Heparin inhibits the binding of basic fibroblast growth factor to cultured human aortic smooth-muscle cells

Françoise BONO, Patrice RIGON, Isabelle LAMARCHE, Pierre SAVI, Véronique SALEL and Jean-Marc HERBERT¹

Sanofi Recherche, 195 Route d'Espagne, 31036 Toulouse, France

Basic fibroblast growth factor (bFGF) and its specific receptors have diverse roles on a variety of cell types, such as the induction of vascular smooth-muscle cell proliferation which contributes to restenosis after coronary balloon angioplasty. bFGF is also known to interact with heparan sulphate proteoglycans present on the cell surface or in the extracellular matrix. In this study, the binding of ¹²⁵I-bFGF to human aortic smooth-muscle cells was investigated. ¹²⁵I-bFGF binding to these cells was reversible and saturable. Scatchard analysis revealed the presence of two distinct binding sites: a high-affinity receptor ($K_d = 38 \pm 7$ pM; 1480 ± 220 sites/cell) and a low-affinity non-saturable binding site ($K_d = 8.0 \pm 2.0$ nM). Pretreatment of the cells with heparinase resulted in a large reduction of ¹²⁵I-bFGF binding to its low-affinity receptors, suggesting that they are heparin-like molecules. The specificity of the low- and high-affinity binding sites for bFGF was determined with acidic FGF, platelet-derived growth

factor-BB and epidermal growth factor, which did not compete for ¹²⁵I-bFGF binding. Expression of FGF receptor isoforms analysed by reverse transcriptase-PCR revealed the presence of only the type-1 receptor. Binding to low-affinity binding sites was antagonized by heparin, suramin, protamine sulphate and platelet factor 4. Unexpectedly, these molecules also reduced the binding of ¹²⁵I-bFGF to its high-affinity sites. Consistent with these results, heparin, suramin, protamine sulphate and platelet factor 4 inhibited bFGF-induced proliferation of human aortic smooth-muscle cells. Heparin abrogated bFGF-induced release of tissue-type plasminogen activator by these cells. These observations suggest that the interaction of bFGF with human aortic smooth-muscle cells is different from that described for other cells such as endothelial cells, in which heparin acts as a potentiating factor of the mitogenic activity of bFGF.

INTRODUCTION

Fibroblast growth factors (FGFs) constitute a family of nine heparin-binding polypeptide growth factors that mediate a broad range of biological activities including induction of proliferation of smooth-muscle cells which contributes to restenosis after coronary angioplasty [1]. Basic (b) FGF (FGF-2) is a potent mitogen for a variety of cells, including vascular endothelial and smooth-muscle cells [2–6]. The biological activities of bFGF are mediated through a family of specific, high-affinity and structurally related membrane-associated tyrosine kinase receptors, and four distinct members of this FGF receptor (FGFR) family derived from separate genes have been identified to date [7–10]. Three of these receptors, FGFR1, FGFR2 and FGFR3, can transduce the bFGF signal *in vitro* and can be alternatively spliced to produce translation products (isoforms) that bind bFGF with different affinities [11]. The temporal and spatial regulation of these isoforms suggest that they probably play a major role in the specificity of FGF activation in different tissues. In addition to binding to high-affinity receptors, bFGF also interacts with lower-affinity cell-surface receptors [12–14]. These sites have been identified as heparan sulphate proteoglycans (HSPGs). The binding of bFGF to HSPGs protects bFGF from proteolytic degradation and may provide a reservoir of active bFGF in the extracellular matrix [15–17]. Recent studies indicate that HSPGs may also facilitate bFGF receptor binding and activation and it has been shown that cells that express FGFR but lack heparan sulphate do not bind or respond to bFGF [18,19]. The addition of exogenous heparin or heparan sulphate restores the binding and mitogenic activity of bFGF, strongly

suggesting that an interaction between heparan sulphate and bFGF is required for the high-affinity binding of bFGF to its receptor and is necessary to elicit a biological response. A dual-receptor system for bFGF has been proposed, which is composed of a low-affinity HSPG receptor which binds bFGF, induces a conformational change in the molecule, and thus presents the ligand to its high-affinity receptor in a biologically active form [20]. However, the heparin-dependence of the bFGF–receptor interaction has been questioned by Roghani et al. [21], who demonstrated that heparin increased the binding affinity of bFGF for FGFR in both myeloid and CHO cells but was not required for bFGF binding to its receptors. Although the importance of HSPG for bFGF binding and biological effect on various non-muscular cell types (mainly endothelial cells and fibroblasts) has been extensively studied and acknowledged [22,23], only a few data have been concerned with its effect on the activity of bFGF in vascular smooth-muscle cells. In the present study, we determined the binding characteristics of bFGF to human aortic smooth-muscle cells (HASMCs) and have identified a potential mechanism by which soluble heparin-like compounds compete with bFGF binding and activity. This is consistent with the model of vascular growth regulation and repair which is controlled by heparan sulphate and related molecules [24–30].

MATERIALS AND METHODS

Materials

HASMCs were purchased from Clonetics (Tebu, Le Perray, France). Dulbecco's modified Eagle's medium (DMEM) and PBS

Abbreviations used: FGF, fibroblast growth factor; a, acidic; b, basic; FGFR, FGF receptor; HSPG, heparan sulphate proteoglycan; HASMCs, human aortic smooth-muscle cells; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; PF4, platelet factor 4; t-PA, tissue-type plasminogen activator.

¹ To whom correspondence should be addressed.

were from Biochrom KG (Polylabo, Strasbourg, France). Foetal-calf serum (FCS), penicillin, streptomycin sulphate and glutamine were from Boehringer-Mannheim (Meylan, Claix, France). bFGF, acidic FGF (aFGF), platelet-derived growth factor-BB (PDGF-BB), epidermal growth factor (EGF), ^{125}I -bFGF (110 $\mu\text{Ci}/\mu\text{g}$) and enhanced chemiluminescence kit were from Amersham (Les Ulis, France). Heparin (172 units/mg; sodium salt), protamine sulphate, chondroitin sulphate, dermatan sulphate, Hepes, soya-bean trypsin inhibitor, bacitracin, heparinase from *Flavobacterium heparinum*, chondroitinase ABC, agarose (type II) and platelet factor 4 (PF4) were from Sigma Chemical Co. (L'Isle d'Abeau, France). Suramin was from Calbiochem (Meudon, France). The ELISA kit Asserachrom t-PA was purchased from Diagnostica Stago (Asnières-sur-Seine, France). The kit FastTrack was purchased from Invitrogen (San Diego, CA, U.S.A.), and DNase I, reverse transcriptase SuperScript II and *Taq* DNA polymerase were from Gibco-BRL (Cergy Pontoise, France). GeneClean silica matrix was from, Bio 101 (La Jolla, CA, U.S.A.). Restriction enzymes were purchased from Gibco-BRL, except *Bst*II, *Sac*II and *Sph*I which were from Promega (Charbonnières, France). Biotinylated B nylon membranes were from Pall (Saint Germain en Laye, France). All other chemicals were of the highest purity grade available and were from Prolabo (Gradignan, France).

Cell culture and growth measurements

HASMCs were routinely cultured in DMEM supplemented with 10 % FCS, 50 units/ml penicillin, 50 $\mu\text{g}/\text{ml}$ streptomycin sulphate and 4 mM glutamine. For measurement of cell growth, cells were seeded in 24-well cluster plates in DMEM+0.2 % FCS ($3 \times 10^4/\text{well}$) for 3 days in a humidified 5 % CO_2 incubator maintained at 37 °C. Culture medium was then removed and cells were seeded in DMEM+0.2 % FCS with various concentrations of bFGF (0–10 nM). The inhibition of proliferation induced by bFGF (1 nM) by various concentrations of heparin, suramin, protamine sulphate and PF4 was also studied under the same experimental conditions. For growth-rate determination, after 24 h in culture, triplicate plates were treated with trypsin, and the cells counted with a Coulter counter (Coultronics). Cells were routinely used between the third and the tenth passage.

Quantification of tissue-type plasminogen activator (t-PA)

For quantification of t-PA released by cultured HASMCs, the cells were cultured under the same experimental conditions. After 24 h in culture in the presence of various concentrations of bFGF (0.03–10 nM) with or without heparin, aliquots (0.1 ml) of medium were frozen at -70°C . Quantitative determination of t-PA in the aliquots of medium was performed with an ELISA kit (Asserachrom t-PA).

^{125}I -bFGF binding to HASMCs

For binding experiments, HASMCs were seeded in 24-well cluster plates ($3 \times 10^4/\text{well}$) in DMEM+10 % FCS and grown to confluence (4–5 days after plating) [$(2-3) \times 10^5$ cells/well]. Subconfluent cultures were incubated at 4 °C for 10 min and washed twice with ice-cold DMEM supplemented with 25 mM Hepes, pH 7.4, containing 0.3 mg/ml soya-bean trypsin inhibitor, 0.5 mg/ml bacitracin and 0.2 % gelatin. Incubations were carried out in 200 μl (total volume) of 25 mM Hepes, pH 7.4, supplemented with 0.3 mg/ml soya-bean trypsin inhibitor, 0.5 mg/ml bacitracin and 0.2 % gelatin (buffer A) which contained different concentrations of ^{125}I -bFGF. Triplicate experiments were carried

out at 7 °C for the indicated period of time. To determine total ^{125}I -bFGF binding, the cells were incubated for 5 min in 1 ml of 1 M NaOH and the extract was counted in a γ counter. Low-affinity binding of bFGF was determined specifically, by salt extraction (5 min with 1 ml of cold 2 M NaCl) before treatment with 1 M NaOH (high-affinity binding of bFGF). Non-specific binding was considered to be the value obtained for high-affinity binding in the presence of a 100-fold excess of unlabelled bFGF. In all experiments, representative wells were treated with trypsin, and cells were counted with a Coulter counter.

Heparinase treatment of HASMCs

To determine the amount of heparin-bound ^{125}I -bFGF in HASMCs, cells were pretreated with heparinase. The medium of subconfluent cultures of HASMCs ($3 \times 10^5/\text{well}$) was removed, and the cells were incubated for 1 h in serum-free DMEM with or without 3 units/ml *Flavobacterium heparinum* heparinase or 100 $\mu\text{g}/\text{ml}$ chondroitinase ABC at 37 °C. At the end of the incubation period, the cells were washed twice with ice-cold buffer A and used for binding experiments as described above.

Reverse transcriptase-PCR/Restriction/Southern-blot analysis of different types of FGFRs

RNA was extracted from HASMCs using FastTrack kit, and samples containing 500 ng of RNA were treated with DNase I and reverse transcriptase SuperScript II to obtain cDNA. PCR was performed as previously described by Xin et al. [31]. Aliquots of each cDNA were amplified in a mixture (100 μl final volume) containing 1/20 of transcript sample, 10 mM Tris/HCl, pH 8.2, 20 mM MgCl_2 , 50 mM KCl, 0.2 mM dNTP, 5 units of *Taq* DNA polymerase and 100 ng of each primer (forward primer, 5'-GARATGGAGRTGATGAAGMTGATYGG-3'; reverse primer, 5'-CCCRAARGACCACACRTCACTCTG-3'). These primers were first designed by Xin et al. [31] to hybridize with all the four types of FGFR. Twenty seven cycles of PCR were performed (95 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min) in a thermal cycler (Trio thermoblock; Biometra, Goettingen, Germany). PCR products were purified by electrophoresis on 2 % agarose (type II) and extracted on silica matrix. Aliquots of cDNA were incubated with various restriction enzymes, chosen with regard to their specificity for the amplified cDNA sequences found in the four FGFRs: *Bst*XI for FGFR1 and FGFR2; *Pst*I for FGFR1; *Pvu*II for FGFR2; *Bst*II and *Ngo*AIV for FGFR3; *Sph*I and *Sac*II for FGFR4. After electrophoresis of the mixture on 2 % agarose gel, cDNA fragments were transferred overnight on nylon membranes in 0.4 M NaOH. Membranes were washed in $2 \times \text{SSC}$ ($1 \times \text{SSC}$ is 0.15 M NaCl/0.015 M sodium citrate) at room temperature, prehybridized for 15 min at 42 °C in KGB buffer (0.1 M Tris/HCl, pH 8.8, 0.5 M NaCl, 1 % gelatin, 1 % casein, 0.1 % Tween) and hybridized for 30 min at 42 °C with horseradish peroxidase-labelled probes specific for each type of FGFR (50 ng/ml). The sequences of these probes have been reported by Xin et al. [31]. Figure 1 summarizes the strategy of PCR, restriction mapping and Southern blotting. Membranes were revealed by enhanced chemiluminescence with an ECL kit.

Expression of data

All data are means of at least triplicate experiments. Percentage inhibition was [(total binding – total binding with inhibitor)/specific binding] $\times 100$. IC_{50} was determined using an indirect Hill plot [32]. The apparent dissociation constant (K_d) and the maximal number of binding sites (B_{max}) were calculated using

Figure 1 Restriction map and hybridization of FGFR reverse transcriptase-PCR products

Reverse transcriptase-PCR-amplified cDNA encoding FGFR1, FGFR2, FGFR3 and FGFR4, obtained as described in the Materials and methods section, were restricted specifically with enzymes at the indicated sites (arrows). Biotinylated probes specific for each FGFR cDNA were hybridized at the specified regions (shaded areas).

Scatchard representation of the experimental data [33]. Data from saturation, competition and association studies were analysed using a non-linear regression program [34].

RESULTS

^{125}I -bFGF binding to HASMCs

As shown in Figure 2, total binding of ^{125}I -bFGF to HASMCs at 7 °C was time-dependent and reached an equilibrium 150 min after the beginning of the experiment. After 270 min of incubation, ^{125}I -bFGF binding was reversible, since the addition of excess unlabelled ligand (75 nM) almost totally dissociated ^{125}I -bFGF from the cells. On the basis of these results, equilibrium binding experiments were performed by setting the incubation time at 180 min.

Binding of ^{125}I -bFGF to HASMCs was dose-dependent (Fig-

Figure 2 Time-course of ^{125}I -bFGF binding to HASMCs

HASMCs (3×10^5 /well) were incubated for various periods of time with ^{125}I -bFGF (45 pM) at 7 °C in the presence (■) (non-specific binding) or absence (●) (total binding) of unlabelled bFGF (75 nM). The arrow indicates the time at which unlabelled bFGF (75 nM) was added to initiate the dissociation process (▲). Each point represents the mean calculated from three experimental determinations.

Figure 3 Saturation of ^{125}I -bFGF binding to HASMCs

(A) Saturation curve: HASMCs (3×10^5 /well) were incubated for 3 h at 7 °C with increasing concentrations of ^{125}I -bFGF. Specific binding is given by the difference between total and non-specific binding determined in the presence of 75 nM unlabelled bFGF. (B) A Scatchard plot of the specific binding of ^{125}I -bFGF was calculated from saturation isotherms determined from at least three independent experiments performed in triplicate.

ure 3A), and non-specific binding, as measured in the presence of excess unlabelled bFGF, was linearly dependent on the concentration of ^{125}I -bFGF (not shown). At all concentrations of ^{125}I -bFGF tested, the non-specific binding varied between 50 and 60 % of total binding. At low bFGF concentrations, binding was determined primarily by high-affinity sites (Figure 3A). The binding data, analysed by the method of Scatchard [33], demonstrated the presence of two binding sites: a high-affinity site with K_d of 38 ± 7 pM and a $B_{\max}$ of 1480 ± 220 sites per cell ($n = 9$) and a low-affinity non-saturable site with a K_d of 8.0 ± 2.0 nM.

As previously shown by Moscatelli [35], low- and high-affinity binding of bFGF could be differentiated by first washing the cells with 2 M NaCl (low-affinity binding sites) and then extracting the cells with NaOH (high-affinity binding sites). When we used this procedure, Scatchard analysis of the dissociation experiments on low-affinity binding sites indicated one site with a K_d of 9.8 ± 2 nM and $490\,000 \pm 90\,000$ sites/cell ($n = 6$). Scatchard analysis on high-affinity sites showed the presence of one binding site with a K_d of 30 ± 9 pM and 1500 ± 320 sites/cell, confirming the data obtained from the saturation experiments shown above (Figure 3). The addition of monoclonal antibodies directed against human bFGF completely inhibited ^{125}I -bFGF binding to both high- and low-affinity sites on HASMCs (not shown). Monoclonal antibodies directed against FGFR1, however, only inhibited the binding of ^{125}I -bFGF to the high-affinity sites without affecting binding to low-affinity sites (not shown). These data therefore show that binding of bFGF occurred via FGFR1

Figure 4 Effect of heparinase on ^{125}I -bFGF binding to HASMCs

HASMCs (3×10^5 /well) were incubated at 37°C for 1 h in serum-free DMEM with ($\diamond$) or without ($\circ$, $\bullet$) 3 units/ml heparinase. The cells were washed and incubated for 3 h at 7°C with increasing concentrations of ^{125}I -bFGF. The amount of ^{125}I -bFGF bound to its high- (A) and low- (B) affinity sites was determined as described in the Materials and methods section. Each point represents the mean calculated from three experimental determinations.

(high-affinity site) and suggest that cell-associated heparan sulphate is involved in low-affinity binding of bFGF, as already found for other cell types.

To investigate this possibility further, HASMCs were treated with heparinase. When this was carried out in conditions that eliminated 90 % of the glycosaminoglycans present at the cell surface (as evidenced after [^3H]glucosamine incorporation; not shown), binding of ^{125}I -bFGF to its high-affinity binding sites was not affected (Figure 4A), whereas binding to the low-affinity sites was greatly reduced (Figure 4B). On the other hand, pretreatment of the cells with 100 $\mu\text{g}/\text{ml}$ chondroitinase ABC did not affect ^{125}I -bFGF binding to its high- or low-affinity sites (not shown).

The specificity of HASMC receptors for bFGF was investigated by competition studies with bFGF, aFGF, PDGF-BB and EGF. bFGF competed with the binding of ^{125}I -bFGF on both low- and high-affinity binding sites (Figure 5). The IC_{50} values were respectively 24.9 ± 5.6 and 0.5 ± 0.1 nM ($n = 3$). For both binding sites, the Hill slope factor was near unity, suggesting a bimolecular reaction. The Shild analysis was linear and of unit slope, indicative of competitive binding. aFGF had little effect on either the low- or high-affinity binding of bFGF to HASMCs and only partially affected ^{125}I -bFGF binding at high concentration (Figure 5). PDGF-BB and EGF did not affect the binding of ^{125}I -bFGF to HASMCs, confirming its specificity for these binding sites on smooth-muscle cells.

Figure 5 Effect of bFGF, aFGF, PDGF and EGF on ^{125}I -bFGF binding to HASMCs

HASMCs (3×10^5 /well) were incubated for 3 h at 7°C with 45 pM ^{125}I -bFGF and increasing concentrations of bFGF ($\circ$), aFGF ($\bullet$), PDGF-BB ($\blacksquare$) or EGF ($\blacktriangle$). The amount of ^{125}I -bFGF bound to its high- (A) and low- (B) affinity sites was determined as described in the Materials and methods section. Each point represents the mean calculated from three experimental determinations.

Effect of heparin on ^{125}I -bFGF binding to HASMCs

The interaction of bFGF with heparan sulphate and related glycosaminoglycans present on the cell surface has been shown to be a prerequisite for the growth factor to bind to its receptors on various cell types [22]. The effects of heparin on the binding of ^{125}I -bFGF to its high- and low-affinity sites on HASMCs were therefore investigated. We found that heparin competed with the binding of ^{125}I -bFGF to its low-affinity sites on HASMCs, but unexpectedly also inhibited the binding of ^{125}I -bFGF to its high-affinity binding sites (Figure 6). Half of the binding of bFGF to its high-affinity sites could be antagonized with 6.0 ± 0.7 $\mu\text{g}/\text{ml}$ heparin. A 10-fold higher concentration was required to compete with ^{125}I -bFGF binding to its low-affinity sites ($\text{IC}_{50} = 48.8 \pm 1.1$ $\mu\text{g}/\text{ml}$) ($n = 3$). This inhibition was competitive for both sites, as demonstrated by Shild analysis, which was linear and of unit slope (not shown).

Suramin, protamine sulphate and PF4 also antagonized the binding of ^{125}I -bFGF to both low- and high-affinity sites of HASMCs (Figure 6). The IC_{50} values for the low- and high-

Figure 6 Effect of heparin, suramin, protamine sulphate and PF4 on ^{125}I -bFGF binding to HASMCs

HASMCs (3×10^5 /well) were incubated for 3 h at 7 °C with 45 pM ^{125}I -bFGF and increasing concentrations of heparin (●), suramin (■), protamine sulphate (▲) or PF4 (▼). The amount of ^{125}I -bFGF bound to its high- (A) and low- (B) affinity sites was determined as described in the Materials and methods section. Each point represents the mean calculated from three experimental determinations.

affinity binding sites respectively were: for suramin, 14.3 ± 1.0 and 21.3 ± 1.1 $\mu\text{g}/\text{ml}$; for protamine sulphate, 8.2 ± 2.4 and 60.3 ± 0.5 $\mu\text{g}/\text{ml}$; for PF4, 2.9 ± 0.8 and 1.3 ± 0.5 $\mu\text{g}/\text{ml}$. Chondroitin and dermatan sulphate, when tested at a high concentration (100 $\mu\text{g}/\text{ml}$), were completely without effect on ^{125}I -bFGF binding to both its high- and low-affinity sites (not shown).

Effect of heparin on bFGF-induced HASMC proliferation

bFGF was analysed for its ability to induce HASMC proliferation. Growth-arrested HASMCs were incubated with various concentrations of bFGF for 24 h. Under these experimental conditions, bFGF stimulated in a dose-dependent manner the growth of HASMCs (Figure 7). The concentration of bFGF required to obtain optimal cell proliferation (A_{max}) was between 3 and 10 nM, with an ED_{50} of around 1 nM. The maximum mitogenic effect of bFGF represented about 60% of the mitogenic effect of 10% FCS (not shown). Under these experimental conditions, heparin, suramin, protamine sulphate and PF4 inhibited in a dose-dependent manner HASMC proliferation induced by bFGF (Figure 8). The IC_{50} values were $24.0 \pm$

Figure 7 Mitogenic effect of bFGF for HASMCs

HASMCs were seeded (3×10^5 /well) in DMEM containing 0.2% FCS for 3 days. Culture medium was then removed and fresh medium was added with various concentrations of bFGF with (○) or without (●) 100 $\mu\text{g}/\text{ml}$ heparin. After 1 day in culture, cells were treated with trypsin and counted. Data are reported as means $\pm$ S.D. ($n = 9$) cell density.

Figure 8 Effect of heparin, suramin, protamine sulphate and PF4 on the mitogenic effect of bFGF for HASMCs

HASMCs were seeded (3×10^4 /well) in DMEM containing 0.2% FCS for 3 days. Culture medium was then removed and cells were seeded in the same medium with bFGF (1 nM) and increasing concentrations of heparin (▲), suramin (■), protamine sulphate (▼) or PF4 (●). After 1 day in culture, cells were treated with trypsin and counted. Data are reported as percentage inhibition of proliferation compared with cells in the controls ($n = 9$).

1.6 $\mu\text{g}/\text{ml}$ for heparin, 25 ± 2.9 $\mu\text{g}/\text{ml}$ for suramin, 30.5 ± 1.5 $\mu\text{g}/\text{ml}$ for protamine sulphate and 1.1 ± 0.1 $\mu\text{g}/\text{ml}$ for PF4.

Effect of heparin on bFGF-induced release of t-PA by HASMCs

bFGF stimulated in a dose-dependent manner the release of t-PA in the culture medium of HASMCs (Figure 9). This effect of bFGF, which represented 60% of the effect of 10% FCS or

Figure 9 Effect of heparin on bFGF-induced t-PA release by HASMCs

HASMCs were seeded (3×10^4 /well) in DMEM containing 0.2% FCS for 3 days. Culture medium was then removed and the cells were seeded in the same medium with various concentrations of bFGF with (○) or without (●) 100 µg/ml heparin. After 1 day in culture, aliquots (0.1 ml) of medium were collected and quantitative determination of t-PA was performed using an ELISA kit. Values are means $\pm$ S.D. for triplicate experiments.

Figure 10 Type of FGFR present on HASMCs

RNA extracted from HASMCs was amplified by reverse transcriptase-PCR as described in the Materials and methods section. PCR products were purified by electrophoresis on agarose (A) and submitted to the action of different restriction enzymes. Restriction products were separated by electrophoresis, transferred to nylon membranes and revealed by Southern blotting (B-E). Areas containing hybridization sites were detected by probes specific for FGFR1 (B), FGFR2 (C), FGFR3 (D) and FGFR4 (E). Samples were as follows: unrestricted material was in lanes 1 and 8; material restricted by *Bst*I in lane 2, by *Ngo*AI in lane 3, by *Sac*II in lane 4, by *Sph*I in lane 5, by *Pvu*II in lane 6, by *Bst*XI in lane 7 and by *Pst*II in lane 9.

PMA (100 nM), was strongly inhibited by heparin (100 µg/ml) (Figure 9).

Determination of the type of FGFR present in HASMCs

After reverse transcriptase-PCR, only one band was obtained on electrophoresis on agarose (Figure 10A) at a molecular mass corresponding to 460 bp. After Southern blotting, probes for the four FGFRs hybridized with the unrestricted cDNA (Figure 10B). However, Southern-blot patterns were not modified when extracts were submitted to the action of restriction enzymes, except for *Bst*XI and *Pst*I, showing that only FGFR1-derived cDNA was actually present.

DISCUSSION

In the present study, we first determined the binding characteristics of bFGF to HASMCs and found the existence of two types of binding site: a high-affinity receptor, which was identified by reverse transcriptase-PCR as FGFR1, and low-affinity binding sites, which were HSPG, as demonstrated by heparinase pretreatment of the cells. Although these results show similarities to binding data for other cell types, such as endothelial cells [23,36], this is the first description of the bFGF-binding characteristics of cultured HASMCs.

The presence of only FGFR1 as the high-affinity binding site for bFGF on these cells confirmed data obtained *in vitro* [31,37] and *in vivo* [38] on rat smooth-muscle cells, providing evidence that, in injured arteries, the ligand/receptor system of bFGF and FGFR1 is involved in the proliferative response of smooth-muscle cells leading to neointima formation.

Besides interacting with its high-affinity receptors, we found that bFGF also interacts with lower-affinity binding sites on HASMCs. These sites have been identified as the HSPG found on the cell surface and in the extracellular matrix [39–42]. Several possibilities have been advanced for the biological importance of bFGF binding to heparan sulphate. (1) The interaction with heparan sulphate protects bFGF from thermal denaturation and from the action of proteases [43, 21]. (2) bFGF bound to HSPG in the matrix or on the cell surface provides a reservoir of growth factor that can subsequently bind to and activate receptors, thereby ensuring long-term response to a brief exposure to bFGF [44]. (3) bFGF can be released from the low-affinity binding sites by an excess of soluble heparin or heparan sulphate or by enzymic degradation of HSPG, generating a soluble heparan sulphate–bFGF complex that can diffuse and activate receptors on cells at sites distant from the point of release [45]. Another role more central to bFGF signal transduction has been proposed for HSPG. It has been suggested that the interaction between bFGF and cell-associated HSPG may be required for the high-affinity binding of bFGF to its receptor and is necessary to elicit a biological response [21]. A dual-receptor system for bFGF has been proposed composed of a low-affinity HSPG receptor that binds bFGF, induces a conformational change in the molecule and thus presents the ligand to its high-affinity receptor in a biologically active form [31]. Therefore, although it is widely accepted that endogenous cell-associated heparin-like compounds facilitate bFGF binding and activity on non-muscular cells [22], apparently conflicting results have been reported for the effects of exogenous preparations of soluble heparin-like compounds on smooth-muscle cells. Indeed, our results and those of others suggest that soluble heparin-like compounds may compete with cell-associated bFGF-binding sites to inhibit bFGF action [30,46,47]. The reason for such a difference remain unclear, but is consistent with the model of vascular growth regulation and repair that is controlled by heparan sulphate and related

molecules [24–29]. Indeed, endothelial and smooth-muscle cells have previously been demonstrated to produce heparin-like compounds that inhibit their growth in culture [25,28,29], and endogenous heparin preparations inhibit neointimal hyperplasia after vascular injury alone [27] or in the presence of bFGF [24]. Consistent with our results are data describing the ability of vascular derived HSPG to inhibit the binding and mitogenic effects of bFGF on vascular smooth-muscle cells, in part by interacting with heparin-binding growth factors in the extracellular environment, preventing binding to cell-surface HSPG [30]. As suggested by others [30,48–50], the effect of soluble heparin on bFGF binding may depend on the level of endogenous heparan sulphate relative to bFGF receptors within the cells and may be complicated by the binding of soluble heparin to bFGF receptors and to cells [48–50]. However, unlike other observations on Balb/c 3T3 cells [30], low concentrations of heparin (1–100 ng/ml) did not facilitate bFGF binding to heparinase-treated or control cells (results not shown), showing that the effect of heparin was not dependent on the level of membrane-associated heparan sulphate.

Since the inhibitory effect of heparin occurred to the same extent on both the high- and low-affinity ^{125}I -bFGF-binding sites, our data are consistent with the hypothesis of a dual-receptor system for bFGF, suggesting that the interaction between bFGF and cell-associated HSPG may be required for the high-affinity binding of bFGF to its receptor and is necessary to elicit a biological response [21]. This hypothesis was further reinforced by the results obtained with suramin, an anti-cancer and angiosuppressive agent that has been described as a potent inhibitor of bFGF binding to cell-bound HSPG on endothelial cells [51]. This possibility was also supported by data showing that PF4, which has been demonstrated to inhibit the binding and activity of bFGF via disruption of the binding of bFGF to cell-associated heparan sulphates [52], also inhibited ^{125}I -bFGF binding to its high-affinity sites on HASMCs.

Smooth-muscle cell proliferation and migration, key steps in atherogenesis and restenosis, are inhibited by heparin [24–30]. However, the underlying mechanism(s) responsible for this effect is still unknown, but the possibility of inhibition of bFGF activity at the vascular level by heparin has been suggested by Lindner et al. [53] who showed, using a rat carotid artery model of balloon catheter injury, that bolus injections of heparin depleted the arterial wall of endogenous bFGF, suggesting that heparin could inhibit smooth-muscle cell proliferation in part by removal of released bFGF from sites of injury.

An early response to myointimal injury is the tightly regulated production of proteases, especially t-PA, required for breakdown of the basement membrane during cell migration and proliferation [54]. The t-PA activity of smooth-muscle cells induced by bFGF was inhibited by heparin. Of interest, we observed that the bFGF-stimulated t-PA activity, but not that induced by a phorbol ester, was inhibited by heparin (not shown), showing that the effect of heparin was indeed specific for bFGF.

Thus, together with previous data, our results suggest that bFGF activity may be tightly controlled by the balance of cell-associated and soluble heparin-like compounds. Our results also indicate that interference with the bFGF–HSPG binding interaction on HASMCs by heparin might be sufficient to inhibit the mitogenic and other functional responses of bFGF on smooth-muscle cells. Moreover, our data provide a rational explanation for the anti-proliferative effect of heparin on these cells, but further studies are needed to determine why heparin behaves as an inhibitor of bFGF binding and activity whereas, in other cell types, the addition of soluble heparin facilitates the effects of bFGF.

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