Different effects of mucosal, bovine lung and chemically modified heparin on selected biological properties of basic fibroblast growth factor

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Heparins from bovine mucosa and lung, and chemically modified heparins were assayed for their capacity to: (i) protect human recombinant basic fibroblast growth factor (bFGF) from tryptic cleavage; (ii) prevent ¹²⁵I-bFGF binding to heparan sulphate proteoglycans present in the extracellular matrix and on the cell surface of fetal bovine aortic endothelial GM 7373 cell cultures; (iii) affect ¹²⁵I-bFGF binding to high-affinity tyrosine kinase FGF receptors present on the cell membrane of GM 7373 cells; (iv) inhibit the mitogenic activity exerted by bFGF in the same cells. The results demonstrate that the potency shown by mucosal heparins in the different assays is a direct function of size, very-low-molecular-mass heparin (2.0 kDa) being significantly less effective on a molar basis than unfractionated heparin (13.6 kDa). Increased flexibility of the backbone structure, as observed in reduced/oxidized heparins of different size, does not affect the capacity of the polysaccharide to interact with bFGF. In contrast, selective 2-O-desulphation, but not 6-O-desulphation, drastically reduced the capacity of heparin to protect bFGF from proteolytic cleavage, to affect its interaction with low- and high-affinity sites,

and to inhibit its mitogenic activity. Two preparations of bovine lung heparin, differing in molecular mass, were as effective as mucosal heparin in the bFGF-tryptic-digestion assay and the endothelial-cell proteoglycan-binding assay, but they were highly inefficient at inhibiting the capacity of bFGF to interact with its tyrosine kinase receptors. Bovine lung heparins were also less effective than mucosal heparin as bFGF antagonists in GM 7373-cell-proliferation assays. N-Desulphated/N-acetylated bovine lung heparin retained only a significant capacity to protect bFGF from tryptic cleavage. The results demonstrate that different chemical features of the heparin molecule, including decrease in molecular mass, selective desulphation, disaccharide composition and clustering, affect differently the capacity of the glycosaminoglycan to interact with bFGF and to influence its biological behaviour in different assays *in vitro* and in endothelial cell cultures. Our findings should aid the design of synthetic oligosaccharides aimed at improving the bioavailability of bFGF when administered *in vivo* as a therapeutic agent.

INTRODUCTION

Basic fibroblast growth factor (bFGF) is an 18 kDa cationic protein which belongs to the family of heparin-binding growth factors [1]. It exerts various biological activities *in vitro* and *in vivo* on different cell types, playing an important role during angiogenesis, wound healing and embryonic development [1]. On responsive cells, bFGF interacts with high-affinity tyrosine kinase FGF receptors responsible for the transduction of the signal across the cell membrane [2], and with low-affinity heparan sulphate proteoglycans (HSPGs) present on the cell surface and in the extracellular matrix (ECM) [3].

The molecular basis and physiological significance of the interaction between bFGF and soluble or immobilized glycosaminoglycans (GAGs) have been subjects of intense investigation over the last few years. X-ray crystallography has identified a region of the bFGF molecule possibly involved in this interaction [4–6], and bFGF-binding sequences have been identified in heparan sulphate and heparin [7–11]. The interaction between bFGF and cell-surface proteoglycans plays an important role in mediating the binding of the growth factor to its tyrosine kinase receptors [12–14]. Also, a heparin-binding domain in FGF receptor 1/flg has been demonstrated to be essential for

receptor–ligand interaction [15], indicating that the bFGF receptor is a ternary complex of heparan sulphate, bFGF and tyrosine kinase receptor.

The variety of *in vitro* and *in vivo* functions of bFGF suggests several potential clinical applications for this compound [16]. However, its use as a therapeutic agent is potentially limited by its capacity to bind to immobilized endothelial HSPGs which may sequester it when administered *in vivo* [17,18]. Theoretically, sequestration of bFGF by ECM should be prevented if the growth factor is administered together with free heparin [19], which may also offer the advantage of protecting bFGF from inactivation and proteolytic degradation [20–23]. Unfortunately, *in vitro* data have shown that free heparin also reduces the rate of bFGF binding to its tyrosine kinase receptors, thus inhibiting its biological activity [24–26].

In the present paper, we have assessed the possibility of dissociating the capacity of free heparin to prevent proteolytic cleavage and HSPG binding of bFGF from its capacity to inhibit FGF receptor interaction and mitogenic activity of the growth factor. To this purpose, natural and chemically modified heparins characterized by different anticoagulant potency and structural features were quantitatively tested in a set of different assays for their ability to protect bFGF from tryptic digestion, to prevent

Abbreviations used: bFGF, basic fibroblast growth factor; FCS, fetal calf serum; FGF receptor, tyrosine kinase fibroblast growth factor receptor; GAG, glycosaminoglycan; ECM, extracellular matrix; HSPG, heparan sulphate proteoglycan.

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Table 1 Summary of activities of different heparins tested

The IC_{50} for each heparin was calculated for each assay from the data shown in Figures 1, 2 and 3. Assay 1, trypsin digestion of bFGF; assay 2, ^{125}I -bFGF binding to low-affinity sites; assay 3, ^{125}I -bFGF binding to high-affinity sites; assay 4, cell proliferation caused by bFGF. Each value is the mean $\pm$ S.E.M. of between two and four independent experiments. IC_{50high}/IC_{50low} is the ratio between the IC_{50} values in assay 3 and assay 2. N.D., not determined; when administered at doses higher than 200 nM, 2.1 kDa heparin exerted a cytotoxic effect on GM 7373 cell cultures maintained in 10% FCS.

Compound	Molecular mass (kDa)	SO ₃ ⁻ /COO ⁻	IC ₅₀ (nM)				IC _{50high} /IC _{50low}
			Assay 1	Assay 2	Assay 3	Assay 4	
Mucosal heparin							
Unmodified	13.6	2.14	84 ± 6	6 ± 1	20 ± 12	600 ± 184	3.3
Reduced/oxidized	11.0	1.81	83 ± 34	4 ± 1	27 ± 6	760 ± 340	6.8
Unmodified	4.0	2.13	297 ± 42	27 ± 5	266 ± 119	3000 ± 500	9.90
Reduced/oxidized	4.0	1.94	238 ± 68	16 ± 2	69 ± 6	7450 ± 2550	4.3
Unmodified	2.1	2.97	385 ± 53	36 ± 11	563 ± 72	N.D.	15.3
2- <i>O</i> -Desulphated	9.6	1.50	850 ± 44	765 ± 333	2086 ± 867	45900 ± 22950	2.7
6- <i>O</i> -Desulphated	10.3	1.50	213 ± 72	25 ± 21	39 ± 5	6612 ± 1243	1.6
Bovine lung heparin							
Unmodified	9.7	2.69	83 ± 34	6 ± 1	238 ± 61	1649 ± 618	39.7
Unmodified	5.9	2.45	238 ± 68	7 ± 1	678 ± 163	1875 ± 1178	96.8
<i>N</i> -Desulphated/ <i>N</i> -acetylated	12.3	1.59	127 ± 82	734 ± 244	5700 ± 1710	4653 ± 1551	7.8

^{125}I -bFGF binding to HSPGs and FGF receptors present in fetal bovine aortic endothelial GM 7373 cell cultures, and to inhibit the mitogenic activity exerted by the growth factor in the same cells.

This study will help in the design of non-anticoagulant synthetic molecules characterized by a high capacity to protect bFGF from inactivation and proteolytic cleavage and to prevent its interaction with HSPGs of the ECM in the absence of a significant effect on the interaction of bFGF with tyrosine kinase receptors and on its biological activity. These molecules should increase the stability and diffusion of bFGF in the extracellular environment in the absence of adverse effects, and thus should represent optimum carriers for *in vivo* bFGF administration.

EXPERIMENTAL

Materials

Human recombinant bFGF was prepared by heparin-Sepharose chromatography of transformed *Escherichia coli* cell extract as described [27].

Mucosal heparin (13.6 kDa) was obtained from a commercial batch preparation of unfractionated sodium heparin from bovine mucosa (1131/900 from Laboratori Derivati Organici S.p.A., Milan, Italy) which was purified as previously described [28]. Bovine lung heparin (9.7 kDa) was obtained from Kabi-Pharmacia-Hepar (Franklin, OH, U.S.A.). The purity of bovine mucosa and lung heparins was greater than 95% as assessed by electrophoresis in acidic buffer [29], uronic acid quantitative determination [30] and h.p.l.c. analysis [28]. ^{13}C -n.m.r. spectroscopy was performed on the two products as described by Casu et al. [31] and showed 78% and 93% N-sulphate glucosamine, 80% and 87% 6-O-sulphate glucosamine, 59% and 84% 2-O-sulphate iduronic acid for mucosal and lung heparin respectively.

Low-molecular-mass (4.0 kDa) and very-low-molecular-mass (2.1 kDa) bovine mucosal heparins and low-molecular-mass (5.9 kDa) bovine lung heparin were obtained by controlled nitrous acid degradation of unfractionated heparins as described elsewhere [31,32].

Reduced/oxidized heparins were prepared by 24 h treatment

of 13.6 kDa and 4.0 kDa bovine mucosal heparins with 0.2 M sodium *m*-periodate at 4 °C in the dark followed by reduction with NaBH₄ as described [31]. This treatment induces cleavage of the C-2-C-3 bonds present in the non-sulphated uronic acid residues, resulting in an increase in backbone flexibility [31].

2-O-desulphated bovine mucosal heparin was obtained by selective 2-O-desulphation of the starting material under alkaline conditions [33]. The ^{13}C -n.m.r. spectrum of the product showed $\approx$ 95% 2-O-desulphation and no 6-O-desulphation. 6-O-desulphated mucosal heparin was obtained by preferential 6-O-desulphation (treatment with dimethyl sulphoxide/10% methanol at 80 °C for 6 h [34]) along with a partial 2-O-desulphation and N-desulphation of the starting material followed by re-N-sulphation. The ^{13}C -n.m.r. spectrum of the product showed $\approx$ 100% 6-O-desulphation, $\approx$ 15% 2-O-desulphation, and a complete re-N-sulphation. Chemical N-desulphation/N-acetylation of bovine lung heparin was carried out on a commercial batch of 15 kDa bovine lung heparin (SO₃⁻/COO⁻ ratio 2.5) obtained from Kabi-Pharmacia-Hepar as described [35].

^{13}C -n.m.r. spectra of unmodified and chemically modified heparins corresponded to those reported in the literature (bovine mucosal and lung heparins [36], low-molecular-mass heparin [37], reduced/oxidized heparins [31], N-desulphated/N-acetylated heparin [38], 2-O-desulphated heparin [33], 6-O-desulphated heparin [39]). Mucosal heparin and its derivatives differed from lung heparins essentially in their content of the prevalent disaccharide sequence L-iduronic acid 2-sulphate $\rightarrow$ D-glucosamine N,6-disulphate (55–70% for mucosal heparin and $>$ 80% for lung heparin), as determined by integration of the relevant ^{13}C -n.m.r. signals [40]. Molecular-mass determination and analysis of SO₃⁻/COO⁻ ratio of the different GAGs tested were performed as described by Harenberg and De Vries [41] and Casu and Gennaro [42] respectively, and the results are shown in Table 1.

Proteolytic digestion and SDS/PAGE

The protective effect of polysaccharides on tryptic digestion of bFGF was evaluated as described [20]. Briefly, 1 μ g portions of the growth factor were equilibrated at 37 °C for 5 min in 50 mM

Tris/HCl, pH 7.5, in the presence of increasing concentrations of the molecule under test. Then 60 ng of trypsin (Sigma, St. Louis, MO, U.S.A.) was added and digestion was allowed to proceed at 37 °C for 3 h. At the end of trypsin digestion, samples were added with an equal volume of SDS/PAGE reducing sample buffer, heated at 100 °C for 2 min and subjected to SDS/PAGE. Polyacrylamide gels (15%) were stained by the silver staining procedure. The only protein band visible on the gel migrated with an apparent molecular mass of 18 kDa and corresponded to undegraded bFGF [20]. The amount of undigested protein in a given lane was estimated by soft-laser scanning. The concentration of heparin able to prevent 50% of growth factor degradation (IC_{50}) was calculated.

Control experiments indicated that all the GAGs under test were unable to protect heat-denatured bFGF from trypsin digestion (results not shown), indicating that the protective effect of heparins depends on a specific interaction with bFGF which occurs only when the growth factor is present in its proper native conformation [20].

Cell cultures and cell-proliferation assay

Transformed fetal bovine aortic endothelial GM 7373 cells, corresponding to described BFA-1c 1BPT multilayered transformed clone [43], were obtained from the National Institute of General Medical Sciences, Human Genetic Mutant Cell Repository (Camden, NJ, U.S.A.). Cells were grown in Eagle's minimal essential medium containing 10% fetal calf serum (FCS), vitamins, essential and non-essential amino acids.

Cell proliferation was assayed on GM 7373 cells as described previously [44]. Briefly, cells were seeded at 70000/cm² in 24-well dishes. Plating efficiency was higher than 90%. After overnight incubation, cells were incubated for 24 h in fresh medium containing 0.4% FCS, 10 ng/ml bFGF and the indicated GAG concentrations. At the end of incubation, cells were treated with trypsin and counted in a Burkert chamber. The heparin concentration able to inhibit 50% of the mitogenic response exerted by bFGF (IC_{50}) was then calculated. In our experimental conditions, control cultures incubated in 0.4% FCS with no addition or with 10 ng/ml bFGF undergo 0.1–0.2 and 0.7–0.8 cell population doublings respectively. Cells grown in 10% FCS undergo 1.0 cell population doubling [44].

¹²⁵I-bFGF-binding assays

bFGF was iodinated as described [45] to a specific radioactivity of 800 c.p.m./fmol. GM 7373 cells were incubated at 4 °C in serum-free medium containing 10 ng/ml ¹²⁵I-bFGF, 0.15% gelatin, 20 mM Hepes buffer (pH 7.5) and the indicated concentration of the GAG under test. After 2 h, the amount of ¹²⁵I-bFGF bound to low- and high-affinity sites was evaluated [46]. Briefly, the medium was removed and the cells were washed once with PBS, twice with 2 M NaCl in 20 mM Hepes buffer (pH 7.5), and twice with 2 M NaCl in 20 mM sodium acetate (pH 4.0). The radioactivity released by the neutral 2 M NaCl wash and by the acidic 2 M NaCl wash represents ¹²⁵I-bFGF bound to low-affinity sites (300000 sites/cell; K_d = 300 pM) and high-affinity sites (20000 sites/cell; K_d = 20 pM) respectively. Previous experiments have demonstrated that this procedure is highly effective in distinguishing between high- and low-affinity bFGF binding in cultured endothelial cells, including GM 7373 cells [25,46]. Non-specific binding was measured in the presence of a 100-fold molar excess of unlabelled bFGF and subtracted from

all the values. The concentrations of heparin required to cause 50% inhibition of ¹²⁵I-bFGF binding to low- and high-affinity sites were then evaluated.

RESULTS

Natural and chemically modified heparins were quantitatively tested in a set of different assays for their capacity to interact with bFGF *in vitro* and to affect its biological behaviour in endothelial cell cultures. In detail, for each polysaccharide we evaluated the capacity to: (i) prevent *in vitro* tryptic digestion of bFGF; (ii) inhibit the binding of ¹²⁵I-bFGF to HSPGs present on the cell surface and in the ECM of endothelial GM 7373 cell cultures; (iii) reduce the rate of binding of ¹²⁵I-bFGF to tyrosine kinase FGF receptors present on the cell membrane of GM 7373 cells; (iv) inhibit the mitogenic activity exerted by bFGF in the same cells. In all experiments, heparins were tested in a wide range of concentrations (usually from 1 nM to 1–10 μM) and the IC_{50} for GAG was then calculated.

Effect of molecular mass and flexibility of mucosal heparin

In a first series of experiments we investigated the influence of size on the capacity of mucosal heparin to interact with bFGF in the different assays. To this purpose, we utilized three unmodified and two reduced/oxidized heparins with different molecular mass ranging from 13.6 to 2.1 kDa, but with similar charge density (Table 1). The 4.0 and 2.1 kDa heparins were obtained by controlled degradation of 13.6 kDa heparin. Reduced/oxidized heparins were prepared from 13.6 kDa and 4.0 kDa heparins by using sodium *m*-periodate treatment to induce cleavage of the C-2–C-3 bond present in the non-sulphated uronic acid residues, thus increasing the backbone flexibility of the molecule.

As shown in Figure 1a, all the heparins tested prevented tryptic digestion of a fixed amount of recombinant bFGF with different potencies. In agreement with previous observations [20], the capacity of heparin to inhibit proteolytic degradation of bFGF was directly related to its size (Table 1). The ability of heparin to prevent ¹²⁵I-bFGF binding to HSPGs and high-affinity FGF receptors present on GM 7373 cells was also directly related to its molecular mass (Figures 1b and 1c and Table 1).

Heparins of different size were assayed for their capacity to affect the mitogenic activity exerted by bFGF in GM 7373 cells (Figure 1d). All the heparins tested inhibited the mitogenic activity of bFGF but with different potencies. In this case also the inhibitory effect was related to the molecular mass of the GAG (Table 1), the only exception being 2.1 kDa heparin which showed a cytotoxic effect when administered to GM 7373 cells at doses higher than 200 nM (see the legend to Figure 1). It should be pointed out that the inhibitory capacity shown by the different heparins in the mitogenic assay does not simply reflect their ability to inhibit ¹²⁵I-bFGF binding to its high-affinity sites. The IC_{50} value for each polysaccharide in this assay is in fact 10–30 times higher than the corresponding value for the receptor-binding assay (Table 1). This may be due to the fact that inhibition of cell proliferation by heparin may depend on much more complex mechanisms than those investigated in a simple receptor-binding assay.

In all the assays, no significant differences were observed among unmodified heparins and the corresponding reduced/oxidized heparins, except for reduced/oxidized 4.0 kDa heparin which was four times more potent than unmodified 4.0 kDa heparin in preventing ¹²⁵I-bFGF binding to FGF receptors (Table 1). This indicates that an increase in backbone flexibility

Figure 1 Effect of size and flexibility of mucosal heparin on different bFGF properties

(a) Portions (1 μ g) of recombinant bFGF were incubated for 3 h at 37 °C with 60 ng of trypsin in the presence of the indicated concentrations of polysaccharide. Undigested bFGF in each sample was estimated by soft-laser scanning of SDS/PAGE gel. Data are expressed as percentage of digested bFGF. Each value is the mean of three to six independent experiments. (b, c) Subconfluent cultures of GM 7373 cells were incubated for 2 h at 4 °C with 10 ng/ml 125 I-bFGF in the presence of the indicated concentrations of polysaccharide. At the end of the incubation 125 I-bFGF bound to low-affinity HSPGs (b) and high-affinity FGF receptors (c) was measured. Data are expressed as percentage of 125 I-bFGF bound to control cultures in the absence of heparin. Each value is the mean of three to six independent experiments in duplicate. (d) Subconfluent cultures of GM 7373 cells were incubated for 24 h at 37 °C with 10 ng/ml bFGF in the presence of the indicated concentrations of heparin. At the end of the incubation, cells were treated with trypsin and counted. Data are expressed as percentage of the proliferation observed in cell cultures treated with bFGF alone. Control experiments showed that the inhibitory effect of the polysaccharides was specific for bFGF-induced cell proliferation, all the polysaccharides being ineffective on the mitogenic response exerted in these cells by 10% FCS, the only exception being 2.1 kDa heparin (results not shown). Each value is the mean of two to four independent experiments in duplicate. ●, 13.6 kDa heparin; ■, 4.0 kDa heparin; ▲, 2.1 kDa heparin; ○, reduced/oxidized 11.0 kDa heparin; □, reduced/oxidized 4.0 kDa heparin.

of the heparin molecule does not significantly affect its capacity to interact with bFGF under different experimental conditions.

As mentioned above, an optimum bFGF carrier should be endowed with the capacity to prevent binding of the growth factor to HSPGs without affecting its interaction with FGF receptors. In this respect, it is interesting to note that all the heparins tested were more potent in inhibiting 125 I-bFGF binding to low-affinity sites than to high-affinity receptors. This can be quantitatively expressed by calculating the ratio between the IC_{50} values for the two binding assays ($IC_{50\text{high}}/IC_{50\text{low}}$ ratio) for each GAG. An $IC_{50\text{high}}/IC_{50\text{low}}$ ratio higher than 1.0, as observed for all the heparins tested (Table 1), indicates the capacity of the GAG to inhibit more efficiently the binding of bFGF to HSPGs than to FGF receptors. Interestingly, the value of this ratio is directly related to heparin size. Indeed, 13.6, 4.0 and 2.1 kDa heparin were respectively 3, 10 and 15 times more potent in

preventing 125 I-bFGF binding to low- than to high-affinity sites (Table 1). This indicates that a reduction in GAG size influences bFGF-FGF receptor interaction more significantly than bFGF-HSPGs interaction. However, reduction in GAG size also results in a decreased capacity to protect bFGF from proteolytic digestion. In conclusion, modifications in molecular mass and/or flexibility of mucosal heparin do not seem to produce a polysaccharide endowed with all the properties required for an optimum carrier of bFGF.

Effect of selective desulphation of bovine mucosal heparin

Sulphate groups play an important role in the recognition of bFGF by heparin. Indeed, complete desulphation of heparin completely prevents interaction of the polysaccharide with the growth factor [3]. Recently, bFGF-binding sequences have been

Figure 2 Effect of selective desulphation of mucosal heparin on different properties of bFGF

Experiments were performed exactly as described in the legend to Figure 1. (a) Trypsin digestion of bFGF; (b) binding of ^{125}I -bFGF to low-affinity sites in GM 7373 cell cultures; (c) binding of ^{125}I -bFGF to high-affinity sites in GM 7373 cell-cultures; (d) mitogenic activity of bFGF in GM 7373 cell cultures. Each value is the mean of two to four independent experiments in duplicate. ●, 13.6 kDa heparin; ▲, 2-O-desulphated heparin; △, 6-O-desulphated heparin.

identified in GAGs of different origin [7–11]. These sequences derive from the clustering of IdoA(2-OSO₃)-containing disaccharide units, where the second hexose is often represented by GlcNSO₃ or GlcNSO₃(6-OSO₃), suggesting that N-sulphation and 2-O-sulphation are essential for bFGF recognition, but 6-O-sulphation is irrelevant [7,11]. On this basis we studied the effect of selective 2-O-desulphation or of 6-O-desulphation on the interaction of heparin with bFGF in our assays.

When tested for capacity to protect bFGF from tryptic cleavage, 6-O-desulphated heparin showed a potency close to that of unmodified heparin, whereas 2-O-desulphated heparin was approximately ten times less potent than mucosal heparin (Figure 2a). It should be pointed out that total charge is similar for the two desulphated heparins [$\text{SO}_3^-/\text{COO}^-$ ratio = 1.5 (Table 1)]. These data indicate that 2-O-sulphation of iduronic acid is important for the binding of GAG to bFGF and protection from tryptic cleavage, whereas 6-O-sulphation of glucosamine is not required.

In keeping with these results, 6-O-desulphated heparin inhibited ^{125}I -bFGF binding to HSPGs and FGF receptors present

in endothelial GM 7373 cell cultures with a potency similar to that of mucosal heparin. 2-O-Desulphated heparin, however, showed a capacity to displace ^{125}I -bFGF from low- and high-affinity sites that was approximately 100 times lower than that of unmodified heparin (Figures 2b and 2c and Table 1). 2-O-Desulphation, as well as 6-O-desulphation, did not affect the value of the $\text{IC}_{50\text{high}}/\text{IC}_{50\text{low}}$ ratio, which remained close to 2–3 (Table 1).

Selectively desulphated heparins were assayed for their capacity to inhibit the mitogenic activity exerted by bFGF in GM 7373 cells (Figure 2d). 2-O-Desulphated heparin, which inhibits ^{125}I -bFGF binding to its receptors poorly, inhibited the mitogenic activity of bFGF only at very high doses compared with unmodified heparin (IC_{50} approx. 46 μM and 0.6 μM for 2-O-desulphated and unmodified heparin respectively). Interestingly, 6-O-desulphated heparin, which displaces ^{125}I -bFGF from its low- and high-affinity receptors with a potency similar to that of unmodified heparin, also showed a 10-fold reduced capacity to inhibit the mitogenic activity of bFGF (IC_{50} 6.6 μM).

In conclusion, our data directly demonstrate the role of 2-O-

Figure 3 Effect of bovine lung-derived heparins on different bFGF properties

Experiments were performed exactly as described in the legend to Figure 1. (a) Trypsin digestion of bFGF; (b) binding of ¹²⁵I-bFGF to low-affinity sites in GM 7373 cell cultures; (c) binding of ¹²⁵I-bFGF to high-affinity sites in GM 7373 cell cultures; (d) mitogenic activity of bFGF in GM 7373 cell cultures. Each value is the mean of two to four independent experiments in duplicate. ●, 13.6 kDa mucosal heparin; ○, 9.7 kDa bovine lung heparin; □, 5.9 kDa bovine lung heparin; △, *N*-desulphated/*N*-acetylated bovine lung heparin.

sulphate groups in bFGF recognition and confirm that 6-O-sulphation represents a neutral structural modification for bFGF interaction. However, selective 2-O-desulphation does not result in a heparin endowed with the characteristics required for the proposed bFGF carrier. Indeed, the reduced inhibitory effect exerted by 2-O-desulphated heparin on the mitogenic activity and FGF receptor-binding capacity of bFGF is paralleled by a decreased capacity to protect the growth factor from tryptic degradation and prevent its binding to immobilized HSPGs.

Bovine lung heparin

The results described above indicate that modifications resulting in alterations of the chemical structure of mucosal heparin (i.e. reduction in molecular mass, increase in flexibility of the backbone structure, selective 2-O- or 6-O-desulphation) do not allow dissociation of the capacity of heparin to prevent proteolytic cleavage and HSPG binding of bFGF from the capacity to inhibit receptor interaction and mitogenic activity of the growth

factor. Mucosal heparin is a complex polysaccharide in which bFGF-binding basic IdoA(2-OSO₃)-containing disaccharide units represents approx. 55–70% of the total sulphated disaccharides [10]. These high-charge-density regions are intercalated with regions of low sulphation. On this basis, we decided to investigate the possibility that the above dissociation could be obtained by utilizing a heparin molecule with less complexity in disaccharide composition and clustering.

Bovine lung heparin shows a homogeneous structure in which IdoA(2-OSO₃)-containing disaccharide units represent up to 90% of the whole GAG [47]. Thus two preparations of bovine lung heparin, differing in size but with similar charge density (Table 1), were compared with mucosal heparin for their capacity to interact with bFGF in our assays. Also, on the basis of published results obtained with chemically modified heparins [3], *N*-desulphated/*N*-acetylated bovine lung heparin was used as a negative control.

As shown in Figure 3(a), 9.7 kDa bovine lung heparin protects bFGF from tryptic cleavage with a potency similar to that of

mucosal heparin. In agreement with the results obtained with low-molecular-mass mucosal heparin, 5.9 kDa bovine lung heparin is approx. three times less efficient than unfractionated heparin. Surprisingly, N-desulphated/N-acetylated bovine lung heparin was as potent as unmodified bovine lung heparin in preventing proteolytic digestion of bFGF.

The 9.7 and 5.9 kDa bovine lung heparins were as potent as mucosal 13.6 kDa heparin in preventing the interaction of bFGF with low-affinity sites present in GM 7373 cell cultures (Figure 3b). In contrast, bovine lung heparins displaced ^{125}I -bFGF from high-affinity FGF receptors with a potency that was significantly lower than that of mucosal heparin (Figure 3c). The 9.7 and 5.9 kDa bovine lung heparins showed values of approx. 50 and 100 respectively for the $\text{IC}_{50\text{high}}/\text{IC}_{50\text{low}}$ ratio (Table 1). This indicates that the two molecules are much more efficient in preventing ^{125}I -bFGF binding to HSPGs than to FGF receptors. Accordingly, the capacity of the two bovine lung heparins to inhibit the mitogenic activity exerted by bFGF in endothelial GM 7373 cells was reduced when compared with mucosal heparin (Figure 3d). As expected, N-desulphated/N-acetylated bovine lung heparin was highly inefficient at preventing ^{125}I -bFGF binding to both its low- and high-affinity sites and in inhibiting its mitogenic activity (Figure 3 and Table 1).

In conclusion, bovine lung heparins have the capacity to protect bFGF from proteolytic cleavage and to prevent its interaction with HSPGs. At the same time, when compared with mucosal heparins, they showed a decreased ability to affect receptor binding and mitogenic activity of bFGF.

DISCUSSION

The variety of *in vitro* and *in vivo* functions of bFGF, in combination with its wide tissue distribution, suggests that this molecule may play an important role in a large spectrum of pathological and physiological processes. On this basis, many potential clinical applications of bFGF and bFGF analogues are apparent [16]. For instance, bFGF has been investigated for its capacity to maintain the integrity of endothelium for the prevention of thrombosis [48] and its angiogenic action has been used to reduce the damage to ischaemic myocardium after coronary obstruction [49]. Moreover, bFGF offers the potential of acting as a direct agent for neuronal regeneration [50] and in the repair of duodenal ulcers [51] and non-healing wounds [52].

However, the use of bFGF as a therapeutic agent is potentially limited by its capacity to bind to immobilized HSPGs of the ECM. This may result in its rapid accumulation in the solid phase [18], preventing it from diffusing into the blood supply and therefore to target cells, thus reducing its effective dosage. However, several observations suggest that the ability of bFGF to interact with free heparin may offer significant advantages for its *in vivo* biodelivery. Indeed, the interaction of bFGF with GAGs in solution protects bFGF from inactivation [21] and proteolytic degradation [20–23] and prevents its binding to immobilized HSPGs, thus increasing its radius of diffusion in the extracellular environment [19]. However, interaction of bFGF with soluble heparin reduces its rate of binding to FGF receptors [25,53], thus inhibiting bFGF-induced proliferation of different endothelial cell lines [24–26].

On these bases, it can be hypothesized that, for efficient *in vivo* biodelivery, bFGF should be administered as a complex with an appropriately designed oligosaccharide characterized by a high capacity to protect bFGF from inactivation and proteolytic cleavage and the ability to prevent its interaction with HSPGs of the ECM, thus increasing its stability and diffusion in the

extracellular environment. At the same time, this oligosaccharide should not affect the interaction of the growth factor with its tyrosine kinase receptors and should not inhibit its biological activity. In the present paper, as a preliminary approach to a rational design of such an oligosaccharide, we have evaluated the possibility that natural heparins from different sources or chemically modified heparins may show at least some of the required characteristics.

In agreement with previous observations [9–11,20], the results demonstrate that heparin size is an important factor in regulating its interaction with bFGF, whereas an increase in flexibility of its backbone structure does not influence its capacity to bind the growth factor. We have also shown that selective 2-O-desulphation, but not 6-O-desulphation, dramatically reduces the capacity of heparin to interact with bFGF in the set of assays used in this study. Moreover, N-desulphation followed by N-acetylation also results in a drastic decrease in heparin potency in all the assays, the only exception being the trypsin-digestion assay which appears to be less sensitive than the others to structural modification of the sulphated polysaccharide (for further discussion of this point see Coltrini et al. [20]). Taken together, these findings demonstrate that 2-O-sulphation and N-sulphation of the polysaccharide are important for the recognition of bFGF by heparin, whereas 6-O-sulphate groups are not required [7]. This is in keeping with the recent identification of bFGF-binding sequences in both heparan sulphate and heparin [7–11]. Indeed, even though these sequences differ in size and affinity (ranging from a minimal low-affinity pentasaccharide sequence [11] to a high-affinity tetradecasaccharide sequence [7]), they all implicate IdoA(2-OSO₃) and GlcNSO₃ residues in bFGF binding, whereas the GlcN 6-O-sulphate groups are irrelevant [7–11]. As discussed recently [11], even though HSPGs represent the authentic cell surface mediator involved in bFGF interaction, bFGF-binding sequences appear to be more abundant in heparin than in heparan sulphate. Together with the availability of the former in amounts useful for therapy, this makes heparin and heparin derivatives, rather than heparan sulphate, interesting tools for the pharmacological modulation of bFGF activity.

The results described indicate that chemical modification of mucosal heparin (such as reduction in molecular mass, selective 2-O-desulphation or N-desulphation) can affect GAG–bFGF interaction. However, no dissociation of the positive effects of this interaction (i.e. protection of bFGF from proteolytic cleavage and inhibition of its binding to immobilized HSPGs) from the negative consequences (i.e. inhibition of bFGF interaction with FGF receptors and inhibition of its mitogenic activity) was observed. This dissociation was, however, obtained using two preparations of bovine lung heparin differing in molecular mass. These preparations retained the capacity to protect bFGF from proteolytic cleavage and to prevent its interaction with HSPGs. At the same time, when compared with mucosal heparins of similar size, they showed a reduced ability to affect binding of the growth factor to its cell membrane receptors. Accordingly, the capacity of bovine lung heparin to inhibit the mitogenic activity of bFGF in endothelial cells was 3-fold less than that of mucosal heparin.

The results obtained with bovine lung heparin raise the question of the mechanism involved in this dissociation. The capacity of a soluble GAG to prevent tryptic cleavage of bFGF depends on its ability to recognize the growth factor and to cause a conformational modification which masks the molecule to the catalytic site of the enzyme. Inhibition of bFGF binding to cellular HSPGs depends instead on the capacity of the soluble polysaccharide to compete with immobilized GAGs for the same heparin-binding domain of the bFGF molecule. The ability

of heparin to affect bFGF binding to FGF receptors, thus inhibiting its biological activity, may depend on more complex mechanisms. Recently, a heparin-binding domain has been characterized in FGF receptor 1/*fg* and it has been demonstrated to be essential for receptor-ligand interaction [15], indicating that the bFGF receptor may exist as a ternary complex of heparin sulphate, bFGF and tyrosine kinase receptor. This raises the possibility that distinct bFGF-binding sequences and FGF-receptor-binding sequences may be present in the carbohydrate portion of HSPGs as well as in free heparin [54]. Thus the capacity of free heparin to inhibit bFGF binding to its high-affinity sites may depend not only on its ability to interact with bFGF, but also on its ability to recognize the FGF receptor. These abilities appear to coexist in mucosal heparin and heparin-derived oligosaccharides of large size (at least 12 sugar residues [9,54]) but not in bovine lung heparin, possibly because of its simpler more homogeneous chemical structure.

In conclusion, our results demonstrate the possibility of dissociating some of the effects exerted by heparin on bFGF by utilizing heparin molecules from different sources. This is of importance in the design of synthetic molecules aimed at improving the bioavailability of bFGF when administered *in vivo* as a therapeutic agent. Moreover, our findings will help to identify oligosaccharide sequences involved not only in bFGF-heparin interaction but also in the more complex bFGF-heparin-FGF-receptor interaction.

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