Heparin affects signaling pathways stimulated by fibroblast growth factor-1 and -2 in type II cells

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Newman, Donna R., Cheng-Ming Li, Rebecca Simmons, Jody Khosla, and Philip L. Sannes. Heparin affects signaling pathways stimulated by fibroblast growth factor 1 and -2 in type II cells. Am J Physiol Lung Cell Mol Physiol 287: L191–L200, 2004.—Undersulfation of the basement membrane matrix of alveolar type II (AT2) cells compared with that of neighboring type I cells is believed to account for some of the known morphological and functional differences between these pneumocytes. Heparin, a model for sulfated components of basement membrane matrices, is known to inhibit fibroblast growth factor (FGF)-2-stimulated DNA synthesis as well as gene expression of FGF-2 and its receptor in AT2 cells. To determine whether these endpoints result from specific effects of heparin on FGF-related signaling pathways, isolated rat AT2 cells were treated with 100 ng/ml FGF-1 or FGF-2 in the presence of up to 500 μg/ml heparin. In addition, experiments were done on cells grown in the presence of 20 mM sodium chloride (sulfation inhibitor). High-dose heparin reduced FGF-1- or FGF-2-stimulated phosphorylation of mitogen-activated protein kinase kinases (MEK1/2), p44/42 mitogen-activated protein kinases (MAPK/ERK1/2), stress-activated protein kinase/c-Jun NH2-terminal kinase, Akt/protein kinase B, and p90RSK. FGF-2-stimulated signaling was more sensitive to heparin’s effects than was signaling stimulated by FGF-1. Heparin had an additive effect on the reduced [3H]thymidine incorporation in FGF-treated AT2 cells caused by inhibition of the MEK/ERK pathway by the MEK inhibitor PD-98059. The data suggest that heparin’s known capacity to alter DNA synthesis and, possibly, other biological endpoints is realized via cross talk between multiple signaling pathways.

extracellular matrix; basement membrane; receptor tyrosine kinase phosphorylation; ERK pathway; fibroblast growth factor signaling

A VARIETY OF FUNCTIONS within the pulmonary alveolus, ranging from epithelial progenitor (6) and producer of surfactant (18) to modulator of regional host defense (38), are carried out by type II pneumocytes (AT2 cells). These various activities are controlled by a number of factors, often in combination, including cytokines and hormones (33, 37) and extracellular matrix (ECM) composition (13). The fibroblast growth factor (FGF) family is particularly relevant in this context, inasmuch as FGF-1, FGF-2, and FGF-7 are mitogens for AT2 cells (15, 25, 30, 31) and stimulants of surfactant production (40) and have important relations with ECMS. FGF-1 and FGF-2 are expressed by AT2 cells, with the former being immunolocalized to the cell cytosol and the latter within the ECM of the alveolar basement membrane (BM) (29). FGF-2 expression has been shown to be upregulated by FGF-1 in these cells (16). The activities of the FGFs are quite sensitive to the sulfated quality and quantity of ECMS; heparan sulfate (HS) side chains of proteoglycans (HSPG) act as low-affinity receptors and are a requisite part of a dual receptor system, which, along with a cell surface tyrosine kinase receptor, transduces ligand activation along appropriate cytosolic and nuclear pathways (9). The sulfated nature of HS has been demonstrated to be crucial in the interaction of FGFs and FGF receptors (20). Many of the FGFs are typically stored/bound within ECMS (chiefly composed of HSPGs), where they are stabilized and protected from extracellular degradation (27). They can be released from these storage sites by enzymatic digestion (27) or by FGF-binding protein, which can act as a shuttling molecule between ECM and cell surfaces (23). These functional characteristics of the FGFs are in large measure due to different affinities for and responses to the variety of sulfated ECMS they may encounter (11). These collective observations present intriguing possibilities with regard to AT2 cells, which reside on a BM known to be quantitatively less sulfated than the remainder of the alveolar BM (28). It has been suggested that such an arrangement may be a key determinant of the varied functions of AT2 cells (28, 30, 31).

FGF-1, FGF-2, and FGF-7 have been studied extensively in AT2 cells, and, in addition to being mitogenic, they also influence a number of different cellular activities, including surfactant production (40). Previous evidence indicates that the model ECM heparin downregulates DNA synthesis in isolated AT2 cells and that this effect is sulfate specific (30, 31). Furthermore, protein expression of FGF-2 itself and gene expression of FGF-1, FGF-2, and FGF receptor (FGFR) type 2 (but not FGFR-1) appear to be downregulated by heparin (17). The signaling events involved in these various outcomes are not fully described in the AT2 cell but likely involve complex interactions among phosphorylated proteins of multiple pathways. The goals of this study were 1) to identify those signaling pathways relevant to FGF-1 and FGF-2 treatments in isolated rat AT2 cells and 2) to determine whether sulfated ECM molecules alter activation of these pathways. To accomplish these goals, primary rat AT2 cells were treated in vitro with FGF-1 or FGF-2 and with or without the model sulfated ECM heparin. In addition, some AT2 cells were cultured in medium with sodium chloride, an inhibitor of sulfation of newly biosynthesized products, and subsequently treated with FGFs and heparin to study the difference undersulfation makes on heparin’s effects on selected signaling pathways. The results indicate that FGF-1 and FGF-2 stimulate signal transduc-
tion via the ERK1/2, stress-activated protein kinase (SAPK)/c-Jun NH2-terminal kinase (JNK), and Akt/protein kinase B (PKB) pathways and that heparin affects elements of these signaling pathways but not others, suggesting some degree of cross talk and specificity in its actions.

MATERIALS AND METHODS

Cell preparation. Rat AT2 cells were isolated from pathogen-free 150- to 200-g Fischer CDF rats (Charles River Laboratory, Wilmington, DE) as described previously (31). Cells were consistently >95% AT2 cells by virtue of their lamellar body content and surfactant protein D expression as previously shown (17). Isolated cells were resuspended in DMEM supplemented with 10% FBS, 100 μM penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B, and 50 μg/ml gentamicin (complete DMEM).

Cell culture and treatment. AT2 cells were plated at a density of 4 × 10^4 cells/cm² in 60-mm tissue culture-treated dishes (TPP, Denville Scientific) that had been coated with 0.06 μg/mm² type I collagen (Sigma, St. Louis, MO). Cells were allowed to attach and spread overnight in complete DMEM in a 5% CO2 atmosphere at 37°C and were cultured in complete DMEM or in hormonally defined, serum-free medium (SFHDM) (13) for an additional 24 h. After 24 h, attached cells were washed twice with DMEM without FBS (neural DMEM) and cultured for 2 h in fresh serum-free DMEM to quiet signal transduction events associated with serum-containing growth factors. Some cells were continuously cultured in medium containing 20 mM sodium chloride (Sigma) after the initial attachment period. After the 2-h quiescence period, cells were treated without a medium change (to avoid triggering nonspecific signal transduction, e.g., through stress-activated or integrin pathway) with 100 ng/ml FGF-1 or 100 ng/ml FGF-2 (R & D Systems, Minneapolis, MN) and with or without high-molecular-weight heparin (13,500; Calbiochem, La Jolla, CA) at a final concentration of 0.05–50 μg/ml. The FGF and heparin treatments were added as simultaneously as possible. Dishes were rocked gently to distribute the additives and incubated at 37°C in 5% CO2 until cells were terminated.

Preparation of cell lysates. Cultures in 60-mm dishes were terminated with an ice-cold PBS rinse and addition of 100–150 μl of cell lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na3VO4, and 1 mM Na2HPO4 in chilled 1.5-ml Eppendorf tubes. Incubation on ice for 30 min was followed by four 5-s bursts of sonication, transfer of chilled 1.5-ml Eppendorf tubes. Incubation on ice for 30 min was followed by four 5-s bursts of sonication, addition of PMSF, and a further 30-min incubation on ice. Samples were centrifuged at 14,000 rpm for 20 min at 4°C, total protein in each supernatant was quantitated by the bicinchoninic acid microassay method (Pierce, Rockford, IL), and lysates were stored at −80°C.

Western blots. Equal amounts (3–5 μg) of cell lysate total protein were subjected to electrophoresis in MOPS running buffer under reducing conditions at 200 V for 50 min on NuPage 4–12% Bis-Tris gels using the Novex X-Cell II system (Invitrogen, Carlsbad, CA). A mixture of 5 μl of SeeBlue Plus 2 molecular weight markers and 1 μl of MagicMark Western Standard markers (Invitrogen) was run in a single lane to assess transfer efficiency and to accurately judge the molecular weights of immunolabeled proteins. Gels were blotted to nitrocellulose membranes at 30 V for 90 min in transfer buffer (Invitrogen) containing antioxidant and 20% methanol. Blots were rinsed once in Tris-buffered saline (TBS), blocked for 1 h in TBS-0.1% Tween (TBS/T)-5% milk, and rinsed three times in TBS/T before transfer in a 1:1 acetic acid-saturated solution at 4°C in TBS/T-5% BSA containing primary antibody diluted 1:1,000–1,250 according to the manufacturer’s suggested protocol (Cell Signaling Technology). After three TBS/T washes, blots were incubated for 1 h at room temperature in horseradish peroxidase (HRP)-conjugated secondary antibody diluted 1:2,000–1:5,000 in TBS/T-milk and washed again (3 times in TBS/T). HRP-labeled proteins were revealed by SuperSignal Pico West (Pierce, Rockford, IL) and documented by subsequent autoradiography. Films were scanned into Adobe Photoshop, and specific bands were combined in Microsoft Powerpoint to construct the Western blots. Scanned bands were quantitated densitometrically using LabWorks Imaging and Analysis Software (UVP, Upland, CA). Integrated optical densities were exported to Microsoft Excel, values were normalized against those of total ERK1/2 or β-actin, and percent increase or decrease was calculated on selected values.

Rabbit polyclonal antibodies (Cell Signaling Technology) to the following selected components of various signaling pathways were used as primary antibodies according to the manufacturer’s protocol:

- phosphorylated (p)-c-Raf (Ser338), p-MEK1/2 (Ser217/221), p-p90RSK (Thr592/Ser635), p-c-Jun (Ser63) II, p-c-Myc (Thr592/Ser63), p-STAT3 (Tyr705), p-p38 MAP kinase (Thr180/Tyr182), phosphorylated SAPK/JNK (ERK-SEK1)/MKK4 (Thr183), phosphorylated phospholipase C-γ1 (p-PLC-γ1; Tyr783), p-MKK3/MKK6 (Ser185/187), p-SAPK/JNK (Thr183/Tyr185), p-Akt/PKB (Ser473), p-p44/42 MAP kinase (p-ERK1/2) (Thr202/Tyr204), and p44/42 MAP kinase (total ERK1/2).

The secondary antibody goat anti-rabbit IgG-HRP conjugate was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Immunodetection was performed using an LKB 1219 scintillation counter (Wallac, Turku, Finland) on a per-well basis. Results were analyzed as previously described (31).

RESULTS

FGF signaling time course. Proteins isolated from AT2 cells treated with 100 ng/ml FGF-1 or FGF-2 were analyzed by Western blot for evidence of stimulation of several major signaling pathways. The FGF receptor tyrosine kinase signaling intermediate c-Raf appeared constitutively active (no-serum lanes) but showed slightly elevated levels of phosphorylation with FGF-1 stimulation that increased with time. FGF-2-stimulated p-c-Raf was lower and did not appear significantly above constitutive levels, except at 2 h (Fig. 1). Phosphorylation of MEK1/2 and ERK1/2 was maximally elevated at 5 min by FGF-1 or FGF-2 and then decreased, with phosphorylation triggered by FGF-2 diminishing more rapidly than that triggered by FGF-1 over the 2-h time course (Fig. 1). Levels of phosphorylated 90-kDa ribosomal S6 kinase (p-p90RSK), a potential downstream target of ERK1/2, also were elevated at 5 min and remained higher than background through 120 min. FGF-1-stimulated phosphorylation of p90RSK appeared somewhat stronger and attenuated more slowly than that triggered by FGF-2. The weaker phosphorylation levels of SAPK/JNK and SEK1/MKK4 were maximal at 5 min of FGF stimulation. FGF-2-stimulated p-SAPK/JNK diminished quickly over the time course and appeared reduced to background levels within...
15 min, whereas p-SEK1/MKK4 levels were very low, even at 5 min, and diminished further over the time course (Fig. 1). Activation of PKB (Akt/PKB) lagged behind the rest; although it was detectable at 5 min in FGF-1- and FGF-2-treated cells, it increased over the 2-h time course. p-PLC-γ1 was detectable in FGF-1 treatments, more so than with FGF-2 (Fig. 1), whereas p-p38 MAPK was only faintly detectable at 5 min and p-phosphatidylinositol 3-kinase 3-kinase was not detected (data not shown).

Heparin dose response. At the 5-min time point, chosen to show the greatest signaling for the majority of the examined phosphoproteins, addition of 500 μg/ml heparin maximally reduced FGF-1- and FGF-2-stimulated phosphorylation of MEK1/2, ERK1/2, SAPK/JNK, p90RSK, and even Akt/PKB, which was not highly stimulated at 5 min (Fig. 2, top). Phosphorylation of c-Raf and PLC-γ appeared less affected by heparin and remained relatively unchanged (Fig. 2, top). Generally, heparin treatment had a greater effect on FGF-2-stimulated phosphorylation events than those triggered by FGF-1 (Fig. 2, top). Weak phosphorylation of SEK1/MKK4 and p38 made any comparison of changes due to heparin difficult (Fig. 2).

Quantification of densitometry of bands normalized against total ERK indicated that high-dose heparin reduced p-p90RSK (−52%) > p-SAPK/JNK (−40%) > p-ERK1/2 (−31%) in FGF-1-treated cells compared with FGF-1 treatments alone (Fig. 2). On the other hand, 5 μg/ml heparin with FGF-1 resulted in normalized increases in p-ERK1/2 (48%) > p-MEK1/2 (42%) > p-p90RSK (16%) > p-SAPK/JNK (15%) above FGF-1 alone (Fig. 2). Additionally, 0.05 μg/ml heparin with FGF-1 increased p-ERK1/2 (57%) > p-MEK1/2 (26%) > p-p90RSK (19%) over FGF-1 alone (Fig. 2). Although the 5-min time point was early in the activation dynamics of p-Akt/PKB, those levels were reduced by 500 μg/ml heparin (−82%) and 5 μg/ml heparin (−11%) in FGF-1-stimulated cells; however, addition of 0.05 μg/ml heparin with FGF-1 increased p-Akt/PKB activation by 51% compared with FGF-1 alone (Fig. 2).

Densitometry of FGF-2-stimulated phosphoprotein targets showed a consistent reduction proportional to the heparin dose. High-dose (500 μg/ml) heparin reduced p-Akt/PKB (−95%) > p-p90RSK (−94%) > p-ERK1/2 (−90%) > p-SAPK/JNK (−70%) > p-MEK1/2 (−59%) compared with FGF-2 alone (Fig. 2). Although not as pronounced, reduction of these targets’ signals in FGF-2-stimulated cells was greater in cells treated with 5 μg/ml heparin than in cells treated with 0.05 μg/ml heparin (Fig. 2); signals at all doses were lower than with FGF-2 alone.

Sulfation affects signaling. Sodium chlorate effectively blocks sulfation of all nascent glycoproteins and glycosaminoglycans, thus reducing the overall level of sulfation within and on the surface of the cell (7). AT2 cells grown in the presence of 20 mM sodium chlorate might therefore be expected to respond differently, on subsequent supplementation with heparin, via those cellular activities that require sulfated molecules and/or utilize sulfated proteoglycans as cofactors. High-dose heparin reduced FGF-1-stimulated levels of p-ERK1/2 (−86%) > p-Akt/PKB (−84%) > p-p90RSK (−78%) > p-MEK1/2 (−68%) > p-SAPK/JNK (−47%) at the 5-min time point compared with FGF-1 alone (Fig. 3A). Similarly, heparin reduced FGF-2-stimulated levels of p-ERK1/2 (−22%) > p-p90RSK (−17%) > p-Akt/PKB (−15%) during the same period compared with FGF-2 alone (Fig. 3A). The inhibition seen with heparin and FGF-1 was reversed with chlorate treatment, inasmuch as increases in p-p90RSK (219%) > p-ERK1/2 (89%) > p-c-Raf (87%) > p-MEK (37%) were noted. This differed with FGF-2-stimulated cells and chlorate, as p-c-Raf was increased (156%), whereas small decreases were seen with p-ERK1/2 (−14%) and p-Akt/PKB (−23%) with heparin treatment. At 45 min, FGF-1-stimulated cells treated with heparin had reduced levels of p-Akt/PKB (−83%) > p-ERK (−68%), p-MEK (−68%), and p-SAPK/JNK (−67%) > p-p90RSK (−58%); Fig. 3B). FGF-2-stimulation resulted in similar decreases in p-ERK1/2 (−75%) > p-p90RSK (−63%) > p-c-Raf (−51%) and p-Akt/PKB (−44%) > p-MEK (−38%) with heparin treatment (Fig. 3B). When FGF-1 treatment was combined with chlorate, however, p-c-Raf (67%), p-ERK1/2 (63%), p-p90RSK (44%), and p-Akt/PKB (39%) were increased by heparin. FGF-2-stimulated cells treated with chlorate and heparin showed decreased levels of p-Akt/PKB.
(-52%) > p-SAP/JNK (-42%) > p-c-Raf (-28%) and p-p90RSK (-30%) but increased levels of p-ERK1/2 (66%; Fig. 3B).

Interestingly, background levels of p-Akt/PKB at 5 min were decreased by FGF treatments and were further reduced by the addition of heparin (Fig. 3A). Chlorate pretreatment had little effect on the background level of p-Akt/PKB in unstimulated cells at 5 min, and the unusual decrease with FGF-1 treatment was unaffected by heparin addition. Chlorate also appeared slightly to increase Akt/PKB activation by FGF-2. At 45 min, p-Akt/PKB activation was stimulated by both FGF treatments (Fig. 3B), a delayed response seen also in the time of heparin addition.
Heparin reduced the FGF-1-stimulated level of p-Akt/PKB to untreated levels. To an even greater extent than in the 5-min samples, at 45 min, growth in 20 mM chlorate reversed heparin’s reductive effects on FGF-1 signaling through Akt/PKB (Fig. 3B).

**[3H]thymidine incorporation.** Treatment of AT2 cells with the MEK1/2 inhibitor U-0126 reduced [3H]thymidine incorporation after FGF-1 (45% of FGF-1 alone) and FGF-2 (45% of FGF-2 alone) treatments (Fig. 4). U-0126 was equally effective in reducing thymidine uptake stimulated by FGF-1 or FGF-2, whereas heparin had a greater effect on FGF-2-stimulated [3H]thymidine incorporation (71% of FGF-2 alone) than on FGF-1-stimulated [3H]thymidine incorporation (87%). Similarly, the MEK inhibitor PD-98059 significantly reduced [3H]thymidine incorporation after FGF-1 (47% of growth factor alone) and FGF-2 (49% of growth factor alone) treatments (Fig. 5). Whereas heparin alone had some suppressive effect on [3H]thymidine incorporation in FGF-2-treated cells (61%), heparin in combination with PD-98059 had an additional and significant suppressive effect (32%) over that of the MEK inhibitor alone (49%). Heparin had little effect, alone or in combination with PD-98059, on cells treated with FGF-1.

**DISCUSSION**

Members of the FGF family of growth factors differ in their affinities for the seven variants of the four forms of FGFRs (24). FGF-1 can signal through all seven receptor variants, whereas FGF-2 favors alternate splice variants of FGFR-1, FGFR-3, and FGFR-4, with each ligand-receptor pair exhibiting different affinities. The predominant signaling cascade(s) activated by a particular FGF depends on the cell type and its receptor expression as well as its immediate environment and stage of development (32). This complexity accounts for the resultant diverse outcomes of FGF signaling on cell division and migration, affecting developmental processes, angiogenesis, wound healing, and tumorigenesis (1, 8). FGFs are known to play a prominent role in lung development (2, 4, 21) as well as in AT2 cell-specific activities (15, 25, 30, 31). Signaling pathways responsible for the potentially varied effects of FGFs on AT2 cells have remained largely unexamined until recently. New evidence has linked FGF-7 (keratinocyte growth factor) with the Akt/PKB and ERK pathways (26) and FGF-10 with the SAPK/JNK pathway (35). The data presented here indicate that FGF-1 and FGF-2 stimulate primarily three major signaling pathways, those via ERK1/2, SAPK/JNK, and Akt/PKB, in primary rat AT2 cells. This is supported by our analysis of phosphorylation of members of the p42/44 MAPK/ERK, SAPK/JNK, and Akt/PKB signaling pathways during 5 min to 2 h of FGF stimulation (Fig. 1; data not shown). Although p-p38 MAPK and p-PLC-γ may indeed be activated by FGFs in other cell types and, to a lesser extent, in AT2 cells, heparin does not appear to affect levels of either of these phosphoproteins in AT2 cells stimulated by FGF-1 or FGF-2.

That the FGFs signal via multiple pathways should not be surprising, insomuch as FGF-1 and FGF-2 are known to initiate or influence a variety of biological outcomes during pulmonary development, including cell proliferation and cell/tissue differentiation (2). FGF-2 has been shown to stimulate cells of the ocular lens via ERK1/2 and SAPK/JNK pathways (34), vascular endothelial cells via p38 MAPK (19), and myoblasts via PLC-γ (32), although it has not been shown to activate all four pathways in the same cell. Our data are consistent with the idea that FGF-1 and FGF-2 activate multiple signaling pathways in AT2 cells, perhaps through kinetically driven interactions with receptor variants that may exist on AT2 cells. In our previous studies of gene expression of FGFR-1 and FGFR-2 in AT2 cells, primers for RT-PCR were chosen that recognize sequences common to both exon III variants (1b-1c and 2b-2c, respectively) of each receptor (17); however, it should be safe to assume that the “b” forms of both receptors were those amplified because of the epithelial expression patterns of AT2 cells (24). Subsequent PCR analysis using specific variant primers confirmed that the b forms are indeed those expressed in our AT2 cells (data not shown). The importance of splice variance common in this family of growth factor receptors may be of critical consideration. It has been shown that the absence of a single exon (exon 16) and resultant reading frame shift of the FGFR-2 transcript delete the major autophosphorylation site (Tyr189) required for interaction of PLC-γ with the full-length FGFR-2 isoform (39). Further work is required to determine what role, if any, FGF receptor splice variation plays in the activation of multiple signaling pathways observed with FGF-1 and FGF-2 in AT2 cells.

The activities of FGFs are closely linked to HSPGs, which effectively bind, sequester, and protect them within ECMs (27). HSPGs also function as the low-affinity receptor portion of a dual-receptor system required for FGF-ligand binding, which triggers autophosphorylation of a tyrosine kinase on the intracellular portion of the receptor to set off the signal cascade. Given that the sulfated nature of HSPG is particularly important in binding events (20), sulfation of HSPGs on the cell surface and within adjacent BMs may be of critical importance to FGF signaling in the context of the pulmonary alveolus. Interestingly, the ECM of the BM microdomains associated with AT2 cells is less sulfated than the ECM associated with AT1 cells (28). We previously proposed that ECM regions of low sulfate content promote responsiveness to growth factors, whereas those with high levels of sulfate retard/inhibit responsiveness (28). In support of this hypothesis, we have demonstrated that desulfated forms of model ECMs (chondroitin sulfate and heparin) promote FGF-1- and FGF-2-stimulated DNA synthesis in isolated AT2 cells (30), whereas normally sulfated high-dose heparin inhibits AT2 cell mitogenesis (31). We reasoned that these different outcomes could be the result of modulation of signaling pathways. Results here indicate that specific elements of the signaling pathways activated by FGF-1 and FGF-2 are sensitive to high concentrations of heparin, perhaps similar to levels encountered in a heavily sulfated ECM, such as that encountered beneath AT1 cells (28) or in pathologically altered ECM (36). It may be significant that moderate to low concentrations of heparin actually enhanced some FGF-1-stimulated elements (p-MEK1/2, p-ERK1/2, p-p90RSK, and p-SAPK/JNK) above FGF-1 alone. This variable sensitivity to different heparin concentrations may be reflective of findings by Fannon et al. (7), who demonstrated that high concentrations of heparin and its absence inhibited FGF binding to cell surfaces, whereas low heparin concentrations enhanced binding. Indeed, the inhibitory profiles observed here may well represent heparin’s capacity to bind or effectively sequester FGFs and, thus, competitively inhibit their ability to bind to their low-affinity...
(HSPG) receptor(s) on cell surfaces. Alternatively, the present studies expand on this concept by showing that the biological activities of FGFs may be more complex than simple receptor binding and may even involve members of multiple pathways subsequent to binding events, in this way influencing multiple downstream events.

Although phosphorylation of the key convergent element c-Raf was less affected, phosphorylation of MEK1/2, ERK1/2, p90RSK, SAPK/JNK, and Akt/PKB showed varied but clearly detectable reduction caused by high concentrations of heparin. This effect was lost when sulfation was prevented in newly biosynthesized HSPGs, as demonstrated here with sodium chlorate.

Fig. 3. Reduction of signaling by heparin reversed by inhibition of normal sulfation with chlorate. AT2 cells were cultured for 48 h in regular medium or medium containing 20 mM sodium chlorate (chlorate-pretreatment lanes) to prevent sulfation of nascent proteins, glycoproteins, and other biomolecules. Signaling events were quieted by 2 h of incubation in serum-free medium with or without sodium chlorate. Phosphorylation events were then stimulated by FGF-1 or FGF-2 alone or in the presence of added heparin (500 μg/ml) for 5 min (A) or 45 min (B). IODs of scanned bands were normalized and expressed as percentage of corresponding β-actin band. Lane order is the same as in blots at top.
chlorate treatment. This may be due to a reduction in the balance of total cellular sulfated HSPGs to a level that, while permitting retention of FGF responsiveness, still allows absorption/adsorption of additional, exogenously added heparin, which tends to behave like an HSPG replacement in low-sulfate environments, thus attaining a critical level of HSPG and HSPG-like molecules necessary in and around cells under homeostatic conditions for optimal biological responsiveness to FGFs (7). In other words, the chlorate-induced inhibition of sulfation slightly reduces FGF-1- and FGF-2-stimulated phosphorylation of some pathway elements, but not dramatically, insomuch as presumably just enough sulfated surface molecules remain to enable receptor activation. The addition of heparin to this environment, which would otherwise inhibit/
reduce receptor-mediated phosphorylation and downstream events, has little or no effect, because it no longer presents an excess of sulfated molecules, falling within the “optimal sulfate concentration range,” which promotes/enables signaling. Our results support the notion that there exists a critical concentration of total cellular sulfate that enables cells to respond to heparin-binding growth factors, below which there is an insufficient amount present and above which there is an excess, resulting in sequestration and/or competitive inhibition. Either extreme leads to reduced signaling and pathway activation.

The link between heparin, intracellular signaling pathways stimulated by the FGFs, and inhibition of DNA synthesis is further supported by results of experiments where treatment of AT2 cells with the specific MEK1/2 inhibitor U-0126 reduced \(^{[3]H}\)thymidine incorporation during exposure to FGF-1 or FGF-2 by 54% or 51%, respectively. In the same assays, 500 µg/ml heparin reduced \(^{[3]H}\)thymidine incorporation in FGF-1-treated cells by 12.5% and in FGF-2-treated cells by 28%, which parallels the greater sensitivity to heparin of FGF-2 signaling events as seen in Western blots probed for the actual MAPK/ERK signal cascade of phosphorylation events (Fig. 2). Notably, when heparin and the MEK inhibitor PD-98059 were added to FGF-2-treated cells together, the effect on \(^{[3]H}\)thymidine incorporation was additive (Fig. 5); this reinforced the notion that heparin influences multiple signaling pathways (i.e., Akt/PI3K; Fig. 2). Evidence for heparin’s inhibition/reduction of signaling via other pathways (p38 MAPK, PLC-γ, and phosphatidylinositol 3-kinase) and its effect on potential downstream targets such as c-Myc and STAT3 was weak or lacking at the time points examined. Involvement of these other pathways in FGF signaling in AT2 cells cannot be completely ruled out, inasmuch as our ability to detect some of the less-abundant phosphorylated proteins (notably the receptors themselves and other signaling intermediates of the receptor tyrosine kinase complex) was limited by the small amount of total proteins recovered in these primary, nontransfected cell lysates. Most signaling protocols recommend the analysis of ≥25 µg of total protein per lane; however, the low yield of total proteins from our primary cells and the necessity of probing multiple blots of the same samples limited us to ~5 µg of total protein per lane for analysis. For the majority of phosphorylated proteins we analyzed and with the use of very sensitive detection reagents, this amount proved sufficient. In some cases (notably p-p38 MAPK and p-PLC-γ), bands were detectable in FGF-treated samples on longer exposure to film but showed no sensitivity to heparin treatment (data not shown). Others were entirely absent after long exposures and were assumed not to be involved in FGF signaling; however, it is possible that some involved proteins fell below our limits of detection.

It has been demonstrated that FGF binding to FGFRs can be inhibited and facilitated by heparin in a concentration-dependent manner (7), such that higher concentrations (similar to the 500 µg/ml used in the present study) inhibited/interfered with binding, whereas low concentrations promoted/facilitated it. Higher concentrations of heparin, therefore, might be expected to prevent the initiation of a signaling cascade. It was therefore of interest that heparin was shown here to have greater effects downstream from the FGFR complex, as evidenced by inhibition seen mainly subsequent to c-Raf, phosphorylation of which was relatively insensitive to high heparin. A postulated bypass of signaling through c-Raf is supported by the observation that fluorescein-labeled heparin gains access to the cell rapidly (within 15 min) via endocytic pathways in vascular smooth muscle cells (3). However, a pretreatment pulse of heparin had little or no effect on subsequent FGF-1-triggered p-MAPK/p-ERK signaling in AT2 cells (unpublished observations), suggesting that, if it does enter the cell, heparin can exit the cell just as rapidly. This argument may be made more compelling in the context of the known shedding of epithelial cell surface HSPGs, constitutively and during inflammation, and their demonstrated retention of significant biological activity (12). We propose that soluble (shed) and insoluble (ECM) sulfated molecules represent major biological modifiers within tissue microenvironments that influence the movements of molecular and cellular constituents and their ultimate cellular effects.

**Fig. 4.** Heparin and U-0126 reduce \(^{[3]H}\)thymidine incorporation in AT2 cells. Pretreatment (1 h) with MEK1/2 inhibitor U-0126 (10 µM) was followed by continuous culture with U-0126 and FGF-1 (100 ng/ml) or FGF-2 (100 ng/ml) in hormonally defined serum-free medium containing 2 µCi/ml \(^{[3]H}\)thymidine. In other samples, high-dose heparin (500 µg/ml) was added simultaneously with FGF-1 or FGF-2 and 2 µCi/ml \(^{[3]H}\)thymidine. \(^{[3]H}\)thymidine incorporation in all samples was assayed in triplicate after 48 h of treatment.

**Fig. 5.** Heparin (Hep) and PD-98059 (PD) reduce \(^{[3]H}\)thymidine incorporation in AT2 cells. Pretreatment (1 h) with MEK inhibitor PD-98059 at 10 µM was followed by continuous culture with inhibitor and FGF-1 (100 ng/ml) or FGF-2 (100 ng/ml) and with or without high-dose heparin (500 µg/ml) in hormonally defined serum-free medium containing 2 µCi/ml \(^{[3]H}\)thymidine. In other samples, cells were treated with FGF-1 or FGF-2 with or without high-dose heparin and 2 µCi/ml \(^{[3]H}\)thymidine with no inhibitor pretreatment. \(^{[3]H}\)thymidine incorporation in all samples was assayed in triplicate after 48 h of treatment and is expressed as percentage of FGF-1- or FGF-2-stimulated counts.
Our data indicate that heparin has surprisingly specific (or at least selective) inhibitory effects on portions of multiple signaling cascades in AT2 cells. Reports of heparin’s effects on signaling in other cell types support this idea. For instance, heparin inhibits c-fos induction by blocking a point upstream from ERK1/2 in mesangial cells (22) and vascular smooth muscle cells (5) and inhibits proliferation of smooth muscle cells by interfering with the PKC pathway via direct inhibition from ERK1/2 in mesangial cells (22) and vascular smooth muscle cells (5). Heparin inhibits c-erbB signaling in other cell types support this idea. For instance, heparin has inhibitory effects on portions of multiple signaling pathways would allow the stimulation of AT2 cell mitogenesis, migration, and specific gene regulation on injury to the alveolus and destruction of alveolar BM as well as the downregulation of such proliferation after repair to maintain alveolar homeostasis.

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REFERENCES


