulfatides (Sasaki et al., 1998), which raised the question as to whether these binding epitopes are related to those involved in heparin binding. This was examined by solid-phase binding assays, as illustrated in Figure 1, in order to determine the concentrations of endostatin and its mutants required for half-maximal binding (Andac et al., 1999a).
Fig. 1. Solid-phase assay of endostatin binding to immobilized sulfatides. Soluble ligands were mouse endostatin (◦) and its mutants R158/169A (●), R158/270A (△), R193/194A (▲), R158/184/270A (▽) and F162A (▼). Binding was detected with a rabbit antiserum against mouse endostatin, which reacted equally well with all soluble ligands used. The assay was carried out in 0.05 M Tris–HCl pH 8.0, 0.11 M NaCl.

1999). These concentrations showed a clear inverse relationship with the NaCl concentrations needed for displacement from heparin (Table I), indicating a considerable overlap of binding epitopes. Typical vessel wall components such as the microfibrillar fibulin-1 and fibulin-2 were also shown to bind endostatin (Sasaki et al., 1998; Miosge et al., 1999). Binding of most mutants to these ligands was also examined in solid-phase binding assays, but no loss of binding activity was observed, except for a 3- to 4-fold reduction in the binding of mutants R158, 169A, H134A/D136A, F165A and L185A (Table I).

Further binding assays were performed with FGF-2, which, as expected, interacted strongly with immobilized heparin–albumin conjugate (half maximal at 2 nM). However, no binding was observed to mouse and human endostatin up to a concentration of 1 μM FGF-2. Surprisingly, mouse and human endostatin up to a concentration of 2 μM did not bind or only weakly bound to heparin–albumin in physiological buffer. This did not allow examination of the possible competition between FGF-2 and endostatin for the same heparin epitopes.

Spatial structure and interpretation of the heparin binding epitope

The interpretation of the mutagenesis data in the context of the crystal structure of mouse endostatin (Hohenester et al., 1998) strongly indicates a major primary and a secondary heparin binding site (Figure 2). R158 and R270, which are spatially close to one another (Cα-Cα distance 10 Å), make the largest contribution and are in close proximity to R155 and R184, which also contribute to a lesser extent. The secondary binding site is constituted by R193 and/or R194, which, notably, are located on the same face of endostatin but at some distance (~30 Å) from R158 and R270. Mutation of several other arginine residues did not have any effect on heparin binding, demonstrating the specificity of binding to the basic surface patch centered on R158 and R270.

The side chains of arginine residues implicated in heparin binding appear to be highly flexible, since they assume different conformations in three distinct crystal structures of mouse endostatin, namely the original structure at pH 5 (Hohenester et al., 1998) and two new crystal forms at neutral pH (E.Hohenester, unpublished data). This observation, together with the known structural and conformational heterogeneity of heparin-like polysaccharides in general (Faham et al., 1996; Salmivirta et al., 1996; DiGabriele et al., 1998; Lindahl et al., 1998; Fry et al., 1999), prevented any attempts to use model building to gain further insights into the endostatin–heparin interaction. Nevertheless, some interesting structural conclusions can be drawn from the mutagenesis results. As shown in Figure 2B, a tetrasaccharide composed of two IdoA–GlcN disaccharide units would roughly match the size of the basic patch defined by R155, R158, R184 and R270. Given the high positive charge density in this region, it can be assumed that endostatin primarily binds
Heparin binding to endostatin

Fig. 3. Dose-dependent binding of heparin to endostatin. Endostatin (2 μg) was incubated with increasing amounts of [3H]heparin, and endostatin-heparin complexes were recovered using the filter trapping assay. Bound heparin was quantified in a liquid scintillation counter. Each data point represents the mean of duplicate incubations. A logarithmic curve fit was made using Cricket Graph v. 1.5.3 (Computer Associates International, Inc.).

Fig. 4. Affinity chromatography of 3H-labeled heparan sulfate chains from bovine kidney on endostatin. The column was loaded with 30 000 d.p.m. and eluted with an NaCl gradient (dashed line). to sulfate-rich stretches of heparan sulfate molecules. The location of a secondary binding region near R193/R194 implies that a considerably longer heparan sulfate sequence (10–12mer) would be required, in order to bind simultaneously to both the primary and secondary sites (see below).

Characterization of the binding region for endostatin on heparin/heparan sulfate

The complexity of the heparin binding epitope on endostatin and the important question of whether heparan sulfate has similar binding properties led us to a more precise study of the carbohydrate ligands involved. This was performed with radioactively labeled ligands used either in a filter trapping assay or in affinity chromatography on an endostatin column. The results showed heparin binding to endostatin in a dose-dependent and saturable manner (Figure 3). The binding was of moderate affinity ($K_d = 0.3 \mu M$), as revealed by Scatchard analysis (data not shown). Samples of heparan sulfate from different tissues were subjected to affinity chromatography on immobilized endostatin. Approximately 50% of bovine kidney heparan sulfate bound to the column and was displaced in a sharp peak at 0.22 M NaCl (Figure 4), thus, at a lower NaCl concentration than that required for endostatin elution from heparin (0.35 M) as shown in Table I. We could also show that 50% of heparan sulfate from lung and 10% from aorta bound to endostatin, indicating the general nature of the interaction. Furthermore, the endostatin mutants R158/270A and R158/184/270A showed >90% reduction in heparan sulfate binding (Figure 5), indicating a close similarity of binding epitopes for heparin and heparan sulfate. Mutation of the secondary heparin binding site (R193/194A) gave a less pronounced reduction in binding, but this reduction was, however, more prominent for heparan sulfate than for heparin (Figure 5).

A series of radiolabeled, even-numbered heparin oligosaccharides was used in both assays for the determination of the minimum length of the endostatin binding heparin domain. The shortest oligomers with appreciable affinity for heparin in the filter trapping assay were 12mers (Figure 6A). Longer (16–20mer) saccharide species showed a 2- to 4-fold increase in binding. Affinity chromatography on endostatin demonstrated that oligosaccharides ranging from 6 to 20 monosaccharides in length all bound to the column but apparently with different strengths (Figure 6B). The NaCl concentrations required for displacement were 0.23–0.28 M NaCl for 6–10mers and 0.33–0.37 M NaCl for 12–20mers. Both assays distinctly discriminated between 10- and 12mers. Collectively, these data suggest that heparin fragments of 12 or more monosaccharide units are required for efficient binding to endostatin. Notably, a heparin 12mer no longer bound significantly to endostatin mutants that had a reduced affinity for full-sized heparin (Figure 5).

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Endostatin inhibition of angiogenesis induced by FGF-2 and VEGF

Angiogenesis in the chorioallantoic membrane (CAM) is efficiently induced by different cytokines and phorbol
14mers (d) analyzed in a scintillation counter.

Effect of angiogenesis inhibitors (Brooks et al. esters, making it a suitable model to examine the effect of angiogenesis inhibitors (Brooks et al., 1994; Friedlander et al., 1995). Using fertilized chicken eggs, with the CAM exposed by a small window in the shell, angiogenesis was strongly induced by applying filter disks containing 0.2 μg/disk of FGF-2 or VEGF onto the CAM. Inclusion of endostatin led to inhibition of angiogenesis to different extents. The inhibition of FGF-2-induced angiogenesis was dose-dependent and close to complete at a 10-fold molar excess (3 μg endostatin; Figure 8; Table II). In contrast, induction of angiogenesis by VEGF165 was not efficiently inhibited by a 10-fold molar excess of endostatin (Figure 8; Table II). Nevertheless, the VEGF/endostatin-treated vessels showed fewer vessel branch points (Table II) as well as a reduced diameter (Figure 8) compared with the buffer control.

Various endostatin mutants were analyzed for their effect on FGF-2-induced angiogenesis in the CAM. Three mutants that had reduced heparin binding displayed loss-of-function and had only a marginal effect on FGF-2-induced CAM angiogenesis, as shown for R158/184/270A in Figure 8 and more quantitatively for R158/270A and R193/194A in Table II. The loss-of-function was almost complete at a 10-fold molar excess of the endostatin mutants, although a slight reduction in the number of branch points, as well as in vessel diameter, was seen to different extents for the different mutants. These data indicate that both primary and secondary heparin binding epitopes are essential for inhibitory activity. Loss of inhibition seems to be specific, since the zinc binding site mutants D207A and H134,D136A were as active as endostatin (Table II). A further mutant, L185A, was also fully active (not shown).

Discussion

The previously reported crystal structure of mouse endostatin demonstrated a compact globular fold with one exposed face particularly rich in Arg residues (Hohenester et al., 1998). From this, and the results of side chain modifications (Sasaki et al., 1998), the localization of the heparin binding epitope was predicted. This has now been confirmed by site-directed mutagenesis. A surprising observation was the existence of a primary binding site centered around Arg158 and Arg270 and a secondary site involving Arg193 and/or Arg194. The spatial relationship of both binding sites clearly indicated that they could be occupied simultaneously by a single heparin molecule. Binding to sulfatides, which has been speculated to facilitate cellular interactions of endostatin (Sasaki et al., 1998), has now been shown to overlap considerably with both heparin binding sites. A similar overlap of heparin and sulfatide binding epitopes was recently shown for the α1LG4 module of laminin α1 chains, which in addition is involved in the binding of the cellular receptor α-dystroglycan (Andac et al., 1999).

The mutants prepared in this study also demonstrated that endostatin binding to the microfibrillar components fibulin-1 and fibulin-2 does not significantly overlap with heparin binding. Binding to the fibrils with a $K_d = 20–100$ nM is considered to be a means of storing endostatin in tissues (Sasaki et al., 1998; Miosge et al., 1999). This may explain why low concentrations of exogenous endostatin (13–130 nM) bind to tissue sections without requiring heparan sulfate (Chang et al., 1999), which, as shown here, has a distinctly lower affinity for heparan sulfate than for fibulins. Heparin binding to endostatin was also not affected by loss of zinc binding. Apart from Asp207, zinc binding involves mainly N-terminal residues of endostatin that are spatially remote from the
Endostatin binds to heparin with moderate affinity ($K_d = 0.3 \mu M$) and, most importantly, also to various heparan sulfates. The latter components have a broad tissue distribution contrary to heparin, which is confined to mast cells. Heparan sulfate substitutions can occur on a large number of proteoglycans, at the cell surface or in the extracellular matrix, and are therefore major candidates for endostatin binding in vivo. They also have a highly versatile and heterogeneous molecular structure compared to heparin (Salmivirta et al., 1996; Lindahl et al., 1998; Lyon and Gallagher, 1998). Nevertheless, the studies using various heparin derivatives along with the mutational analysis may provide some insights into the mode of interaction between endostatin and heparan sulfate. The compact primary site is non-contiguous in sequence as found for several other heparin-binding proteins (Lander, 1994; Spillmann and Lindahl, 1994) and matches a 4–6mer saccharide structure. Simultaneous binding of this region and of the secondary binding site (Arg193,194) would require a longer 10–12mer. Affinity chromatography on endostatin showed moderate binding of 6–10mers (Figure 6) in reasonable agreement with predictions for the primary binding site (Figure 2). A distinct increase in binding strength was observed for 12mers suggesting occupation of both binding sites.

Similar extended saccharide sequences, presumably involving at least two interaction sites, have previously been implicated in the binding of other proteins such as the FGF-2–FGF receptor complex (Guimond et al., 1993; Pye et al., 1998), the herpes simplex gC glycoprotein (Feyzi et al., 1997b), fibronectin (Sharma et al., 1999) and the IL-8 dimer (Spillmann et al., 1998). A heparan sulfate sequence specifically tailored for interactions with endostatin would not necessarily be composed exclusively of contiguous N-sulfated disaccharide units, but could conceivably contain two N-sulfated domains for the interaction with both endostatin binding sites that are separated by one or more N-acetylated disaccharide units. Further evidence showed the importance of both 2-O- and 6-O-sulfate groups for endostatin binding as also found for the other interactions specified above. This also supports the notion that polar interactions between various sulfate

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**Table II.** Inhibitory effects of endostatin (ES) and endostatin mutants on chick chorioallantoic membrane angiogenesis induced by FGF-2 or VEGF

<table>
<thead>
<tr>
<th>Inducer</th>
<th>Inhibitor</th>
<th>No. of embryos</th>
<th>Angiogenesis score$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>none</td>
<td>23</td>
<td>1.0</td>
</tr>
<tr>
<td>FGF-2, 0.2 μg</td>
<td>none</td>
<td>15</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>ES, 0.3 μg</td>
<td>5</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>ES, 3 μg</td>
<td>6</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>ES, 30 μg</td>
<td>3</td>
<td>1.2</td>
</tr>
<tr>
<td>VEGF, 0.2 μg</td>
<td>none</td>
<td>9</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>ES, 3 μg</td>
<td>6</td>
<td>3.3</td>
</tr>
<tr>
<td>FGF-2, 0.2 μg</td>
<td>R158/184/270A, 3 μg</td>
<td>5</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>R193/194A, 3 μg</td>
<td>5</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>D207A, 3 μg</td>
<td>7</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>H134A,D136A, 3 μg</td>
<td>5</td>
<td>1.1</td>
</tr>
</tbody>
</table>

$^a$Score from 1 (low) to 4 (high) according to Friedlander et al. (1995). Average values of the embryos are recorded. The variability was <20%.
groups and arginines make major contributions to endostatin–heparin binding. In addition, other residues may provide hydrogen bonds, such as shown for complexes between heparin oligosaccharides and FGFs (Faham et al., 1996; DiGabriele et al., 1998). A more precise analysis of the endostatin binding epitopes will require similar co-crystallizations of endostatin and suitable heparin oligosaccharides, which have not yet been successful.

Both the primary and secondary heparin binding sites of endostatin were also found to be crucial for endostatin inhibition of CAM angiogenesis induced by FGF-2. Nearly complete inhibition required a 10-fold molar excess of endostatin over FGF-2, while the same amounts of mutants R193/194A, R158/270A and R158/184/270A were inactive. These and other data allow some speculations as to how endostatin interferes with angiogenesis induced by FGF-2. Induction of angiogenesis by FGF-2 is most likely to be dependent on binding to the FGF tyrosine kinase receptors. This process requires heparan sulfate co-receptors, which bind directly to FGF-2 and promote the productive interaction with its tyrosine kinase receptor (Galzle et al., 1997; Klint and Claesson-Welsh, 1999). While the detailed molecular mechanism behind this effect still remains unclear in part (DiGabriele et al., 1998; Hulttala et al., 1999; Venkataraman et al., 1999), it definitively involves a 10–12mer heparan sulfate sequence that is considerably longer than the 5mer structure required to simply bind the growth factor (Guimond et al., 1993; Herr et al., 1997; Moy et al., 1997). This oligosaccharide size is remarkably similar to that predicted for the concerted occupation of the primary and secondary heparin binding sites on endostatin. Since we were not able to detect a direct interaction between FGF-2 and endostatin it is tempting to postulate that endostatin competes with FGF-2 and/or its receptor for the same 12mer binding sites on the heparan sulfate chains. The relatively high affinity of these chains for FGF-2 with $K_d = 0.03–0.2$ nM (Rahmoune et al., 1998) compared with that for endostatin would explain the need for an excess of endostatin for inhibition. Other possibilities include co-receptor function(s) of heparan sulfate required for specific signaling events induced by endostatin. Heparan sulfate proteoglycans could potentially promote a variety of such interactions through their ability to capture and thus locally concentrate soluble ligands (Lander, 1998) such as endostatin.

Contrary to the effects on FGF-2-induced angiogenesis, endostatin showed only little inhibition of angiogenesis induced by VEGF165. This form of the cytokine binds heparin and heparan sulfates, probably via two basic clusters in its C-terminal module (Fairbrother et al., 1998). Yet the co-receptor function of this interaction is still controversial and the most accepted model of action involves clustering of VEGF receptors 1 and 2 by disulfide-bonded VEGF dimers (Neufeld et al., 1999). There are also notable differences in other aspects of angiogenesis induced by FGF-2 or VEGF that are connected to the expression of either αβ3 or αβ5 integrins, and a difference in sensitivity to a protein kinase C antagonist (Friedlander et al., 1995).

A strong reduction in the zinc content of endostatin by two different mutations had no effect on its potential to inhibit angiogenesis. This single zinc has a structural function in the N-terminal region and is very likely to have no catalytic role (Ding et al., 1998; E.Hohenester, unpublished data). Similar mutations introduced into bacterial endostatin were shown to reduce its ability to suppress tumor growth in mice (Boehm et al., 1998). This recombinant material was highly insoluble and therefore used in high amounts (50 mg/kg), and it is not yet clear whether the loss of zinc caused a refolding problem or interfered with biological activity. Other studies with VEGF-stimulated umbilical vein endothelial cells demonstrated very efficient inhibition of migration by endostatin, which was not lost by mutation of the primary and secondary heparin binding site or deletion of the zinc binding site (Yamaguchi et al., 1999). Furthermore, this zinc mutant was as efficient as wild-type endostatin in the inhibition of a renal carcinoma in mice.

In summary, our data provide the first evidence of a particular biological function of the heparin binding epitope of endostatin. Recent data indicate that the same epitope is important for the induction of tyrosine kinase activity in endothelial cells, which led to the phosphorylation of several adaptor molecules (J.Dixelius, H.Larsson, T.Sasaki, L.Lü, A.Engström, R.Timpl, M.Welsh, L.Claesson-Welsh, manuscript submitted). As already discussed, there may be other mechanisms for the interaction of endostatin with endothelial cells, which, apart from leading to inhibition of migration (Yamaguchi et al., 1999), may also induce apoptosis (Dhanabal et al., 1999b). This suggests a versatile repertoire of endostatin for cellular interactions.

**Materials and methods**

**Transfection vectors and mutations**

Episomal expression vectors for mouse and human endostatin were used to transfect human kidney EBNA-293 cells (Sasaki et al., 1998). The mouse vector was used as a template to introduce single to triple point mutations by appropriate pairs of overlapping oligonucleotides by fusion PCR (Vallejo et al., 1994; Pöschl et al., 1996). Cycle sequencing using Dye Terminator Cycle Sequencing Ready Reaction Kit (ABI) was used to confirm the correct introduction of the mutations. All vectors were used to transfect EBNA-293 cells and to obtain serum-free culture medium containing the recombinant proteins.

**Protein purification and characterization**

Wild-type endostatin was purified by heparin–Sepharose and Superox 12 chromatography (Sasaki et al., 1998). The procedure for purifying the mutants was similar except that serum-free culture medium (0.3–0.5 I) was dialyzed against 0.05 M Tris–HCl pH 7.4, and passed over a heparin-Sepharose column (2.5 × 20 cm) or a 5 ml heparin HiTrap column (Pharmacia) equilibrated in the same buffer. Elution was then performed with a linear 0–0.6 M NaCl gradient. An analytical 1 ml heparin HiTrap column was used to determine the concentration of NaCl required for displacement with a precision of ± 0.01 M NaCl (Andac et al., 1999). Purified proteins were characterized by SDS–PAGE and protein concentrations were determined on a Biotronik LC3000 analyzer after hydrolysis with 6 M HCl (16 h, 110°C). Electrospray ionization mass spectroscopy was used for molecular mass determination. Zinc contents were determined by atomic emission spectroscopy in argon plasma (ICP Spectro equipment). ELISA titration with a polyclonal rabbit antiserum against mouse endostatin (Sasaki et al., 1998) and several rat monoclonal antibodies (our unpublished data) followed established protocols.

**Glycosaminoglycan preparations**

Heparin from pig intestinal mucosa was radiolabeled by $^3$H-acetylation of free amino groups (specific activity $0.34 \times 10^6$ d.p.m./nmol disaccharide) as described previously (Höök et al., 1982). The preparations of O-desulfated heparin dodecasaccharides were as described earlier.
In the 2-O-desulfated heparin preparation, 83% of all GlcNSO 3 6-O-sulfate groups, but also of ~30% of the IdoA residues with NaB₃H₄ (Amersham Pharmacia Biotech), yielding labeled saccharides were radiolabeled by reduction of their anhydromannose residues with NaBH₄ (Amersham Pharmacia Biotech), yielding labeled 2,5-anhydromannitol units. Labeled oligosaccharides were separated on a column of Bio-Gel P-10 (Bio-Rad; 1 × 190 cm) in 0.5 M NaH₂HCO₃ to fractions corresponding to 6–14mers and larger oligosaccharides, which were all pooled, desalted and stored at –20°C until further use.

The specific radioactivities of the saccharides were determined by scintillation counting and saccharide quantification using the carbazole reaction for hexuronic acid (Bitter and Muir, 1962).

Binding studies

For analytical nitrocellulose trapping experiments (Maccarana and Lindahl, 1993), 2 μg of endostatin were incubated with radiolabeled saccharides in 100 μl of phosphate-buffered saline (PBS pH 7.4) containing 0.1 mg/ml bovine serum albumin (Sigma) at room temperature for 2 h. After the incubation, the mixture was rapidly passed through a nitrocellulose filter (Sartorius, diameter 25 mm, pore size 0.45 μm) previously washed with PBS using a vacuum-assisted filtering apparatus, followed by two washes with 5 ml of PBS. Proteins and protein-bound saccharides bind to the filter, whereas free saccharides pass through. Bound saccharides were released from the filter with 2 ml of 2 M NaCl and radiolabeled saccharides were measured in a scintillation spectrometer.

Recombinant mouse endostatin was covalently immobilized onto CH-Sepharose (Amersham Pharmacia Biotech) matrix according to the manufacturer’s instructions. Prior to immobilization, endostatin (0.5 mg) was incubated with a 4-fold molar excess of heparin in 0.5 ml of 0.2 M ammonium acetate pH 7 for 1 h in order to protect the heparin binding sites of endostatin. To prevent binding of heparin to the column, the heparin preparation used had previously been cleaved with HNO₂ at pH 3.9 (Safaiyan et al., 1998). The resulting oligosaccharides were radiolabeled by reduction of their anhydromannose residues with NaBH₄ (Amersham Pharmacia Biotech), yielding labeled 2,5-anhydromannitol units. Labeled oligosaccharides were separated on a column of Bio-Gel P-10 (Bio-Rad; 1 × 190 cm) in 0.5 M NaH₂HCO₃ to fractions corresponding to 6–14mers and larger oligosaccharides, which were all pooled, desalted and stored at –20°C until further use.

The specific radioactivities of the saccharides were determined by scintillation counting and saccharide quantification using the carbazole reaction for hexuronic acid (Bitter and Muir, 1962).

Recognition of heparin–sulfate–protein complexes by VEGF 165

VEGF 165 (Peprotech Inc.) was used to determine binding of FGF-2 to immobilized heparin–sulfate–protein complexes. Solid-phase binding assays in neutral buffer of physiological ionic strength and 0.14–1.0 M NaCl were used to determine binding of FGF-2 to immobilized heparin–sulfate–protein complexes. Solid-phase binding assays in neutral buffer of physiological ionic strength and 0.14–1.0 M NaCl were used to determine binding of FGF-2 to immobilized heparin–sulfate–protein complexes. Solid-phase binding assays in neutral buffer of physiological ionic strength and 0.14–1.0 M NaCl were used to determine binding of FGF-2 to immobilized heparin–sulfate–protein complexes. Solid-phase binding assays in neutral buffer of physiological ionic strength and 0.14–1.0 M NaCl were used to determine binding of FGF-2 to immobilized heparin–sulfate–protein complexes.

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