Heparan sulphate proteoglycans modulate fibroblast growth factor-2 binding through a lipid raft-mediated mechanism

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We investigated how lipid raft association of HSPG (heparan sulphate proteoglycans) modulates FGF-2 (fibroblast growth factor-2/basic fibroblast growth factor) interactions with vascular smooth-muscle cells. When lipid rafts were disrupted with sterol-binding agents, methyl- β -cyclodextrin and filipin, FGF-2 binding to HSPG was reduced 2–5-fold, yet the amount and turnover of cell-surface HSPG were unaffected. Approx. 50–65 % of bound FGF-2 was in lipid raft-associated fractions based on insolubility in cold Triton X-100 and flotation in OptiPrep density gradients, and this level was increased with higher FGF-2 concentrations. Less FGF-2 (50–90 %) was associated in raft fractions when cholesterol was depleted or HSPG were degraded with heparinase III. To investigate how lipid raft–HSPG interactions

altered binding, we compared the rates of FGF-2 dissociation with native, M β CD (methyl- β -cyclodextrin)- and filipin-treated cells. We found that FGF-2 dissociation rates were increased when lipid rafts were disrupted. These results suggest that localization of HSPG within lipid rafts creates high local concentrations of binding sites such that dissociation of FGF-2 is hindered. The localization of FGF-2 and HSPG to lipid rafts also correlated with the activation of protein kinase C α . Thus raft association of HSPG might create growth factor traps resulting in increased binding and signal transduction to enhance cell sensitivity.

Key words: cholesterol, FGF (fibroblast growth factor), heparan sulphate, Triton X-100 insolubility.

INTRODUCTION

FGF-2 (fibroblast growth factor-2/basic fibroblast growth factor) represents the best-characterized member of the larger family of heparin-binding growth factors [1]. FGF-2 has been implicated in stimulating VSMC (vascular smooth-muscle cells) proliferation after balloon injury and in mediating vascular remodelling *in vivo* [2,3]. At the cell surface, FGF-2 binds to tyrosine kinase receptors as well as HSPG (heparan sulphate proteoglycans) [4]. HSPG have been shown to enhance interactions of FGF-2 with its receptors through the generation of high-affinity ternary complexes [5]. In addition, recent reports have demonstrated that FGF-2 can act directly to aggregate cell-surface HSPG and induce PKC α (protein kinase C α) activation, suggesting receptor signalling activities of HSPG that might not depend on FGF receptor activation [6]. These findings, coupled with the long-standing observations that heparin and heparan sulphate are potent regulators of VSMC proliferation [7,8], suggest a complex mode of growth regulation by HSPG and growth factors within the blood vessel wall.

HSPG consist of a protein core and at least one heparan sulphate polysaccharide chain. Approx. 20 HSPG core proteins have been characterized with most being associated with the cell surface as members of the syndecan or glypican families [9,10]. Additionally, HSPG function in the extracellular matrix (i.e. perlecan) as sites for growth factor storage, where they protect growth factors from denaturation and degradation and modulate macromolecular transport and dimerization [11–13].

The structural determinants for binding of growth factors to HSPG have only begun to be defined, and current results coupled with the structural complexity of heparan sulphate suggest the

existence of a large number of distinct protein binding sites [14]. Indeed, HSPG have been shown to participate in the cell binding, uptake and catabolism of a wide variety of ligands, including lipoproteins, infectious agents and enzymes [15,16]. For example, the ability of HSPG to facilitate lipoprotein uptake and retention within the blood vessel wall is believed to be central to the development of atherosclerosis. Thus HSPG appear to hold a key position in two major cell-signalling pathways, lipoprotein retention and growth factor action, implicated in the development of vascular disease.

Recent results suggest that cell-surface HSPG are not uniformly distributed but instead appear to be localized to cholesterol-rich lipid raft domains [17,18]. Electron microscopic analyses have suggested that FGF-2 is internalized via HSPG within non-coated flask-like invaginations and caveolar-like structures rather than in clathrin-coated pits [19]. Syndecan-1, -2 and -4 have been shown to associate with detergent-insoluble membrane microdomains, and FGF-2 induces clustering and redistribution of syndecan-4 into membrane rafts [17,18]. Lipid rafts are cholesterol-dependent membrane domains with high sphingolipid content. These specialized domains are resistant to solubilization in non-ionic detergents such as Triton X-100 at low temperature [20]. It appears that a subset of membrane proteins possess affinity for raft domains; however, the specific properties that dictate protein preference to rafts are yet to be defined. Thus it is possible that the binding of heparin-binding growth factors to cell-surface HSPG could induce clustering within membrane rafts, where unique signal-transduction events could be activated.

The aim of the present study was to determine if HSPG localization within cholesterol-rich membrane rafts alters FGF-2

Abbreviations used: CS, calf serum; ECM, extracellular matrix; EGF, epidermal growth factor; FGF-2, fibroblast growth factor-2/basic fibroblast growth factor; GAG, glycosaminoglycan; HB-EGF, heparin-binding EGF-like growth factor; Hep III, heparinase III; HSPG, heparan sulphate proteoglycans; M β CD, methyl- β -cyclodextrin; PKC α , protein kinase C α ; SMC, smooth-muscle cells; VSMC, vascular SMC.

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binding to VSMC. FGF-2 binding to HSPG sites and its receptor decreased when lipid rafts were disrupted with the sterol-binding agents, methyl- β -cyclodextrin (M β CD) and filipin. The kinetics of FGF-2 dissociation was much more rapid in the presence of sterol-binding agents. Therefore FGF-2 binding and subsequent signalling appear to depend on lipid raft association of HSPG. These findings suggest an important mechanism for HSPG-facilitated binding of FGF-2, whereby the functionality of heparin-binding growth factors is dependent on localization to lipid rafts through HSPG interactions. These results also suggest a more general fundamental process whereby the localization of binding sites to microdomains on the cell surface could represent a mechanism used by cells to enhance their effective sensitivity in detecting dilute ligands present in their extracellular environment.

EXPERIMENTAL

Materials

Recombinant human FGF-2 was from Chiron (Mountain View, CA, U.S.A.). Hep III (heparinase III) from *Flavobacterium heparinum* was a gift from Dr E. Denholm at Biomarin Technologies (Montreal, Canada). $^{35}\text{SO}_4^-$ sulphate, ^{125}I -Bolton-Hunter, Na^{125}I and $[^3\text{H}]$ cholesterol were from PerkinElmer (Boston, MA, U.S.A.). Murine recombinant EGF (epidermal growth factor) was from Invitrogen (Carlsbad, CA, U.S.A.) and HB-EGF (heparin-binding-EGF-like growth factor) was from R&D Systems (Minneapolis, MN, U.S.A.). Mouse monoclonal antibody to a neo-epitope of HSPG, exposed after Hep III digestion (clone 3G10), was from Cape Cod Assoc. (Ijamsville, MD, U.S.A.). Mouse monoclonal antibody against phospho-PKCa was from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Mouse monoclonal antibody to transferrin receptor was from Zymed Laboratories (San Francisco, CA, U.S.A.). Mouse monoclonal antibody to flotillin-1 was from BD Biosciences Pharmingen (San Diego, CA, U.S.A.). Mouse monoclonal horseradish peroxidase-linked antibody was from Amersham Biosciences (Piscataway, NJ, U.S.A.). M β CD, water-soluble cholesterol (complexed with M β CD), filipin and nystatin were from Sigma (St. Louis, MO, U.S.A.). Sphingomyelinase from *Staphylococcus aureus* was from Calbiochem (San Diego, CA, U.S.A.). Cell-culture reagents were from Invitrogen. ^{125}I -FGF-2 and ^{125}I -HB-EGF were prepared using a modified Bolton-Hunter procedure [21]. ^{125}I -EGF was prepared using Iodobeads (Pierce Endogen; Rockford, IL, U.S.A.) [22].

VSMC culture

Bovine aortic VSMC were from Coriell Cell Repositories (Camden, NJ, U.S.A.). SMC (smooth-muscle cells, passages 6–15) were used at confluence as described previously [23]. SMC were maintained in Dulbecco's modified Eagle's medium low glucose, supplemented with 10 % (v/v) bovine calf serum (CS), penicillin (100 units/ml), streptomycin (100 $\mu\text{g}/\text{ml}$) and glutamine (2 mM). Cell numbers were determined quantitatively for each experiment with a Coulter counter (Model Z2; Coulter Corp., Miami, FL, U.S.A.).

Sterol-binding-agent treatments

To disrupt lipid raft formation, the cholesterol-binding agents, M β CD and filipin, were used [24,25]. SMC were pretreated with the indicated concentrations of M β CD for 2 h at 37 °C or filipin for 30 min at 37 °C before conducting binding assays with radiolabelled growth factor. Drug treatment was maintained during the 2.5 h binding incubation. Native SMC showed $95.1 \pm$

3.4 % viability when compared with 94.4 ± 4.4 and 89.9 ± 4.0 % for 10 mM M β CD- and 5 $\mu\text{g}/\text{ml}$ filipin-treated cells (by Trypan Blue). Cell number and morphology were similar for non-treated and treated cells.

FGF-2 binding

Equilibrium binding of ^{125}I -FGF-2 to SMC was conducted essentially as described in [21,23]. SMC were plated at 75 000 cells/2 cm² and cultured for 2 days in 10 % CS-Dulbecco's modified Eagle's medium. After ^{125}I -FGF-2 addition, cells were incubated for 2.5 h at 4 °C for cell-surface binding to occur. To extract HSPG-bound growth factor, a high-salt neutral-pH buffer (2 M NaCl/20 mM Hepes, pH 7.4) was used. To remove the receptor-bound growth factor, a high-salt low-pH extraction (2 M NaCl/20 mM sodium acetate, pH 4) was performed for 5 min. In some experiments, cells were digested with Hep III (0.1 unit/ml, 1 h at 37 °C) before performing the binding assay. Bound ^{125}I was quantified using an Auto-Gamma Cobra II gamma counter (Packard, Meriden, CT, U.S.A.).

FGF-2 dissociation kinetics

The dissociation kinetics of FGF-2 was determined with control, 5 $\mu\text{g}/\text{ml}$ filipin- or 10 mM M β CD-treated SMC [21]. ^{125}I -FGF-2 (0.28 nM) was bound to the surface of SMC for 2.5 h at 4 °C. Cell layers were washed and the new binding medium with or without 100 $\mu\text{g}/\text{ml}$ heparin was added to each well. Heparin was included in the binding medium to prevent rebinding of ^{125}I -FGF-2 that had been released from its primary binding site. Dissociation of ^{125}I -FGF-2 was performed for the indicated time after which the cells were washed once, and ^{125}I -FGF-2 bound to HSPG was extracted with high-salt buffer as described above. The amount of ^{125}I -FGF-2 bound to HSPG at each time point was normalized to the amount bound at zero time for each treatment. ^{125}I -FGF-2 bound to HSPG was plotted against the time of dissociation and fitted to a generalized equation for exponential decay that included a term to describe an irreversibly absorbed fraction of FGF-2 to determine observed dissociation constants according to $B_t = B_0 e^{-k_{\text{off}} t} + B_0 x$, where B_t and B_0 represent the amount of FGF-2 bound to HSPG sites at each time point t and at zero time respectively. k_{off} and x represent the observed first-order dissociation rate constant and the factor that describes the fraction of B_0 that was irreversibly absorbed (generally 0.1–0.15). The data were fitted using the Levenberg-Marquardt algorithm (non-linear least-squares method) with the program KaleidaGraph version 3.51 (Synergy Software, Reading, PA, U.S.A.). The apparent dissociation constants for each condition were calculated from the average of five separate experiments.

Triton X-100 extraction and immunoblot analysis

Confluent SMC were treated with either 10 mM M β CD or 5 $\mu\text{g}/\text{ml}$ filipin and incubated with ^{125}I -FGF-2 for 2.5 h at 4 °C. After three washes with binding buffer, the cells were extracted in 50 mM Tris (pH 7.4), 200 mM sucrose, 150 mM NaCl, 1 % (v/v) Triton X-100 and 2 mM PMSF for 30 min at 4 °C [25]. Extracts were centrifuged at 10 000 g for 10 min at 4 °C to separate the Triton X-100-insoluble (pellet) from the -soluble fraction. ^{125}I -growth factor was quantified by a gamma counter.

HSPG in whole cell lysates and Triton X-100-soluble and -insoluble fractions were analysed by SDS/PAGE. To generate the epitope for the 3G10 antibody on HSPG core proteins, cells were digested with Hep III (0.1 unit/ml, 1 h or 0.5 unit/ml, 15 min at 37 °C) before extraction. Fractions were solubilized in boiling Laemmli sample buffer [62.5 mM Tris, pH 6.8/10 % (v/v)

glycerol/2.3 % (w/v) SDS/5 % (v/v) 1-mercaptoethanol/0.01 % Bromophenol Blue] or lysis buffer (1 % Triton X-100/0.15 M NaCl/0.01 M Tris, pH 7.5/1 mM EDTA/1 mM EGTA/0.5 % Nonidet P40/1 mM PMSF/2 mM Na₃VO₄). Samples were subjected to SDS/PAGE (10 % gel) and transferred on to Immobilon-P membrane (Millipore, Bedford, MA, U.S.A.), and HSPG core proteins visualized with 3G10 (1 µg/ml, 1:1000 dilution) antibody by enhanced chemiluminescence [26,27]. As controls, a non-raft-associated protein, the transferrin receptor [anti-transferrin receptor (1 µg/ml), 1:500, 1 h at room temperature (23 °C)] and a raft-associated protein, flotillin-1 (anti-flotillin-1 (1 µg/ml), 1:250, 1 h at room temperature), in the Triton X-100 fractions were analysed. Transferrin receptor was exclusively in the soluble fraction and flotillin-1 was in the insoluble fraction (results not shown).

The Triton X-100-insoluble fraction was further separated into lipid raft and cytoskeletal components by successive detergent extraction with 1 % Triton X-100, 10 mM Tris (pH 7.6), 500 mM NaCl, 60 mM β-octylglucoside, 2 mM PMSF and 2 mM Na₃VO₄ on ice for 20 min [28]. Approx. 7 % of FGF-2 bound to the cell surface was associated with the cytoskeletal fraction; thus the vast majority (approx. 80 %) of the bound FGF-2 in the Triton X-100-insoluble fraction appears to represent lipid-raft-associated FGF-2 (results not shown).

Isolation of lipid raft membrane fractions

Raft membranes were isolated from VSMC using flotation on discontinuous OptiPrep density gradients [29,30]. Samples were prepared from confluent VSMC cultured in 10 cm dishes. VSMC were treated with or without 0.1 unit/ml Hep III digestion for 1 h at 37 °C and/or with or without 10 µg/ml FGF-2 for 2.5 h at 4 °C. Cells were washed once with cold PBS and lysed for 1 h on ice in 50 mM Tris (pH 7.5), 5 M NaCl, 1 % Triton X-100, 10 mM Na₃VO₄, 1 mM PMSF, 1 mM EDTA, 1 mM EGTA and protease inhibitor cocktail (Sigma). For flotation-gradient centrifugation, 270 µl of the cell lysate (100 µg of protein) was mixed with 530 µl of 60 % OptiPrep (Axis-Shield, Oslo, Norway) to produce a final concentration of 40 % OptiPrep, and transferred to the bottom of a microcentrifuge tube. The lysate was overlaid with five layers (0.6 ml each) of OptiPrep from 35 to 15 %. The samples were centrifuged in a TST60.4 rotor (Sorvall, Newtown, CT, U.S.A.) at 140 000 g for 18 h at 4 °C. Twelve fractions of 310 µl were collected from the top of the gradient. A 30 µl sample of each fraction was mixed with 6 µl of 5 × Laemmli sample buffer and proteins separated on 10 % (stub HSPG) or 15 % (FGF-2 and flotillin-1) SDS/polyacrylamide gel, transferred on to PVDF membrane and analysed by Western immunoblotting for HSPG (3G10), FGF-2 and flotillin-1.

Proteoglycan analysis

VSMC were metabolically radiolabelled with 100 µCi/ml [³⁵S]-sulphate overnight (18–20 h) and extracted and analysed by filtration through cationic nylon as described previously [31,32]. The labelling medium was removed and the cells were washed with 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, and the labelled proteoglycans were sequentially extracted from the extracellular matrix (ECM) and cell surface. ECM proteoglycans were extracted in 10 mM Tris/HCl (pH 7.4), 1 M urea, 10 mM EDTA and 1 mM dithiothreitol, and the cells collected by centrifugation at 12 000 g for 15 min. The cells were further extracted in 10 mM Tris/HCl (pH 8.0), 8 M urea, 10 mM sodium sulphate, 0.1 % Triton X-100 and 1 mM PMSF for 10 min on ice. [³⁵S]-sulphate-labelled proteoglycans in the ECM and cell fractions were

determined quantitatively using cationic nylon filtration [31]. The relative amount of heparan [³⁵S]-sulphate was determined by cleavage with nitrous acid. Filters were subjected to two 90 min incubations in fresh nitrous acid (0.48 M sodium nitrite combined with 3.6 M acetic acid), followed by a 60 min wash with 50 mM Tris/HCl (pH 8.0) and 0.65 M NaCl. Heparan [³⁵S]-sulphate levels were calculated as the difference between total ³⁵S-labelled proteoglycan and that resistant to nitrous acid cleavage.

In some experiments, cell-surface heparan [³⁵S]-sulphate levels were measured by digesting the cells with Hep III or trypsin. In these experiments, VSMC were radiolabelled overnight (18–20 h) with 50 µCi/ml [³⁵S]-sulphate, the labelling medium was removed, the cells washed twice with binding buffer and incubated in binding buffer alone or in buffer containing 1 unit/ml Hep III or in 0.05 % trypsin, 0.53 mM EDTA for 5 min at 37 °C. After the 5 min digestion period, the media were collected and the cells solubilized in 0.1 M NaOH. ³⁵S levels in the media and the cell layers were determined by counting in a liquid-scintillation counter. In some experiments, ³⁵S-labelled cells were subjected to unlabelled Triton X-100 solubility separation to assess the relative amount of ³⁵S-labelled material in raft and non-raft fractions.

PKCα Western-blot analysis

To measure the activation of PKCα by FGF-2, VSMC were plated at 750 000 cells/9.6 cm² and grown to confluence. The media were changed to 0.5 % CS 24 h before the experiment to generate quiescent VSMC. Cells were pretreated with either 10 mM MβCD for 2 h at 37 °C or 5 µg/ml filipin for 30 min at 37 °C. VSMC were washed with a warm binding buffer and FGF-2 (0.22 nM) added for various time points. Binding buffer with FGF-2 was removed and the cells were extracted in 50 mM Tris (pH 7.4), 200 mM sucrose, 150 mM NaCl, 1 % Triton X-100 and 2 mM PMSF for 30 min at 4 °C [25]. Extracts were centrifuged at 10 000 g for 10 min at 4 °C to separate the Triton X-100-insoluble (pellet) from the soluble fraction. Equal cell equivalents were subjected to SDS/PAGE (10 % gel) and Western-blot analysis was performed using anti-phospho-PKCα (1 µg/ml; 1:1000 dilution) for 1 h at room temperature.

Cholesterol efflux

To determine the effects of MβCD on cholesterol efflux, cells were labelled with [³H]-cholesterol (2 µCi/ml) overnight at 37 °C; the labelling medium was removed and the cells were treated with or without MβCD (10 mM) for 2 h at 37 °C. The media were collected and the cells were extracted with hexane/propan-2-ol (3:2, v/v) and [³H]-cholesterol levels were measured in a Tri-Carb Model 1500 scintillation counter (Packard). The 2 h MβCD treatment resulted in the release of 56.1 ± 2.0 % of the cell-associated cholesterol, whereas only 2.7 ± 0.7 % of the cell-associated cholesterol was released after 2 h in media alone.

RESULTS

Sterol-binding agents reduce FGF-2 binding to SMC

HSPG have been identified as critical cell-surface-binding sites for a wide range of heparin-binding growth factors. Recent observations that HSPG can be localized to cholesterol-rich lipid raft domains on the plasma membrane [17,18] suggest additional mechanisms whereby HSPG might control growth factor interactions. To determine if localization of HSPG within lipid rafts has an impact on FGF-2 binding to SMC, we treated cells with MβCD and filipin, which are known disruptors of lipid rafts.

Figure 1 FGF-2 binding to HSPG sites and its receptors decreases when lipid rafts are disrupted

SMC were pretreated with increasing doses of MβCD (**A**) for 2 h at 37 °C or filipin (**B**) for 30 min at 37 °C. ¹²⁵I-FGF-2 (0.28 nM) was added and equilibrium cell-surface binding of FGF-2 conducted for 2.5 h at 4 °C. The amount of FGF-2 bound to HSPG sites was determined quantitatively by extraction with 2 M NaCl and 20 mM Hepes, pH 7.4 (●). FGF-2 binding to its receptor was measured by extraction with 2 M NaCl and 20 mM sodium acetate, pH 4 (■). Results (means ± S.E.M.) shown are representative of two independent experiments performed in triplicate.

After treatment, binding of ¹²⁵I-FGF-2 to cell-surface HSPG was measured. Both agents caused a significant reduction in the level of ¹²⁵I-FGF-2 bound to HSPG (Figure 1). MβCD reduced FGF binding by as much as 90 % (Figure 1A), whereas filipin reduced binding by approx. 50 % (Figure 1B). An analysis of ¹²⁵I-FGF-2 binding over a wide range of concentrations revealed that both agents appeared to cause a reduction in the observed affinity and number of available FGF-2 binding sites on SMC (results not shown). Binding of FGF-2 to its cell-surface receptors was also decreased by treatment with the sterol-binding agents, consistent with the co-receptor function of HSPG (Figure 1). Importantly, the effects of MβCD on FGF-2 binding could be partially reversed by adding water-soluble cholesterol back to MβCD-treated cells (Table 1).

The effects of MβCD and filipin were neither specific to FGF-2 nor to SMC. Both agents reduced the binding of another heparin-binding growth factor, HB-EGF, to HSPG on SMC, and both agents reduced the binding of HB-EGF and FGF-2 to HSPG on bovine aortic endothelial cells (results not shown). To ensure that these effects were not unique to the specific agents tested, cells were also treated with an additional sterol-binding agent, nystatin

Table 1 Cholesterol reverses the MβCD effect on FGF-2 binding to SMC

SMC were pretreated with and without MβCD (10 mM) for 1.5 h at 37 °C. The cells were washed and treated with MβCD (10 mM) and/or cholesterol (10 μg/ml; water-soluble cholesterol complexed with MβCD) for an additional 2 h. After the treatments, ¹²⁵I-FGF-2 (0.28 nM) was allowed to bind for 2.5 h at 4 °C. The amount of FGF-2 bound to HSPG sites was determined quantitatively by extraction with 2 M NaCl and 20 mM Hepes (pH 7.4), and the receptor-bound FGF-2 was extracted with 2 M NaCl and 20 mM sodium acetate (pH 4). Results presented are the average of experiments performed in triplicate and expressed as means ± S.E.M.

Treatment		FGF-2-bound (fmol/10 ⁵ cells)	
MβCD	Cholesterol	HSPG-bound	Receptor-bound
–	–	14.8 ± 3.8	6.3 ± 0.3
–	+	14.4 ± 1.8	5.8 ± 0.4
+	–	2.7 ± 0.2	1.2 ± 0.1
+	+	10.5 ± 0.3	2.5 ± 0.3

(10 μg/ml for 30 min at 37 °C) or with sphingomyelinase (50 m-units/ml for 1 h at 37 °C), an enzyme that degrades sphingolipids, and FGF-2 binding was measured. Both treatments caused a reduction in FGF-2 binding to HSPG on VSMC (43.4 ± 5.5 and 32.9 ± 6.5 % reduction for nystatin and sphingomyelinase respectively). Thus disruption of lipid rafts alters heparin-binding growth factor binding, possibly by altering the physical localization and availability of HSPG on the cell surface.

Sterol-binding agents do not alter proteoglycan levels

A decrease in the effective binding of heparin-binding growth factors could result, in part, from the physical loss of HSPG from the cell surface during treatment with the raft-disrupting agents. To evaluate the level of cell-surface HSPG under the various treatment conditions, SMC were metabolically radiolabelled with ³⁵SO₄, and total ³⁵S-labelled proteoglycan as well as heparan [³⁵S]sulphate were measured (Figure 2). To evaluate the level of cell-surface heparan sulphate, ³⁵S-labelled SMC were subjected to treatment with Hep III, to solubilize specifically heparan sulphate or were digested with trypsin to cleave and release HSPG core proteins. Neither MβCD nor filipin treatment altered the level of total ³⁵S, nor the amount of ³⁵S released by Hep III or trypsin (Figure 2A). As an additional measure of the effects of these drugs on proteoglycan levels, ³⁵S-labelled cells were separated into ECM and cell-associated fractions and subjected to cationic nylon filtration to separate proteoglycans from other sulphate-labelled macromolecules as well as unincorporated sulphate. The filters were also subjected to nitrous acid treatment to evaluate the relative levels of heparan sulphate. Again, neither drug altered total or HSPG levels and, moreover, the relative distribution between ECM versus cell-associated ³⁵S-labelled proteoglycan was unaffected by the treatments (Figure 2B). The above analyses demonstrate that heparan sulphate levels were unchanged by the various treatments; however, it is still possible that proteoglycan turnover was altered during the treatment period. To assess turnover, ³⁵S-labelled cells were chased in medium with or without drugs containing no [³⁵S]sulphate for 2 h. Total ³⁵S-proteoglycan decreased to a similar extent under all three conditions (Figure 2C). Thus reduced growth factor binding to HSPG by MβCD and filipin was not a reflection of significant changes in the levels or turnover of cell-surface HSPG.

HSPG and FGF-2 associate with lipid rafts

To evaluate whether HSPG might localize to lipid rafts, we examined the HSPG core protein profile in Triton X-100-insoluble

Figure 2 Proteoglycan levels in cells treated with raft-disrupting agents

SMC were radiolabelled overnight with $^{35}\text{SO}_4$ (A, 50 $\mu\text{Ci/ml}$; B, C, 100 $\mu\text{Ci/ml}$). The cells were treated with M β CD or filipin and ^{35}S -proteoglycan and heparan sulphate levels measured as described in the Experimental section. (A) Cell-surface heparan sulphate levels were assessed by measuring the levels of ^{35}S released by Hep III or trypsin. M β CD (10 mM) was added to ^{35}S -labelled cells for the final 2 h and filipin (5 $\mu\text{g/ml}$) for the final 0.5 h and the cells were incubated with media alone or media containing Hep III (HS) or trypsin for 5 min. The media were collected and the radioactivity was measured. The cells were also extracted and radioactivity was measured (indicated as Total). Results (means $\pm$ S.E.M.) shown are representative of two independent experiments performed in triplicate. Black bars, untreated cells; drug-treated (M β CD or filipin) cells by the shaded bars. (B) ^{35}S -proteoglycan and ^{35}S -heparan sulphate levels were measured in SMC after treatment with M β CD and filipin. ^{35}S -labelled SMC were treated with M β CD (10 mM) for 2 h and filipin (5 $\mu\text{g/ml}$) for 0.5 h, and the ^{35}S -proteoglycan

Figure 3 HSPG core protein distribution in Triton X-100-insoluble fractions

(A) SMC were treated with 0.5 unit/ml Hep III (+ Hep III) or without enzyme (– Hep III) for 15 min at 37 °C. Whole cell lysates (W), Triton X-100-soluble (S) and -insoluble (I) fractions of SMC were isolated as described in the Experimental section. Samples were run on 10 % SDS/polyacrylamide gel and subjected to Western-blot analysis using antibody 3G10 (1 $\mu\text{g/ml}$) and anti-mouse horseradish peroxidase-linked secondary antibody (0.2 $\mu\text{g/ml}$). (B) Triton X-100-insoluble fractions of non-treated (NT), 10 mM M β CD (2 h at 37 °C) and 5 $\mu\text{g/ml}$ filipin (30 min at 37 °C) treated SMC were isolated as described in the Experimental section. Cells were treated with $\pm$ 0.1 unit/ml Hep III for 1 h at 37 °C and extracts were run on SDS/polyacrylamide gel, blotted and probed with antibody 3G10 as described above. NT, not treated.

fractions by Western-blot analysis using antibody clone 3G10 (Figure 3). Antibody 3G10 is a pan-HSPG core protein antibody that detects HSPG core proteins as a result of the generation of a neo-epitope after Hep III digestions. Thus 3G10 reactivity was created on cell-surface-exposed HSPG core proteins by subjecting cells to Hep III treatment before extraction and separation into Triton X-100-soluble and -insoluble fractions. Three to four major bands with apparent molecular masses of approx. 37–39, 53 and 76 kDa were observed in whole cell extracts from Hep III-treated cells (Figure 3A). There were also some high-molecular-mass (> 150 kDa) bands of lower intensity, which most probably represent extracellular matrix HSPG (i.e. perlecan), that were not solubilized by Triton X-100. In the Triton X-100-soluble fractions [Figure 3A, (S)], the 53 and 76 kDa bands were prominent, whereas the 37–39 and 76 kDa bands appeared to be enriched in the Triton X-100-insoluble fractions [Figure 3A, (I)]. M β CD

extracted from the cell layer into 'ECM' and 'Cell' fractions as described in the Experimental section. ^{35}S -proteoglycan was isolated on cationic nylon filters and the filters treated with and without nitrous acid to determine the levels of total ^{35}S -proteoglycan (black bars) and ^{35}S -heparan sulphate (shaded bars) in each sample. Means $\pm$ S.E.M. for quadruplicate samples are shown. (C) Turnover of ^{35}S -proteoglycan during drug treatment was assessed by subjecting $^{35}\text{SO}_4$ -labelled SMC to a 2 h chase period in the absence (black bars) or presence of 10 mM M β CD (shaded bars) or 5 $\mu\text{g/ml}$ filipin (for the last 0.5 h; hatched bars). Total cell-associated ^{35}S -proteoglycan was measured in cells that were not subject to the chase (time = 0) and compared with those that were (chase = 2 h; means $\pm$ S.E.M. for quadruplicate samples). The difference was calculated by subtracting the ^{35}S level from the chase samples from the zero time values (Difference). NT, not treated.

Figure 4 HSPG and FGF-2 association with lipid rafts

SMC were treated with 0.1 unit/ml Hep III for 1 h at 37 °C. FGF-2 (0.56 μ M) was added to SMC and allowed to bind for 2.5 h at 4 °C. Lipid raft fractions were isolated by flotation gradient centrifugation as described in the Experimental section. **(A)** Samples (30 μ l of each fraction) were run on 10% SDS/polyacrylamide gel and subjected to Western-blot analysis using antibody 3G10 (1 μ g/ml) and anti-mouse horseradish peroxidase-linked secondary antibody (0.2 μ g/ml). **(B, C)** Samples were run on 15% SDS/polyacrylamide gel and subjected to Western-blot analysis using antibody to 1 μ g/ml flotillin-1 **(B)** or 1 μ g/ml FGF-2 **(C)** and anti-mouse horseradish peroxidase-linked secondary antibody (0.4 μ g/ml). NT, not treated with Hep III.

and filipin treatment decreased the levels of HSPG core proteins in the Triton X-100-insoluble fractions (Figure 3B). Densitometric analysis revealed an approx. 40 and 60% decrease in the intensities of the 76 and 37–39 kDa core proteins respectively in the M β CD and filipin treatment conditions. A corresponding increase in the level of the 37–39 kDa core proteins in the soluble fraction was also observed in M β CD- and filipin-treated cells ($17 \pm 2\%$ increase from two separate experiments; results not shown). Similar changes in the relative distribution of the total pool of 35 S-labelled proteoglycans (not treated with Hep III) were observed where there was a $23 \pm 7\%$ increase in soluble and a $16 \pm 5\%$ decrease in Triton X-100-insoluble 35 S levels after drug treatment (mean of four separate experiments; total $n = 15$).

The presence of HSPG in the insoluble fraction of cold Triton X-100 extracts suggests that these molecules might be present within lipid rafts in SMC. To evaluate raft association, Triton

X-100 extracts of SMC were applied to discontinuous OptiPrep density gradients (5–40%). The light buoyant density of membrane rafts allows them to float, thus separating them from soluble and cytoskeleton-associated material in the Triton X-100 extracts. Cells were treated with and without Hep III before extraction to generate the 3G10 epitope on HSPG core proteins. Antibody 3G10 revealed that a significant fraction of HSPG core proteins (37–39 and 76 kDa) floated to the low-density fractions together with the lipid raft marker flotillin-1 (Figure 4). The 76 kDa HSPG core protein was observed in all fractions, with the highest levels being in fraction 5, whereas the 37–39 kDa core proteins were predominantly in fraction 5. However, it is important to note that the blots had to be overexposed with respect to the 76 kDa band to visualize the 37–39 kDa bands properly. Thus the results presented obscure the relative enrichment of the 76 kDa core protein in the raft fractions. To determine if FGF-2 bound to SMC

Table 2 Disruption of growth factors in Triton X-100-soluble and -insoluble fractions

^{125}I -FGF-2 (0.56 nM), ^{125}I -HB-EGF (0.83 nM) and ^{125}I -EGF (1.67 nM) were allowed to bind to SMC for 2.5 h at 4 °C. Unbound growth factor was removed by washing the cells three times with binding buffer. Cells were lysed in cold 1 % Triton X-100 and extracts were separated into soluble and insoluble fractions by centrifugation. The amount of radiolabelled growth factor in Triton X-100-insoluble fractions (lipid-raft-associated components) and Triton X-100-soluble fractions (non-lipid-raft-associated components) was determined quantitatively. The amount of ^{125}I -growth factor was normalized to the total amount bound (100 %). Results presented are the average of experiments performed in triplicate and expressed as means $\pm$ S.E.M.

Growth factor	% of total bound	
	Insoluble	Soluble
FGF-2	45.8 $\pm$ 1.60	54.2 $\pm$ 2.07
HB-EGF	48.8 $\pm$ 3.93	51.2 $\pm$ 8.26
EGF	24.4 $\pm$ 2.60	75.6 $\pm$ 11.4

became associated with lipid rafts via HSPG interactions, FGF-2 was allowed to bind to the cells before extraction and density flotation gradient analyses. Western-blot analysis of gradient fractions with anti-FGF-2 revealed that a significant fraction of the FGF-2 floated to the low-density fraction 5, whereas a portion remained at the bottom of the gradient, probably because it was solubilized (i.e. dissociated from its binding sites during extraction and centrifugation) or cytoskeleton-associated (Figure 4C). The association of FGF-2 with the buoyant fraction was HSPG-dependent as no FGF-2 was detected in fraction 5 from cells treated with Hep III. Protein assays were also conducted on each fraction to reveal that approx. 6 % of the total protein in the cell extract floated up to fraction 5, whereas most of it (> 50 %) remained on the bottom of the gradient (fractions 9–12). Thus the enrichment of HSPG core proteins and FGF-2 in fraction 5 probably represents the specific association of these molecules with lipid raft membranes in SMC.

Growth factor distribution in Triton X-100 fractions

To identify further the role of HSPG in mediating growth factor association with lipid rafts, we analysed the relative distribution of SMC-bound FGF-2, HB-EGF and EGF within unlabelled Triton X-100-insoluble fractions. ^{125}I -growth factors, FGF-2 (0.56 nM), HB-EGF (0.83 nM) and EGF (1.67 nM), were bound to SMC at 4 °C and the cells were lysed in unlabelled 1 % Triton X-100 and separated into soluble and insoluble fractions by centrifugation. Approx. 50 % of the bound FGF-2 and HB-EGF was localized to the Triton X-100-insoluble fraction (Table 2). Most (80 %) of the non-heparin-binding growth factor, EGF, was found in the Triton X-100-soluble fraction. Interestingly, approx. 80 % of the total SMC protein was in the soluble fraction, suggesting that the EGF profile reflects the general distribution of protein under these extraction conditions. Thus the relative enrichment of FGF-2 and HB-EGF in the insoluble fraction probably reflects the association of these heparin-binding growth factors with raft domains by binding to HSPG.

To evaluate further the role of HSPG, cells were treated with Hep III to degrade heparan sulphate chains, before conducting growth factor binding and detergent extraction. Hep III pre-treatment significantly reduced the amount of bound FGF-2 that associated with the Triton X-100-insoluble fraction (Table 3). Moreover, selective removal of HSPG-bound growth factor by treatment with high ionic strength buffer (2 M NaCl) before detergent extraction resulted in the reduction of insoluble FGF-2

Table 3 Triton X-100 insolubility of SMC-bound FGF-2 after Hep III treatment or high-salt neutral-pH extraction

SMC were treated with $\pm$ 0.1 unit/ml Hep III for 1 h at 37 °C. ^{125}I -FGF-2 (0.56 nM) was allowed to bind to SMC for 2.5 h at 4 °C. In a parallel set of SMC not treated with Hep III, the HSPG-bound FGF-2 was extracted with 2 M NaCl and 20 mM Hepes (pH 7.4) before Triton X-100 extraction. The level of ^{125}I -FGF-2 in the Triton X-100-insoluble fractions was measured and normalized to non-treated SMC (NT, 100 % bound). Results presented are the average of experiments performed in triplicate and expressed as means $\pm$ S.E.M.

Treatment	% of control-bound FGF-2
No treatment (control)	100 $\pm$ 3.49
+ Hep III treatment	13.6 $\pm$ 5.39
+ Salt extraction	18.5 $\pm$ 1.21

Figure 5 Effect of FGF-2 concentration on the level of FGF-2 in the Triton X-100-insoluble fraction

^{125}I -FGF-2 (0.139–55.6 nM) was added to SMC and allowed to bind for 2.5 h at 4 °C. The levels of bound ^{125}I -FGF-2 in Triton X-100 insoluble (○) and soluble fractions (●) were measured. The amount of FGF-2 in each fraction was normalized to the total amount of FGF-2 bound at each concentration. The results presented are average of experiments performed in triplicate and expressed as means $\pm$ S.E.M.

(Table 3). Hence, interaction of FGF-2 with HSPG appears to be responsible for the association of FGF-2 with the unlabelled Triton X-100-insoluble fraction of SMC.

The association of syndecan-4 with lipid rafts is facilitated by FGF-2 [17,18]. Thus we investigated whether the relative association of bound FGF-2 within the Triton-insoluble fraction was sensitive to FGF-2 concentration. FGF-2 (0.14–55.6 nM) was bound to SMC at 4 °C, and cells were separated into Triton X-100-soluble and -insoluble fractions (Figure 5). As the FGF-2 concentration increased, the relative amount of bound FGF-2 associated with the insoluble fraction increased steadily from approx. 45 % at the lowest dose tested to a maximum of approx. 65 %. Consistent with a mechanism involving lipid rafts, M β CD and filipin decreased the relative amount of bound FGF-2 that was associated with the insoluble fraction (Figure 6). Thus FGF-2 binding appears to facilitate its own association within lipid rafts, thereby increasing the efficiency of FGF-2 binding and retention by HSPG.

Filipin and M β CD increase the rate of FGF-2 dissociation

The clustering of HSPG within microdomains on the cell surface could have significant effects on the effective binding of growth

Figure 6 Effects of M β CD and filipin on FGF-2 levels in the Triton X-100-insoluble fraction

SMC were pretreated with increasing concentrations of M β CD (5–10 mM) for 2 h at 37 °C (**A**) or with filipin (1–5 μ g/ml) for 30 min at 37 °C (**B**). 125 I-FGF-2 (5.6 nM) was allowed to bind for 2.5 h at 4 °C. The amount of bound 125 I-FGF-2 in the Triton X-100-insoluble (open bars) and -soluble (black bars) fractions was measured. The results presented are the average of experiments performed in triplicate and expressed as means $\pm$ S.E.M.

factors. For example, theoretical considerations indicate that receptor clustering can significantly alter the observed rate constants for ligand binding [33]. One effect that has been documented is the ability of high concentrations of unoccupied binding sites to decrease ligand dissociation from the cell as a result of rebinding of dissociated ligand before it can escape from the cell surface. This effect has been implicated as an underlying reason for the differences in binding constants measured with isolated receptors in solution versus those with receptors localized to the plasma membrane [34]. Similarly, clustering of binding sites similar to HSPG within microdomains on the cell surface, in comparison with a situation where the receptors are evenly dispersed, could further enhance the capture of ligands such as FGF-2 once they have diffused to and encountered the cell surface. This type of process could play the important role of keeping FGF-2 within a raft until effective cell signalling and internalization have occurred.

To determine whether raft association of HSPG provides an environment where significant rebinding of FGF-2 can impact on FGF-2 dissociation, we measured observed rates of FGF-2 dissociation from SMC (control, M α CD- and filipin-treated) in the presence and absence of high concentrations of soluble heparin to block rebinding of dissociated FGF-2 (Figure 7). In the presence of heparin, all treatment groups showed rapid FGF-2 dissociation from HSPG binding sites (Figure 7B; observed $k_{\text{off}} = 0.300$, 0.250 and 0.176 min $^{-1}$ for control, M α CD- and filipin-treated cells respectively). However, in the absence of heparin, the dissociation of FGF-2 from HSPG sites on control cells was significantly lowered ($k_{\text{off}} = 0.004 \pm 0.002$ min $^{-1}$), suggesting that

Figure 7 Dissociation rate of FGF-2 increases when lipid rafts are disrupted

Dissociation kinetics of FGF-2 in non-treated (●, ○), M β CD-treated (10 mM, 2 h; ■, □) and filipin-treated (5 μ g/ml, 30 min; ◆, ◇) SMC in the (**A**) absence (filled symbols), and (**B**) presence (open symbols) of heparin (100 μ g/ml). 125 I-FGF-2 (0.28 nM) was allowed to bind to the cell surface for 2.5 h at 4 °C. Unbound growth factor was removed and the dissociation of bound FGF-2 was examined over a time period of 2 h. The amount of FGF-2 bound to HSPG sites was determined quantitatively by extraction with 2 M NaCl and 20 mM Hepes, pH 7.4. The results presented are the average of experiments performed in triplicate and expressed as means $\pm$ S.E.M.

significant rebinding of dissociated FGF-2 was occurring (Figure 7A). In contrast, dissociation of FGF-2 from filipin- and M α CD-treated cells was relatively rapid even in the absence of heparin (Figure 7A; $k_{\text{off}} = 0.129$ and 0.023 min $^{-1}$ for filipin- and M α CD-treated cells respectively). Thus rebinding appears to play a significant role in retaining FGF-2 on SMC and, moreover, this process seems to depend on lipid rafts.

FGF-2 stimulates PKC α activation

The above results indicate that HSPG clustering in lipid rafts results in prolonged FGF-2–HSPG interactions. The increased FGF-2 binding to HSPG might in turn lead to altered signalling through HSPG. While relatively little is known concerning HSPG-mediated signalling, several studies have shown that FGF-2 can stimulate PKC α activity through syndecan-4 [6,35]. Thus we investigated the role of lipid rafts in FGF-2-mediated PKC α signalling. Cells were treated with FGF-2 for various periods, and the

Figure 8 FGF-2 induced PKC α activation

VSMC were pretreated with 10 mM M β CD for 2 h at 37 °C or with 5 μ g/ml filipin for 30 min at 37 °C. FGF-2 (1 ng/ml, 0.056 nM) was added to VSMC for various time points within a 1 h time period at 37 °C. Triton X-100-soluble (A) and -insoluble (B) fractions were isolated as described in the Experimental section. Equal cell equivalents were loaded and run on 10% SDS/polyacrylamide gels and subjected to SDS/PAGE and Western-blot analysis. Blots were probed with an anti-phospho-specific PKC α antibody (1 μ g/ml). Subsequently, blots were incubated with horseradish peroxidase-linked anti-rabbit IgG (0.4 μ g/ml). Immunoreactive bands were visualized by ECL[®].

levels of phospho-PKC α (activated PKC α) in the Triton X-100-soluble and -insoluble (raft) fractions were measured (Figure 8). FGF-2-treated SMC showed higher levels of activated PKC α in the Triton X-100-soluble fraction, and a transient enrichment of activated PKC α was observed in the Triton X-100-insoluble fractions from FGF-2-treated cells. When lipid rafts were disrupted with M β CD or filipin, the FGF-2-mediated increase in the level of activated PKC α was significantly attenuated. Thus it appears that HSPG–lipid raft interactions contribute to modulating FGF-2-mediated HSPG signalling pathways.

DISCUSSION

Recent breakthroughs in proteoglycan research have revealed that HSPG are critical players in regulating cell function [36–38]. Several studies showed that HSPG are necessary for effective FGF-2 binding and subsequent downstream signalling through its receptor tyrosine kinase proteins [14,39]. Although the ability of HSPG to modulate FGF-2 binding to its receptors has received considerable attention, recent evidence also suggests that FGF-2 might signal directly through its interactions with HSPG, specifically syndecan-4 [6,35,40]. Moreover, evidence indicating that HSPG are localized in lipid raft microdomains suggests that non-uniform plasma-membrane distribution of HSPG could play a role in the modulation of heparin-binding growth factors [17,18,41]. In

the present study, we show that localization of HSPG in lipid rafts stabilizes cell-surface binding of FGF-2. Indeed, cholesterol depletion of SMC decreased FGF-2 binding without altering HSPG levels in the cell. Binding of FGF-2 to HSPG within lipid rafts appears to lengthen the residence time of FGF-2 at the cell surface, as reflected by an overall reduction in FGF-2 dissociation kinetics. The raft association of FGF-2–HSPG also appears to correlate with increased activated PKC α , suggesting a possible consequence of HSPG raft association with FGF-2-mediated signalling. Thus the physical localization of binding sites within microdomains on the plasma membrane could be a general mechanism by which cells increase their effective sensitivity to dilute biological signalling ligands in the extracellular space.

In these studies, we used two sterol-binding agents, M β CD and filipin, to manipulate lipid raft levels. M β CD is a cyclic poly-saccharide that binds cholesterol within its hydrophobic pore and chelates cholesterol from the plasma membrane [24]. Filipin is a polyene antifungal agent that sequesters cholesterol on the plasma membrane [42]. Thus these agents are chemically unrelated and act through very distinct mechanisms, yet have in common the ability to disrupt raft structures. In addition, the raft-disrupting agents, nystatin and sphingomyelinase, also decreased FGF-2 binding to HSPG. Therefore the consistent finding that all of these agents reduced FGF-2 binding to HSPG, without altering HSPG levels, strongly supports that this effect is a reflection of their ability to disrupt lipid rafts and is unlikely to be the result of non-raft-related activities. However, it is interesting to note that M β CD and filipin did show some differences in their ability to modulate FGF-2 binding to HSPG. Whereas M β CD caused a greater reduction in the amount of FGF-2 bound under equilibrium conditions (Figure 1), filipin caused a greater increase in the observed rate of FGF-2 release from HSPG-binding sites on cells (Figure 7). Thus it is unlikely that the effects of these agents on FGF-2 binding are solely the product of the decreased dissociation rate. For example, it is possible that raft association of HSPG might alter heparan sulphate structure such that cryptic binding sites are made available. For example, when HSPG are not within rafts, cell-surface heparin-binding proteins that are excluded from rafts might occupy protein binding sites on heparan sulphate chains. Hence, raft association of HSPG could destabilize these interactions making protein binding sites on heparan sulphate available for growth factor binding. In any case, it is clear that altering lipid raft structure and HSPG distribution has a marked impact on effective binding of FGF-2.

HSPG localization in lipid rafts might be an important mechanism to facilitate ligand binding. Recent evidence has shown that syndecan-1, -2 and -4 are localized to Triton X-100-insoluble fractions [17,18,41]. Moreover, syndecan-1 clustering in rafts has been suggested to be important for ligand internalization [17]. Recent studies have also shown that syndecan-4 clustering in lipid rafts can be induced by FGF-2 [18]. Taken together with the present results, FGF-2-mediated clustering of HSPG within rafts might create sites for increased growth factor capture by effectively changing the binding kinetics (Figure 9). The association and dissociation kinetics for FGF-2 binding to HSPG are rapid; in particular, the dissociation of FGF-2 from HSPG when re-binding is prevented is significantly faster than the rate observed for bound FGF-2 to be released from cells [21,33,34]. These findings indicate that once an FGF-2 molecule has become bound to an HSPG site on the cell surface, it probably dissociates and rebinds to neighbouring HSPG sites a significant number of times before dissociating from the cell surface. Therefore the observed binding affinity for FGF-2, and possibly other heparin-binding growth factors, is probably affected significantly by the density of HSPG on cells [43]. Hence,

Figure 9 Schematic representation of FGF-2-HSPG-lipid raft clustering

In native SMC, FGF-2 (filled circles) binds to cell-surface HSPG and this complex is localized in lipid raft microdomains. This 'clustering' effect facilitates further FGF-2 rebinding events to neighbouring HSPG causing an apparent increase in FGF-2 concentration in the local cellular microenvironment. The observed dissociation of FGF-2 from the cell surface is lowered resulting in increased retention of the growth factor and HSPG in lipid rafts. When the lipid rafts are disrupted by cholesterol depletion, the HSPG become dispersed in the membrane so that FGF-2-HSPG complexes are no longer 'clustered', thus allowing FGF-2-HSPG interactions to dissociate more rapidly.

clustering of HSPG could effectively increase the concentration of available growth factor binding sites within the immediate environment surrounding a growth factor that has dissociated from a primary binding site. In a sense, clusters of HSPG could act as growth factor traps, resulting in increased observed binding to cells and potentially increased signal transduction through HSPG. This process would be sensitive to plasma-membrane cholesterol levels as depletion of cholesterol could result in a more even distribution of HSPG and decreased growth factor binding. With recent evidence that the phosphorylation of syndecan-4 and the activation of PKC α are regulated by FGF-2 binding [6,44], it is clear that HSPG can directly participate in growth factor signalling. Thus increased FGF-2 binding to HSPG via lipid raft association could have marked consequences on cell signalling.

HSPG have been suggested to function as receptors in other systems as well. Notably, HSPG interact with several atherogenic lipoproteins directly mediating their uptake through a mechanism that involves clustering of HSPG (i.e. syndecan-1) and internalization through a non-coated pit-mediated pathway involving lipid rafts [41]. Whereas the role of lipid raft microdomains in the plasma membrane has received considerable attention, emerging evidence has also identified sphingolipid and cholesterol-rich lipid rafts within internal membrane compartments such as the Golgi apparatus, suggesting additional roles for lipid rafts in cholesterol homeostasis and vesicular trafficking [46,47]. Indeed, lipid raft-mediated clustering of HSPG may provide a link between physiological cholesterol levels and intracellular signalling pathways involved in the vascular response to heparin-binding growth factors associated with cardiovascular disease.

The process described here, where localization of binding sites (HSPG) on the plasma membrane alters the observed binding of ligand (FGF-2), might have general implications for a wide range of ligand-receptor pairs. It has been known for some time that cells use specific surface receptors to interact with extracellular stimuli; however, theoretical considerations reveal that the cell-surface density and affinity of these receptors is often insufficient to provide optimal cell sensitivity [48]. Moreover, it is clear that it is not sufficient if the number of receptors present on a cell as well as the intrinsic affinity of those receptors for ligand is known to predict cellular responsiveness. One reason for this could be that complex processes involving non-uniform distribution of binding sites on the cell surface might play major roles in controlling binding and activation. The example provided here

with FGF-2 and HSPG might represent one of many situations where localization of binding sites to microdomains on the cell surface can be used by cells to enhance their effective sensitivity to dilute ligands.

In summary, our results show that localization of HSPG-FGF-2 complexes in lipid rafts alters cell-surface binding and dissociation kinetics of FGF-2 in VSMC. Disruption of lipid raft formation resulted in reduced binding and increased dissociation rates of FGF-2 from the cell surface, and decreased FGF-2-mediated PKC α activation. Lipid raft-mediated clustering might function as a mechanism to regulate heparin-binding growth factors. More generally, the localization of ligand binding sites within specialized domains on cell surfaces may be a critical mechanism for enhancing cell sensitivity to external stimuli.

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