The Morphogenic Properties of Oligomeric Endostatin Are Dependent on Cell Surface Heparan Sulfate*

Andrew Clamp‡, Fiona H. Blackhall‡, Audrey Henrioud‡, Gordon C. Jayson‡, Kashi Javaherian‡, Jeff Esko‡, John T. Gallagher‡, and Catherine L. R. Merry†1

From the Department of Medical Oncology, ‡Cancer Research UK and the University of Manchester, Christie Hospital NHS Trust, Manchester, M20 4BX United Kingdom, the †Department of Surgery, Children’s Hospital, Harvard Medical School, Boston, Massachusetts 02115, and the ‡Department of Cellular and Molecular Medicine, University of California, La Jolla, California 92039-0687

Endostatin has attracted considerable attention because of its ability to inhibit angiogenesis. This property of monomeric endostatin contrasts with that of the trimeric endostatin moiety generated from the intact C-terminal domain of collagen XVIII that induces a promigratory phenotype in endothelial cells. This activity is inhibited by monomeric endostatin. In this study we demonstrate that the effect of oligomeric endostatin can also be inhibited by exogenous glycosaminoglycans in a size-dependent manner, with heparin oligosaccharides containing more than 20 monosaccharide residues having optimal inhibitory activity. Oligomeric endostatin was also found to induce morphological changes in Chinese hamster ovary cells, an epithelial cell line. This novel observation allowed the utilization of a panel of Chinese hamster ovary cell mutants with defined glycosaminoglycan biosynthetic defects. The action of oligomeric endostatin on these cells was shown to be dependent on cell surface glycosaminoglycans, principally heparan sulfate with N- and 6-O-sulfation of glucosamine residues rather than idurionate 2-O-sulfation being important for bioactivity. The responsiveness of a cell line (pgsE-606) with globally reduced heparan sulfate sulfation and shortened S domains, however, indicates that overall heparan sulfate domain patterning is the key determinant of the bioactivity of oligomeric endostatin. Purified heparin-monomeric endostatin constructs generated by zero-length cross-linking techniques were found to be unable to inhibit the action of oligomeric endostatin. This indicates a mechanism for the perturbation of oligomeric endostatin action by its monomeric counterpart via competition for glycosaminoglycan attachment sites at the cell surface.

Endothelial and epithelial cells interact closely with the basement membrane that, as well as providing structural support, modulates cell behavior (1). Recently, much work has focused on the functional roles of the basement membrane collagens, particularly their C-terminal non-collagenous domains (2).

Endostatin is a cryptic C-terminal fragment of collagen XVIII released by matrix metalloproteases, cathepsin L, and elastase (3, 4). This potent inhibitor of angiogenesis has been shown to induce tumor regression in mice (5) and humans (6). Whereas endostatin in its monomeric form inhibits endothelial cell proliferation and migration in vitro (7, 8), the trimeric first non-collagenous domain of collagen XVIII (NC1) is a motility factor that causes dissociation and scattering of endothelial cells from capillary tubules that form spontaneously on Matrigel, a complex basement membrane preparation (9). This effect is inhibited by tubule preincubation with endostatin monomer. The mitogenic activity of trimeric endostatin is retained by an artificially created endostatin dimer, indicating that oligomerization of the endostatin domain is essential. This phenomenon has also been shown to occur in Caenorhabditis elegans where the NC1 trimer of the collagen XVIII homologue cle-1 induces neuronal migration, which is inhibited by the monomer (10) indicating an evolutionarily conserved negative feedback loop.

Due to its potent anti-angiogenic effects, much work has focused on the mechanism of action of endostatin. Whereas several putative downstream signaling pathways have been described, a high affinity cell surface receptor remains elusive. Two endothelial cell surface binding sites have been distinguished by Scatchard analysis with $K_d$ values of 18 and 200 pM, respectively (11). The low affinity receptor was identified as the heparan sulfate (HS)$^2$ component of the glypic family of cell surface heparan sulfate proteoglycans.

The glycosaminoglycan (GAG) HS is a linear polyanionic molecule composed of repeating disaccharide units found attached to cell surface and extracellular matrix proteins. HS biosynthesis occurs in the Golgi and is initiated by the formation of a polymer of alternating glucuronic acid (GlcA) and N-acetylgalactosamine (GlcNAc) residues. This nascent chain is then subject to a series of incomplete modifications; de-N-acetylation and N-sulfation of GlcNAc to generate N-sulfoglucosamine (GlcNS), epimerization of GlcA to iduronic acid (IdoA) and O-sulfation at C-2 of the uronic acids, C-6, and rarely C-3 of the glucosamine residues. These enzymatic modifications are both incomplete and interdependent, resulting in a mature HS chain composed of relatively short but highly heterogeneous domains of high sulfation (S domains) flanked by regions of intermediate sulfation (NA/NS domains) distributed at regular intervals within an N-acetylated backbone. The polymorphic S domains form the principal recognition sites for HS-binding proteins (12).

The crystal structure of endostatin identifies two basic clusters of surface-exposed arginine residues (13) that have been designated the primary and secondary GAG-binding sites (14). Initial studies indicated that a saccharide of at least 10 monosaccharide units in length is required to bind simultaneously to both sites and suggested that both 2-O- and 6-O-sulfate groups are necessary for this interaction (14). Subsequently, Ricard-Blum et al. (15), using surface plasmon resonance,
Oligomeric Endostatin and GAGs

indicated that N- and 2-O-sulfation in particular are critical for the interaction of endostatin with heparin. Endostatin also fails to bind to a melanoma cell line transfected with a 2-O-sulfotransferase antisense construct resulting in a 90% reduction in 2-O-sulfation in HS (14). Experiments using the physiologically relevant ligand endothelial cell HS in filter-binding assays have, however, questioned these findings (16). By assessing the physiologically relevant ligand endothelial cell HS and short S domain remnants. This suggests that the structural motif required for endostatin binding need not consist of a contiguous sulfated sequence, provided that negatively charged domains are present which can interact with the primary and secondary heparin-binding sites simultaneously.

This result confirmed the findings of Kreuger et al. (17), who identified a region composed of two short sulfated domains separated by a single unsulfated disaccharide (“SAS” domain) as being the likely histologically relevant ligand for endostatin. This patterning is distinct from that identified in many other HS-ligand interactions, typified by some of the best studied examples, FGF-1 and FGF-2 (18, 19).

Whereas the studies described above have increased our understanding of the interactions between endostatin and GAGs, the majority have been conducted in cell-free systems. In this paper we describe the development of a novel cellular bioassay that has enabled us to explore the interactions between oligomeric endostatin and cell surface glycosaminoglycans for the first time and probe the mechanism by which monomeric endostatin inhibits its oligomeric counterpart.

EXPERIMENTAL PROCEDURES

Materials—Tinzaparin was from Leo Pharmaceuticals (Princes Risborough, UK). Bio-Gel P-10 columns were obtained from Bio-Rad. PD10 desalting columns and Sephadex G-25 were purchased from Amersham Biosciences. Cell culture media and sera were from Invitrogen. Matrigel was acquired from BD Biosciences. Fluorescently labeled antibodies and phalloidin were purchased from Molecular Probes. The porcine mucosal heparin, murine anti-vinculin, and goat anti-rabbit antibodies and phalloidin were purchased from Amersham Biosciences. Cell culture media and sera were from Invitro-Risborough, UK). Bio-Gel P-10 columns were obtained from Bio-Rad. Oligosaccharides, 20,000 IU of tinzaparin was fractionated by size exclusion chromatography using a 240/110 column run at 10 ml/h in 0.2 M ammonium bicarbonate. 2.5-l ml fractions were collected. Eluted material was monitored at 232 nm and individual peaks were pooled and desalted by lyophilization followed by redissolution in water and passage through a PD10 desalting column.

Cell Culture—Bovine aortic endothelial (BAE) cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 15% (v/v) donor calf serum and 300 μg/ml glutamine at 37 °C in humidified air containing 5% CO2. Cells were evaluated in the angiogenesis assay before passage 14. The medium was changed every 48 h and cells were routinely subcultured in a 1:3 split ratio every 5 days.

Chinese hamster ovary (CHO) cell lines were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum (FCS) and 300 μg/ml glutamine under identical conditions. Cells were split every 2 or 3 days in a 1:5 ratio.

In Vitro Angiogenesis Assays—This was performed as described previously (9). 24-Well plates were coated with 250 μl of Matrigel/well at 4 °C and then incubated at 37 °C for 20 min to facilitate Matrigel solidification. Wells were then seeded with 50,000 BAE cells in 1 ml of assay medium (Dulbecco’s modified Eagle’s medium with 5% donor calf serum and 300 μg/ml glutamine) and the plates were incubated at 37 °C for 16 h allowing the formation of capillary-like endothelial tubules. Recombinant human endostatin dimer (HED) (9) was then added to a final concentration of 50 nM. Where relevant, human endostatin monomer (HEM) (9) at 3000 nM or variable concentrations of intact heparin or size–defined oligosaccharides were added 30 min prior to the addition of HED. Loss of tubule morphology was monitored at 24 and 40 h by phase-contrast microscopy and the cells were photographed at ×100 magnification using a digital imaging system.

The degree of tubule breakdown and cell scattering was also scored as a proportion of that caused by HED alone by two independent observers on a six-point scale. Good interobserver correlation was achieved.

Epithelial Cell Scatter Assays—The assays were conducted using CHO cells on a Matrigel substratum in a similar manner to the in vitro angiogenesis assays, with the following modifications. 50,000 CHO cells per well were seeded in standard growth medium and HED was added immediately, or after 16 h incubation. HEM/GAGs were added simultaneously in relevant experiments. The degree of cell morphological change was assessed by imaging at 24 and 40 h. A panel of CHOC cell GAG biosynthetic mutant lines was utilized (see Table 1).

Immunofluorescence—CHO cells were seeded onto a glass coverslip coated with 100 μl of Matrigel. After coincubation with HED for 40 h, the cells were fixed with 3.7% paraformaldehyde for 10 min and then permeabilized with 0.1% Triton X-100 for 10 min. After blocking nonspecific antibody binding with 10% FCS for 1 h, the coverslips were incubated with monoclonal murine anti-human vinculin antibody, added at 1:200 for 1 h in PBS with 1% FCS and then washed extensively with 1% FCS in PBS. An Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibody in 1% FCS/PBS was then added at 1:200 for 1 h and then after extensive rinsing, 5 μl of Alexa Fluor 633-conjugated phalloidin diluted to 200 μl in PBS, 1% FCS was added for 30 min. After rinsing, the samples were mounted in Prolong® anti-fade and allowed to dry at 4 °C for 48 h prior to imaging by confocal microscopy. Images were overlaid using Adobe Photoshop version 6.0.

Zero-length Cross-linking—This was performed as described previously (20, 21). Briefly, heparin oligosaccharides were incubated in coupling buffer (0.1 M NaCl, 0.1 M MES, pH 6.0) for 15 min at 25 °C with the cross-linkers 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and sulfo-N-hydroxysuccinimide (S-NHS) in a 27:23 mm ratio. Oligosaccharide activation was terminated by size separation on a 1-ml Sephadex G-25 column. Activated oligosaccharides were then incubated with endostatin in coupling buffer overnight at 25 °C. 1 μl of 1 M hydroxylamine was then added to reconstitute any reduced oligosaccharide hydroxyl groups and cross-linked products were stored at −20 °C prior to analysis.

Products of cross-linking reactions were analyzed by standard SDS-PAGE on a 4-15% gel and Western blotting. After blocking with 15% skimmed milk powder for 1 h, the nitrocellulose membrane was incubated overnight with a 1:1000 dilution of a polyclonal rabbit anti-human...
endostatin antibody (R7012) (9). After rinsing 3 times with PBS, 0.05% (v/v) Tween 20, the membrane was incubated for 1 h with a 1:500 dilution of the goat anti-rabbit horseradish peroxidase-conjugated secondary antibody. Endostatin was then visualized by enhanced chemiluminescence according to the manufacturer’s protocol. An assessment of the proportion of endostatin that had been cross-linked was made by densitometry using a UVItec gel analysis system and associated software.

**Purification of Heparin Cross-linked Endostatin Monomer**—Cross-linked constructs were separated from unreacted HEM by size exclusion high performance liquid chromatography using a 7.5 × 300-mm TSKG4000PW column run at 0.5 ml/min in 1 M sodium chloride, pH 7.0. The chromatographic profile was monitored at 210 nm and fractions corresponding to peaks were pooled. Due to co-elution of uncross-linked oligosaccharides with the cross-linked constructs, the sample was dialyzed into 5 mM Tris-HCl, pH 7.4, and lyophilized followed by resuspension in heparinase buffer (0.1 M sodium acetate, 0.1 M calcium acetate, pH 7.0). 0.1 IU of heparinase I and II were then added and incubated for 24 h at 37 °C. Full digestion of non-protected cross-linked oligosaccharide (and therefore contaminating free oligosaccharides) was then confirmed by Western blotting. To determine the effect of the purification procedure on protein integrity, the uncross-linked HEM purified by chromatography was subjected to the same experimental protocol.

**RESULTS**

**Matrigel in Vitro Angiogenesis Assays**—We used an in vitro angiogenesis assay to investigate the interaction between dimeric HED and GAGs. Endothelial cells plated onto the basement membrane preparation Matrigel spontaneously form capillary-like tubules. Initial experiments confirmed the findings of Kuo et al. (9). Endothelial cell migration and capillary tubule breakdown are induced by the addition of HED and this breakdown is inhibited by monomeric endostatin (HEM) in a dose-dependent manner (data not shown, but see Fig. 7). Intact porcine mucosal heparin (17–19 kDa, ~dp60) also inhibits the migratory effect of HED (Fig. 1). Addition of 50 nM HED induces endothelial cell migration away from capillary tubules (Fig. 1, A and B). Heparin alone has no effect on tubule integrity (Fig. 1C) but inhibits the pro-migratory action of HED in a dose-dependent fashion (data not shown, but see Fig. 7).
Oligomeric Endostatin and GAGs

shown) with complete inhibition at a final concentration of 200 μg/ml (Fig. 1D).

We then explored whether the inhibitory effect of heparin could be reproduced by heparin-derived saccharides. A panel of size-defined fragments (dp4–28, where dp = degree of polymerization, i.e. disaccharide = dp2) was prepared from the low molecular weight heparin derivative tinzaparin by gel filtration chromatography (22).

Oligosaccharides (200 μg/ml) were added to preformed capillary tubules at 16 h after plating simultaneously with HED (50 nM). Partial inhibition of cell migration at 24 h was noted for all oligosaccharides (dp4 and upards) but the degree of inhibition increased with oligosaccharide size, particularly above dp20 (50% inhibition), with dp28s showing equivalent activity to heparin (85% inhibition) (see Figs. 1, E–H, and 2).

Whereas the experiments described above demonstrate a biologically significant interaction between HED and heparin oligosaccharides, they do not directly address the role of cell surface GAG species. We therefore developed a novel assay to allow us to investigate this.

Dimeric Endostatin Can Induce Morphological Changes in Chinese Hamster Ovary Cells—Although the actions of endostatin were initially thought to be confined to endothelial cells, recent experiments have demonstrated the endostatin/NCl1 domain of collagen XVIII activity on human carcinoma cell lines (9, 23) and neuronal cells (10). We therefore explored the action of HED on CHO epithelial cell lines that have been chemically mutated and selected to create a panel of lines with defined GAG biosynthetic defects (see Table 1). This panel provides an attractive system to directly address the role of cell surface GAG species and sulfation patterns on HED activity. It also avoids the inherent problems generated by the inhibition of multiple sulfate-dependent processes associated with using chlorate treatment as a means of inhibiting GAG synthesis.

HED (50 nM) was added to wild-type CHO K1 cells 30 min after plating on Matrigel. This induced a change in morphology from rounded cells to more elongated cells with long cellular projections and intercellular bridging (Fig. 3C). These changes could be inhibited by coincubation with HEM at 3000 nM (data not shown). Similar changes in morphology were also seen when HED was added 16 h after plating, indicating that alterations in cell morphology were not due to interference with initial cell attachment to the underlying matrix. Consistent with the endothelial assay of Kuo et al. (9), no morphological changes were noted when CHO K1 cells plated on tissue culture plastic/laminin were stimulated with HED. No overt changes in cell number after HED exposure were noted on multiple assays, consistent with the lack of an effect on proliferation noted in endothelial cells (9).

To further elucidate the changes in cytoskeletal architecture induced by HED, the actin cytoskeleton and cell-matrix adhesions were examined in quiescent and HED-stimulated CHO-K1 cells plated on Matrigel. Unstimulated cells form tight multicellular aggregates (Fig. 3B), whereas HED-stimulated cells (Fig. 3D) become elongated with the formation of extended cytoplasmic projections characterized by the concentration of vinculin staining in focal complexes at their tips.

Glycosaminoglycans Can Inhibit the Effects of HED in an Epithelial Cell Line—We then investigated whether this HED-induced phenotype could be inhibited with heparin and size-defined heparin oligosaccharides as observed in endothelial cells. Heparin (50 μg/ml, Fig. 4D) completely inhibited development of the HED phenotype and in common with the findings in endothelial cells, this inhibition was size-dependent with dp12 saccharides showing weak inhibition (Fig. 4B) and dp20 fragments (Fig. 4C), being almost as inhibitory as heparin.

To determine whether the ability to inhibit HED was specific to heparin, other GAGs were also tested. At 50 μg/ml, the more physiologically relevant ligand HS (Fig. 4F) demonstrated a similar ability to heparin to inhibit HED (Fig. 4D). Dermatan sulfate (DS) (Fig. 4F) was quantitatively less potent, whereas chondroitin sulfate (CS) (data not shown) was unable to inhibit HED.

The Presence of Cell Surface Heparan Sulfate Is Critical for the Development of the HED-induced Phenotype—In Fig. 5, B and C, it can be seen that cell lines that lack cell surface GAGs due to deficiency in either xylosyl transferase (745) or GlcATI (pgsG-224), enzymes involved in the initiation of GAG chain synthesis, do not respond to HED. Of note, pgsG-224 cells that have been transfected with wild-type GlcATI and re-express cell surface GAGs (24) have restored responsiveness to HED (Fig. 5D). The pgsD-677 mutant line (Fig. 5E), which is specifically deficient in cell surface HS but synthesizes DS and CS demonstrates a strikingly diminished phenotype with fewer and shorter cell projections and a lack of intercellular bridges. The wild-type response cannot be reproduced in this cell line by increasing the concentration of HED by 10-fold (data not shown). This suggests that DS/CS are weak promoters of HED but cannot compensate for the lack of cell surface HS.

Finally, two interesting mutant cell lines with modified HS sulfation patterns were examined. The pgsE-606 cell line lacks NDST-1 activity and so synthesizes HS with globally decreased sulfation and short S domains. When stimulated with HED, these cells demonstrate a similar phenotype to wild-type CHO-K1 cells although longer intercellular

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**TABLE 1**

| CHO cell line | Mutant enzyme | GAG phenotype | Ref. |
|--------------|---------------|---------------|-----|
| CHO K1       | Xylosyl transferase | Wild-type, normal cell surface GAGs | 39 |
| CHO 745      | Glucuronyl transferase 1 (GlcAT-1) | No cell surface GAGs | 24 |
| CHO pgsG-224 | pgsG-224 transfected with GlcAT-1 | “Normal” GAG phenotype | 24 |
| CHO GTX-GFP  | EXT-1 | Lack of cell-surface HS | 40, 41 |
| CHO pgsD-677 | 2-O-Sulfotransferase | No 2 O-sulfation on HS | 25 |
| CHO pgsF-17  | N-Deacetylase sulfotransferase I | Globally decreased HS sulfation | 29 |

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**FIGURE 2.** The inhibitory effect of heparin oligosaccharides on HED-induced migration of BAE cells is size-dependent. Size-defined oligosaccharides (200 μg/ml) were added to pre-formed capillary tubules simultaneously with HED (50 nM) at 16 h. Each experiment was scored at 24 h by two independent observers who compared the degree of tube breakdown and cell scattering with that caused by HED alone on a 6-point scale and the results were combined. The results are displayed as a mean scoring ± 1 S.D.
bridges and cords of cells form (Fig. 5F). GAG 2-O-sulfation has previously been implicated in HEM binding to a melanoma cell line (11). We therefore investigated the effects of HED on the CHO pgsF-17 cell line, which has 1% of wild-type HS 2-O-sulfation (25). As illustrated in Fig. 5G these cells respond apparently normally to HED, indicating that 2-O-sulfation is not essential for the activity of HED in this system.

Multiple Endostatin Subunits Can Interact with the Same Oligosaccharide Chain Simultaneously—As the divergent effects of monomeric and oligomeric endostatin are dependent on GAG binding, we explored the stoichiometry of the endostatin/GAG interaction using two-step zero-length cross-linking (26). This technique enables the formation of covalent bonds between GAGs and proteins specifically at the site of their physiological interaction (27).

HEM was cross-linked to a series of size-defined heparin oligosaccharides using a cross-linking reaction mixture in which activated oligosaccharides were present in a 40:1 molar excess over HEM. Fig. 6 shows a Western blot of the constructs formed when HEM is cross-linked to dp 4, 6, 8, 12, 16, and 22 species. No cross-linking is detected with dp4. Although for all other saccharides cross-linking is incomplete, substantial amounts of cross-linked products were observed. An estimate of the proportion of HEM cross-linked into each construct was obtained by band densitometry. In addition to the native endostatin monomer, a second band (band A) is seen above the uncross-linked HEM; the size of this increases stepwise with oligosaccharide length. These products have a mass consistent with a 1:1 HEM:oligosaccharide ratio. Of note, further products are seen when HEM is cross-linked to dp8 and above. A second series of bands (band B) is consistent with 2 HEM molecules linked to a single oligosaccharide chain. The intensity of band B increases with oligosaccharide chain length consistent with a higher proportion of HEM forming 2:1 complexes and notably a product (band C) consistent with a 3:1 HEM:oligosaccharide ratio is present.
seen when a dp22 oligosaccharide is used. Protein multimerization on a single oligosaccharide ligand has not been observed when the zero-length cross-linking technique is applied to other GAG-binding growth factors including FGF-2 (20), HGF (21), and thrombospondin. Multimerization is also notable given the excess of activated oligosaccharides present in the cross-linking reaction that should be bias toward the formation of 1:1 complexes.

**HEM with an Occupied GAG-binding Site Lacks the Ability to Inhibit HED**—HEM with a decreased affinity for heparin due to mutations in the GAG-binding sites fails to inhibit FGF-2-induced angiogenesis (14) and also cannot inhibit the promigratory effects of HED on CHO K1 cells (Fig. 7A). We wanted to determine whether GAG binding alone induces a change in HEM allowing it to engage directly with downstream signaling receptors to block tube formation by HED or whether it must interact with GAGs at the cell surface to inhibit HED. This latter possibility would indicate that the mechanism of HEM inhibition of HED is via competition for access to cell surface HS. To investigate this, we separated the cross-linked products formed by co-incubating HEM with activated dp24s from uncross-linked HEM (Fig. 7B). 51% of the HEM contained in the initial cross-linking reaction had formed cross-linked complexes. This fractionation was confirmed by Western blotting (Fig. 7C). Whereas untreated protein was easily removed by chromatography, unreacted oligosaccharide co-eluted with cross-linked HEM (data not shown). To remove this, the cross-linked products were incubated overnight with heparinase I and II. As can be seen in Fig. 7C, a shift is seen in the migration of the cross-linked products consistent with complete digestion of GAG present in the sample apart from the portion protected from enzymatic lysis by the GAG-binding site of HEM. The proportion of monomeric to dimeric cross-linked products as determined by densitometry was not altered significantly by exposure to heparinase (Fig. 7C).

The heparinase-trimmed cross-linked product was then assessed in the CHO cell assay for its ability to inhibit the morphogenic properties of HED. Fig. 7, D and E, illustrate quiescent CHO K1 cells and those stimulated by HED. Fig. 7F demonstrates that the addition of HEM (3000 nM) 30 min prior to HED completely inhibits the morphogenetic effects of HED. In contrast, when isolated cross-linked HEM was added prior to HED, the morphogenic properties of HED (Fig. 7E) were retained, with cellular elongation, intercellular bridges, and lamellipodia formation occurring (Fig. 7H). This result indicates that HEM binds to cell surface GAG to inhibit the actions of HED in this assay.

**Exogenous Heparin Is Unable to Recover HED Activity in the Cell Surface HS-deficient 745 Cell Line**—To further probe the requirement of endostatin for the cell surface as opposed to matrix GAG, we investigated whether we could recover HED bioactivity in the 745 cell line with exogenous heparin. Concentrations of heparin that failed to inhibit completely the activity of HED in wild-type CHO K1 cells were added with HED to 745 cells (Fig. 8). Fig. 8C demonstrated that 100 ng/ml

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3 M. Lyon and C. Mulatero, unpublished data.
heparin is unable to inhibit the morphogenic properties of HED on K1 cells, whereas 1 mg/ml heparin is only partly inhibitory (Fig. 8E). Neither of these concentrations nor 50 mg/ml heparin are able to recover any HED bioactivity on 745 cells (Fig. 8, D, F, and H). This result further supports the requirement of endostatin for cell surface rather than matrix GAG.

**DISCUSSION**

In this study we provide the first biological evidence that the activity of oligomeric endostatin is dependent on the presence of cell surface GAGs. Dimeric endostatin induces migration of endothelial and epithelial cells plated on Matrigel; a process that is inhibited by co-incubation with exogenous GAGs, principally heparin and HS, in a manner dependent on their size, sulfation pattern, and saccharide composition. Importantly, we show that the presence of cell surface HS is absolutely necessary for HED to exert its full effect. The CHO 745 and pgsG-224 cell lines that are completely devoid of cell surface GAGs fail to respond to HED. CHO pgsD-677 cells that have normal cell surface CS/DS but lack HS have a markedly attenuated response to HED. Zero-length cross-linking experiments have indicated that binding to cell surface GAGs is critical for the ability of monomeric endostatin to inhibit its oligomeric counterpart as occupation of GAG-binding sites on the monomer leads to loss of this activity. Non-inhibitory concentrations of exogenous heparin are also unable to restore any HED bioactivity in the CHO 745 cell line.

**Oligosaccharide Length and HED Inhibition**—Biochemical studies using affinity chromatography (18) and filter binding assays (16, 14) have indicated that an oligosaccharide of dp10–12 is necessary to bind to monomeric endostatin with moderate affinity and that this length of oligosaccharide would be required to fully occupy both parts of the heparin-binding site (13, 14).

In this study, although shorter oligosaccharides have a modest ability to inhibit the effects of HED, heparin-derived oligosaccharides >dp20 in size were most effective. This suggests that the relatively low affinity
Oligomeric Endostatin and GAGs

noted for smaller oligosaccharides in cell-free assays is not sufficient to affect activity in biological systems. Alternatively, both endostatin subunits in the dimer may need to be bound by the same oligosaccharide for full biological activity.

FIGURE 6. Multiple HEM subunits can be cross-linked to a single oligosaccharide chain. HEM was co-incubated with activated oligosaccharides of increasing size (dp 4, 6, 8, 12, 16, and 22) with a 40:1 molar excess of activated oligosaccharides over HEM. The products formed were then separated on a 15% SDS-polyacrylamide gel. After transfer to a nitrocellulose membrane, the blot was probed with the polyclonal rabbit anti-human endostatin R7012 primary antibody and visualized with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody as detailed under "Experimental Procedures." Cross-linking is incomplete. Products consistent with a 1:1 HEM:oligosaccharide ratio are formed with dp6 and above (band A). Products consistent with a 2:1 complex are seen with dp8s and above, but the proportional amount formed increases with oligosaccharide length (band B). A 3:1 product is formed with an activated dp22 (band C). The proportion of complexes formed in each cross-linking reaction as determined by densitometry is given in the accompanying table.

FIGURE 7. Purified cross-linked HEM:dp24 constructs are unable to inhibit the morphogenic properties of HED. A, CHO K1 cells were preincubated with HEM (R158A,R270A) prior to the addition of HED. This primary heparin-binding site mutant is unable to inhibit HED. B, cross-linked products were separated from unreacted HEM by passage over a 7.5 mm × 30-cm tskg4000pw size-exclusion high performance liquid chromatography column in 1 M NaCl eluted at 0.5 ml/min. The chromatogram was monitored at 210 nm. Aliquots corresponding to peaks a and b were pooled. C, Western blot of peaks a (lane 2) and b (lane 3) and heparinase digested peak a (lane 4). Lane 1 contains HEM. The blot was probed with the polyclonal rabbit anti-human endostatin R7012 primary antibody and visualized with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody. The proportion of HEM in each band as determined by densitometry is given in the accompanying table. The Matrigel epithelial cell scatter assay was used to assay for activity of the cross-linked HEM:dp24 product. Images were taken at 40 h at ×200 magnification. D, unstimulated K1 cells. E, CHO K1 cells stimulated by HED (3000 nM) prior to HED stimulation. F, CHO K1 cells preincubated with HEM (3000 nM) prior to HED stimulation. G, CHO K1 cells preincubated with processed unreacted HEM (peak b) prior to HED stimulation (3000 nM). H, CHO K1 cells preincubated with purified cross-linked HEM:dp24 (3000 nM) prior to HED stimulation.

HS Sulfation, Domain Patterning, and the Activity of HED—Analysis of the monomeric endostatin/HS interaction by surface plasmon resonance has suggested that GAG N- and 2-O-sulfation are important (15) and a previous research (11) demonstrated that a 90% reduction of HS
Oligomeric Endostatin and GAGs

The addition of exogenous heparin at non-inhibitory concentrations does not induce a response to HED in CHO 745 cells that lack cell surface heparan sulfate. CHO K1 (A, C, E, G, and I) and CHO 745 (B, D, F, H, and J) cells were plated on Matrigel at 50,000 cells/ml. A and B, cells alone; C and D, stimulated with HED (50 nM) alone; or in the presence of heparin at 100 ng/ml (E and F), 1 µg/ml (G and H), or 50 µg/ml (I and J). Cells were imaged at 24 h after addition of HED. The response of K1 cells to HED can be inhibited by exogenous heparin in a dose-dependent manner. 745 cells do not respond to HED and a response cannot be induced with non-inhibitory concentrations of exogenous heparin.

2-O-sulfation abrogated cell surface endostatin binding. Notably, we show that two cell lines with markedly altered HS sulfation patterns display morphological changes on stimulation with HED. The HED-induced phenotype is unaffected by the absence of HS 2-O-sulfation as CHO pgsF-17 cells respond identically to wild-type CHO K1 cells. Our results are consistent with data previously published (16) that have shown that HS derived from the 2-OST gene trap mouse has no detectable difference in affinity for monomeric endostatin compared with wild-type murine HS. Of note HS from both the 2-OST knock-out mouse (28) and the pgsF-17 cell line (25) have increases in 6-O- and N-sulfation that maintain some of the charge-density characteristics and domain structure of the HS chain. These may compensate for the lack of 2-O-sulfation and enable endostatin binding to occur. These results indicate that 2-O-sulfation is not essential for the bioactivity of HED.

The pgsE-606 cell line that lacks NDST-1 activity also exhibits marked morphological changes after stimulation with HED. The HS on the surface of these cells has globally reduced sulfation with levels of N-, 6-O-, and 2-O-sulfation being 50, 59, and 34%, respectively, of those in wild-type K1 cells (29). This argues against a simplistic “charge threshold” model in which increasing total sulfation within HS allows increased HED bioactivity via binding to the cell surface, and alternatively suggests that the HS domain patterning is the critical determinant. The analysis of pgsE-606 HS by low pH nitrous acid depolymerization indicates that a greater proportion of O-sulfate groups exist in non-contiguous sulfated regions than in K1 cells (29, 30). This patterning is identical to that seen in the NA/NS “transition zones” that are positioned between the highly modified S domains and unmodified NA domains in mammalian HS (31). These hypervariable regions are known to contain over 50% of 6-O-sulfate groups in mammalian HS but are devoid of 2-O-sulfation (32) and may have an important bearing on HS interactions with its protein ligands. Indeed such NA/NS domains in conjunction with short S domain “stubs” have recently been demonstrated to bind monomeric endostatin (16) and inhibit its anti-angiogenic actions (18), suggesting that these sequences rather than extended S domains comprise the authentic HS binding site for endostatin.

Dermatan Sulfate and HED—The ability of DS to inhibit partially the activity of HED indicates that there may be a direct interaction between endostatin and DS. Although DS is biosynthetically unrelated to HS it possesses some similar organizational features, in particular, the presence of iduronate-rich sulfated domains of variable length. Although 6-O-sulfation is rare in mammalian DS, it should be noted that other growth factors requiring 6-O-sulfation for their activity, in particular hepatocyte growth factor (21, 33) and FGF-2 (34), bind to and are activated by DS. A physiological interaction between DS and endostatin cannot therefore be ruled out and clearly needs to be explored further.

Multiple Endostatin Monomeric Units Can Bind to the Same Oligosaccharide Chain—The results of our zero-length cross-linking experiments indicate that multiple monomeric endostatin molecules can bind to a single oligosaccharide molecule. Although 1:1 complexes are the major products formed, HEM:oligosaccharide 2:1 constructs are seen with heparin oligosaccharides as small as dp8, and 3:1 constructs are formed with heparin dp22s (Fig. 6). These are formed despite the 40:1 molar excess of GAG to HEM used in the cross-linking reactions that should bias toward the formation of 1:1 complexes. This result suggests co-operativity in the binding of monomeric endostatin subunits to GAG chains with the interaction of one endostatin molecule increasing the likelihood of a second monomer binding to the same oligosaccharide. Pertinent to this, Sasaki et al. (14) showed a 4-fold increased affinity of monomeric endostatin for dp20 oligosaccharides when compared with dp12s. It is also interesting to note that heparinase digestion fails to cleave between the two endostatin monomers cross-linked to a heparin-derived dp24 indicating the close apposition of the endostatin molecules within this construct (Fig. 7B). Kinetic studies comparing the binding of monomeric and oligomeric endostatin to defined GAG ligates will address the possibility of cooperative binding.

FIGURE 8. The addition of exogenous heparin at non-inhibitory concentrations does not induce a response to HED in CHO 745 cells that lack cell surface heparan sulfate. CHO K1 (A, C, E, G, and I) and CHO 745 (B, D, F, H, and J) cells were plated on Matrigel at 50,000 cells/ml. A and B, cells alone; C and D, stimulated with HED (50 nM) alone; or in the presence of heparin at 100 ng/ml (E and F), 1 µg/ml (G and H), or 50 µg/ml (I and J). Cells were imaged at 24 h after addition of HED. The response of K1 cells to HED can be inhibited by exogenous heparin in a dose-dependent manner. 745 cells do not respond to HED and a response cannot be induced with non-inhibitory concentrations of exogenous heparin.
This clustering of HEM molecules on a single oligosaccharide also suggests that HEM could inhibit the actions of HED by competing with it for cell surface binding sites on GAGs, and would explain why a 40-fold molar excess is required for the complete inhibition of the actions of HED by HEM in an endothelial cell assay (9). Although it has previously been suggested that the anti-angiogenic effects of monomeric endostatin are purely due to its masking of HS epitopes required for the activity of pro-angiogenic growth factors (14), recent data do not support this as endostatin does not bind to HS dodecasaccharides that activate FG-2 (17). The observation that purified cross-linked constructs formed between HEM and heparin-derived dp24s are unable to inhibit the morphogenetic properties of HED (Fig. 7) does, however, support direct competition for cell surface GAG-binding sites as one explanation for the mechanism of action of HEM.

Do Cell Surface GAGs Support HED Function by the Formation of a Trimolecular Complex?—Our results highlight the importance of cell surface GAGs for the activity of oligomeric endostatin. Indeed the demonstration that non-inhibitory concentrations of exogenous heparin are unable to recover HED activity in the 745 cell line (Fig. 8) suggests that direct binding to cell surface HS is required absolutely.

Scatchard analysis demonstrated that monomeric endostatin binds to endothelial cells at both high and low affinity sites (11) with glypicans HS being the low affinity receptor. It is tempting to speculate that like some other GAG-binding growth factors such as FG-2 (35), a trimolecular complex is formed at the cell surface between HED, a HS proteoglycan, and a currently unidentified high affinity receptor that allows intracellular signaling and acquisition of a promigratory state.

Recent work has indicated that both glypicans HS and the $\alpha_5\beta_1$ integrin serve as cell surface binding partners for monomeric endostatin in endothelial cells and that interference with either of these interactions prevents downstream cytoskeletal changes normally induced by endostatin (36). The cell surface proteoglycan syndecan-4 plays a key role in the formation and maintenance of focal adhesions (37), which would also make it an attractive potential binding partner for oligomeric endostatin.

The phenotypic effects induced by HED require the presence of a complex basement membrane preparation and endostatin has been shown to bind laminin at a domain distinct from that which binds GAGs (38) raising a second possibility for the mechanism of action of HED, namely that oligomeric endostatin functions by cross-linking cell surface HS proteoglycans to basement membrane laminin. This could have significant effects on the morphology and motility of cells.

In conclusion, we have clearly demonstrated that in both endothelial and epithelial cell lines oligomeric endostatin induces a migratory phenotype that is absolutely dependent on the presence of cell surface GAGs, principally HS. HS domain patterning appears to be of critical importance for its interaction with endostatin. HEM may inhibit the actions of HED by competition for binding sites within GAGs. Further work is required to tease out the nature of the interaction between cell surface GAGs and oligomeric endostatin and to investigate the physiologically important role of oligomeric versus monomeric endostatin as a system for maintaining the integrity of endothelial and epithelial cell monolayers.

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