Heparan Sulfate Proteoglycans as Regulators of Fibroblast Growth Factor-2 Receptor Binding in Breast Carcinomas

Christoph Mundhenke, Kristy Meyer, Sally Drew, and Andreas Friedl

From the Department of Pathology and Laboratory Medicine, University of Wisconsin–Madison, Madison, Wisconsin

Binding of fibroblast growth factors (FGFs) to their tyrosine kinase-signaling receptors (FGFRs) requires heparan sulfate (HS). HS proteoglycans (HSPGs) determine mitogenic responses of breast carcinoma cells to FGF-2 *in vitro*. For this study, we examined the role of HSPGs as modulators of FGF-2 binding to FGFR-1 *in situ* and *in vitro*. During stepwise reconstitution of the FGF-2/HSPG/FGFR-1 complex *in situ*, we identified an elevated ability of breast carcinoma cell HSPGs to promote receptor complex formation compared to normal breast epithelium. HSPGs isolated from the MCF-7 breast-carcinoma cell line were then fractionated according to their ability to assemble the FGF-2 receptor complex. All MCF-7 HSPGs are decorated with HS chains similarly capable of promoting FGF-2 receptor complex formation. In this *in vitro* model, syndecan-1 and syndecan-4 are the cell surface HSPGs contributing most to the complex formation. Relative expression levels of these syndecans in human breast carcinoma tissues correlate well with receptor complex formation *in situ*, indicating that in breast carcinomas, core protein levels determine FGF-2 receptor complex formation. However, variances in syndecan expression levels do not explain the difference in FGF-2 receptor complex formation between normal and malignant epithelial cells, suggesting that alterations in HS structure occur during malignant transformation. (*Am J Pathol* 2002, 160:185–194)

The fibroblast growth factor (FGF) family of cytokines is currently comprised of at least 23 members with diverse functions in cell growth and differentiation.^{1,2} FGFs signal through transmembrane receptor tyrosine kinases (RTKs, specifically FGFR-1 through FGFR-4).³ Multiple roles have been proposed for FGFs in cancer origin and progression.^{4,5} The most studied FGF family member is FGF-2 (basic FGF). Apart from being a potent angiogenic factor, this growth factor also acts directly on breast carcinoma cells.^{6,7} Interestingly, both growth stimulatory

and inhibitory effects have been reported, suggesting that additional modulators regulate FGF-2 signaling.^{8–13}

Stable binding of FGFs to their RTKs and signaling requires the presence of heparan sulfate (HS) or heparin (a specialized mast cell HS).^{14,15} The assembly of a ternary signaling complex comprising two FGFs, two RTK molecules, and HS is made possible by HS-binding motifs on both the FGF ligand and on the RTK.^{16–18} Mounting evidence indicates that HS is not just anionic glue stabilizing the receptor complex, but rather a specific regulator of FGF signaling.^{19,20} Heparan sulfate is initially synthesized as a simple polysaccharide chain consisting of repeating glucuronic acid and *N*-acetyl-glucosamine units. This monotonous chain is subsequently modified in the Golgi by a battery of enzymes that catalyze epimerization reactions and sulfate substitutions in various positions.^{21,22} Mature HS is a highly complex and diverse molecule, and the potential information density contained within these polysaccharides has been estimated to exceed that of DNA or polypeptides.²³ The HS-binding regions of FGF ligand and RTK require different structural motifs within the HS chains for binding.^{24,25} These differences explain why HS can either stimulate or inhibit signaling. A HS species that binds both FGF ligand and RTK will act as a stimulator, whereas a HS that only binds FGF ligand will act as an inhibitor of signaling by sequestering the growth factor. Using novel *in situ* binding assays, we have recently described specific interactions of HSPGs with members of the FGF family and characterized HSPGs as promoters or inhibitors of FGF receptor binding.^{20,26}

Virtually all HS exists in covalent linkage to proteins, the HS proteoglycans (HSPGs). Cell surface HSPGs are anchored in the cell membrane either via a transmembrane domain (syndecans), or by glycosyl-phosphoinositol linkage (glypicans).²⁷ Literature reports on which HSPG core protein is responsible for FGF-2 signaling are contradictory. Some investigators have identified the ex-

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Address reprint requests to Andreas Friedl, M.D., Department of Pathology and Laboratory Medicine, Clinical Sciences Center, K4-850, 600 Highland Ave., Madison, WI 52792-8550. E-mail: afriedl@facstaff.wisc.edu.

tracellular basement membrane component perlecan as the HSPG most actively promoting FGF-2 signaling, whereas cell surface forms were inhibitory.²⁸ Others found that syndecans-1, -2, and -4, and glypican-1 can also potently stimulate FGF-2 signaling.^{29–31} Likely, different HSPGs take over the role of FGF-2 co-receptors depending on cell type and tissue context.

Convincing experimental evidence indicates that in the normal breast gland as well as in carcinomas, response to FGFs is regulated by HSPGs.^{13,32,33} Tight regulation is achieved by a balance of HSPGs that either stimulate or inhibit FGF binding to its FGFR. Our knowledge about HSPG expression in breast carcinomas is very limited. Recently, reduced syndecan-1 expression has been reported in carcinomas of the breast *in vivo*, but the impact of this alteration on FGF-2 signaling was not examined.³⁴ The goal of this project was to investigate HSPGs as modulators of FGF-2 signaling in human breast cancer by addressing the following unresolved questions: 1) Do breast carcinoma HSPGs differ from normal breast epithelial cell HSPGs in their capacity to promote FGF-2 binding to FGFR-1? 2) What breast carcinoma HSPG species are involved in FGF-2 receptor complex formation? 3) Does the regulation of FGF receptor binding occur at the level of HS synthesis or core protein expression?

Materials and Methods

Tissue Samples and Slide Preparation

Twelve randomly selected cases of infiltrating breast carcinomas were used for all tissue-based assays. Institutional Human Subjects Committee approval was obtained for these studies (protocol no. 1995-314). For each case, one routinely processed paraffin block was chosen from the pathology archives that contained both carcinoma and benign glandular tissue. Four- μ m sections were mounted on charged slides (Plus slides; Fisher Scientific, Pittsburgh, PA), baked, deparaffinized, and rehydrated.

FGF Receptor Complex Reconstitution in Situ

The assays were performed with paraffin-embedded sections essentially as previously described for cryosections.^{20,26,35} Briefly, after blocking, sections were incubated with FGF-2 (10 nmol/L; kindly provided by B. Olwin, University of Colorado, Boulder, CO). Bound FGF-2 was detected with anti-FGF-2 monoclonal antibody (clone DE6; kindly provided by DuPont, Wilmington, DE). Alternatively, the FGF-2-binding step was followed by incubation with FR1-AP (30 nmol/L), a soluble fusion protein, containing the extracellular domain of FGFR-1 and alkaline phosphatase (AP) as enzyme tag.³⁶ Bound FR1-AP was detected with monoclonal anti-AP antibody (Sigma, St. Louis, MO). Either signal was visualized with the peroxidase-based Envision Plus detection kit (DAKO, Carpinteria, CA). Controls included omission of the FGF-2 incubation step and treatment of the tissue sections with heparitinase (mixture of generic heparitinase, catalog no.

100703-3; and heparitinase II, catalog no. 100705-1, both EC 4.2.2.8 and both at 10.0 mIU/ml; Seikagaku, Falmouth, MA) before FGF-2 incubation.

Immunohistochemistry

For heat-induced epitope retrieval, the slides were placed in 1 mmol/L of disodium ethylenediaminetetraacetic acid solution, pH 8, in a 92°C water bath for 20 minutes. The slides were then transferred to a Ventana ES automated immunohistochemical stainer (Ventana Medical Systems, Tucson, AZ) and all subsequent steps were performed on the instrument according to the manufacturer's instructions. Anti-syndecan-1 antibody (clone BB4; Serotec, distributed by Harlan, Indianapolis, IN) was applied at a concentration of 4 μ g/ml and anti-syndecan-4 antibody (clone 8G3; kindly provided by G. David, University of Leuven, Leuven, Belgium) at 5 μ g/ml for 28 minutes at 42°C. Muscle-specific actin was detected on a limited number of slides with a monoclonal antibody (HHF35, Biocare, Walnut Creek, CA) using a prediluted preparation for 20 minutes at 42°C. Incubation with the DAB Basic detection system (Ventana) was followed by a pale Harris hematoxylin counterstain as the final step in the automated procedure. At completion, the slides were removed from the stainer, rinsed free of coverslip oil, dehydrated through graded alcohols and cleared in xylene. Slides were coverslipped with a synthetic-mounting medium.

All HSPGs regardless of the core protein were detected with monoclonal antibody 3G10 (5 μ g/ml, Seikagaku). Before antibody labeling, the slides were treated with heparitinase (12 mIU/ml, EC 4.2.2.8, catalog no. 100703-3; Seikagaku) at 37°C for a total of 4 hours, replacing the enzyme after 2 hours to generate the unsaturated uronate residues that are recognized by this antibody. The signal was visualized with Alexa-546-conjugated secondary antibody (Molecular Probes, Eugene, OR) using an epifluorescence microscope (Olympus BX51) equipped with a SPOT RT slider chilled charge-coupled device digital camera (Diagnostic Instruments, Sterling Heights, MI).

Slide Evaluation and Statistical Analysis

The intensity of all labeling reactions was graded on a scale from 0 (no staining beyond background) to 4 (strong staining). The predominant signal intensity (seen in the majority of cells) was recorded to characterize each tumor case. Analysis of the cases was performed in a blinded fashion. Student's *t*-test was used to evaluate differences between data sets (eg, between normal and malignant cells) and linear regression analysis was used to compare correlations between data sets (eg, between FGF receptor complex assembly and syndecan labeling). Microsoft Excel and Statview programs were used for these analyses. Bright-field microscopic images were acquired with an Olympus BX40 microscope equipped with an SV-Micro digital camera (SoundVision, Great Neck, NY) and an Apple Macintosh G3 computer.

Western Analysis of Whole Cell Extracts (Characterization of Anti-Syndecan-4 Antibody)

T47D breast carcinoma cells (kindly provided by Dr. Michael Gould, Department of Oncology, University of Wisconsin, Madison, WI), were grown in an 100-mm tissue culture dish to 80% confluency. The dish was placed on ice and the cells were washed twice with cold phosphate-buffered saline. One ml of cold lysis buffer [136 mmol/L NaCl, 2.7 mmol/L KCl, 1 mmol/L Na_2HPO_4 , 1.8 mmol/L KH_2PO_4 , 1% (w/v) Triton X100, 0.1% (w/v) sodium dodecyl sulfate, 5 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L phenylmethyl sulfonyl fluoride, 1 mmol/L *N*-ethylmaleimide, 1 $\mu\text{g/ml}$ leupeptin] was added for 10 minutes and the lysate was scraped into an Eppendorf tube. After centrifugation at 14,000 rpm in a microcentrifuge at 4°C, protein was precipitated by adding methanol (−20°C, 2.5-fold volume) and by placing the sample in a −20°C freezer for 2 hours. The sample was again centrifuged at 14,000 rpm for 10 minutes at 4°C, and the supernatant was discarded. The pellet was washed twice with ice-cold acetone, dried, and then resuspended in 50-ml heparitinase buffer (50 mmol/L HEPES, 50 mmol/L NaOAc, 150 mmol/L NaCl, 5 mmol/L CaCl_2). After treatment with heparitinase (2.4 mIU/ml, EC 4.2.2.8, catalog no. 100703-3; Seikagaku), and chondroitinase (100 mIU/ml, EC 4.2.2.4, catalog no. 190334; ICN, Costa Mesa, CA) to degrade all glycosaminoglycan (GAG) chains, the sample was analyzed with a 3.5 to 15% (w/v) Tris-borate polyacrylamide gradient gel. The polyvinylidene difluoride membrane was probed with anti-syndecan-4 antibody (10 $\mu\text{g/ml}$, clone 8G3) and horseradish peroxidase-conjugated secondary antibody.

HSPG Fractionation

MCF-7 cells (kindly provided by Dr. Michael Gould) were grown in Dulbecco's modified Eagle's medium substituted with 10% (v/v) calf serum, L-glutamine, and penicillin/streptomycin (eg, 10 150- μm dishes). Two additional plates of cells were grown under identical conditions for cell counting. At ~80% confluency, the cells were placed on ice, washed three times with cold HEPES saline buffer (30 mmol/L HEPES, 150 mmol/L NaCl, pH 7.4) and extracted with TUT buffer [10 mmol/L Tris, 8 mol/L urea, 0.1% (w/v) Triton X-100, 1 mmol/L Na_2SO_4 , phenylmethyl sulfonyl fluoride, *N*-ethylmaleimide, pH 8.0]. After sonication, DEAE beads previously equilibrated with TUT buffer were added (200 μl of beads per 10^7 cells) and the tubes were placed on a rotator overnight at 4°C. After washing with Tris-buffered saline/ethylenediaminetetraacetic acid buffer (10 mmol/L Tris, 150 mmol/L NaCl, 0.5 mmol/L ethylenediaminetetraacetic acid, pH 7.4), HSPGs were removed from the beads with elution buffer [100 mmol/L HEPES, 1 mol/L NaCl, 10 mmol/L CaCl_2 , 20 mmol/L NaOAc, 0.2 mg/ml β -casein, 0.5% (w/v) CHAPS, pH 6.5] and the eluate was diluted with double-distilled H_2O to reduce the NaCl concentration to 200 mmol/L for the *in vitro* binding experiment. Conditioned medium from COS-7 cells selected for high expression of FR1-AP was

incubated with anti-AP-agarose beads (33 μl of bead volume per 2 ml of conditioned medium; catalog no. A-2080, Sigma) to generate a solid FGFR-1 matrix. The beads were then washed with 1 mol/L NaCl, to remove any potentially prebound HSPGs stemming from the COS-7 cells, and were blocked with β -casein [0.2% (w/v)]. A 2×10^6 cell equivalent of extracted HSPGs was combined with 0.32 μg of FGF-2 and added to 33 μl of FR1-AP/anti-AP agarose beads and incubated overnight on a rotator at 4°C, after the volume had been adjusted to 1 ml. After centrifugation, the supernatant was decanted and HSPGs contained in the supernatant were concentrated with a DEAE column. All samples including crude HSPG extracts were then digested with heparitinase and chondroitinase for a total of 6 hours, replenishing the enzymes after 3 hours. Negative controls included omission of FGF-2 from the incubation mixture and digestion of the HSPG preparation with heparitinase before the binding step. The conditions for the enzyme treatments were the same as for the sample preparation (see above). The samples were analyzed with a 3.5 to 15% (w/v) Tris-borate polyacrylamide gradient gel. After transfer, the polyvinylidene difluoride membrane was blocked with 50 mg/ml of milk and stained with anti- δ HS antibody (0.03 $\mu\text{g/ml}$, clone 3G10).³⁷ Core proteins were detected with antisyndecan-1 (20 $\mu\text{g/ml}$, clone BB4) and anti-syndecan-4 (10 $\mu\text{g/ml}$, clone 8G3) antibodies. A horseradish peroxidase conjugated secondary antibody and SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL) served for chemiluminescent detection.

Results

Breast Carcinoma HSPGs Have an Increased Ability to Promote Assembly of the FGF-2/FGFR-1 Complex

HSPGs of invasive breast carcinomas and of adjacent normal breast gland were analyzed with respect to their role as modulators of FGFR-1 binding. Previously, we have described and validated assays that allow the characterization of HSPGs as co-stimulators or inhibitors of FGF-2 signaling in intact tissues.^{26,35} This analysis is done by a stepwise reconstitution of the FGF-signaling complex *in situ*. The assay was initially developed for frozen sections, but we have now successfully applied it to routinely formalin-fixed and paraffin-embedded tissues. This modification allows us to examine archival material, greatly expanding the utility of the test.

In the first part of the assay, the binding interaction between tissue HSPGs and FGF-2 is examined. Tissue sections are incubated with recombinant FGF-2 and bound growth factor is detected with an anti-FGF-2 antibody (schematically shown in Figure 1A). In 9 of 12 cases examined, carcinoma cells show moderate to strong (3+ or 4+) FGF-2 binding. Normal ductal and acinar epithelial cells (which were present for evaluation in 10 of 12 cases in these experiments) bind less FGF-2 than carcinoma cells (one example of carcinoma cells surrounding nor-

Figure 2. Semiquantitative measurement of FGF-2 and FR1-AP binding to normal and malignant epithelial cells: The binding signal detected in tissue sections of breast carcinomas and of adjacent normal epithelial cells (see Figure 1, A to F) was graded on a scale of 0 (no signal beyond background staining) to 4 (strong signal). Each line represents one patient. The *P* value calculated by paired *t*-test analysis is indicated. Abbreviations: Nml, normal epithelial cells; Ca, carcinoma.

mal duct shown in Figure 1B). The difference between normal tissue and cancer is highly significant ($P = 0.0003$) (Figure 2A). FGF-2 binding is mediated by HS contained in the tissues because treatment with heparitinase greatly reduces binding (Figure 1C). Heparitinase (EC 4.2.2.8) specifically cleaves hexosaminidic linkages with glucuronic acid: $\rightarrow 4)-\alpha\text{-D-GlcNpR}(1\rightarrow 4)-\beta\text{-D-Gl-$

cAp(1 $\rightarrow$) (where R = *N*-acetamido or *N*-sulfo).³⁸ Therefore, this enzyme degrades specifically HS, but does not digest other GAGs or heparin, a specialized and highly sulfated mast cell HS.

Binding of FGF to HS is necessary but not sufficient to promote signaling. In addition, HS must have the ability to stabilize FGF binding to FGFR, likely by using an HS-binding site on the receptor. This activity of tissue HSPGs was tested by first incubating tissue sections with FGF-2, and then with the soluble FGFR-1 construct FR1-AP. This fusion protein consists of the extracellular (ligand and HS-binding) portion of FGFR-1 and of placental AP as tag. Bound soluble receptor is detected with anti-AP antibodies (schematically shown in Figure 1D). Breast carcinoma cell-associated HSPGs show a significantly ($P = 0.002$) increased ability to promote formation of the FGF-2/HSPG/FGFR-1 signaling complex *in situ* compared to normal breast gland epithelial cells (Figure 1, E and M, and Figure 2B). FR1-AP localizes predominantly to the cell surface, as would be expected from a binding interaction with cell surface HSPGs, but cytoplasmic binding is also seen (Figure 1M). FR1-AP binding is FGF-2-dependent, because omission of the growth factor incubation step completely abolishes binding (Figure 1F). Also, heparitinase treatment fully abolishes FR1-AP binding (not shown). As expected, there is a significant ($P < 0.025$) correlation between FGF-2 and FR1-AP binding, but this relationship is not perfect, because in 2 of 12 cases no FR1-AP binding is detected despite strong FGF-2 binding (not shown). This observation supports the notion that FGF-2 binding is required but not sufficient for FR1-AP binding.

Breast Carcinoma Cell HSPG Core Proteins Are Decorated with HS Chains Similarly Capable of Promoting the FGF-2/FGFR-1 Complex—Syndecan-1 and Syndecan-4 Are the Most Abundant Cell Surface HSPGs Contributing to the Receptor Complex

Because breast carcinoma cells express a variety of HSPGs, we decided to examine the question whether all or specific HSPGs are capable of promoting FGF receptor complex assembly. Initially, we characterized HSPGs pro-

Figure 1. A–F: Reconstitution of FGF receptor complex *in situ*. **A:** Schematic representation of FGF-2 binding to tissue HSPGs *in situ*. HSPGs labeled “2” bind FGF-2, HSPGs labeled “1” fail to bind FGF-2. **B:** FGF-2 (10 nmol/L) binding to HSPGs in section of infiltrating carcinoma. Bound FGF-2 is detected with anti-FGF-2 mAb DE-6 and visualized with horseradish peroxidase. Note binding of FGF-2 to carcinoma cells (CA) and stroma, whereas a benign duct (ND) lacks FGF-2 binding. **C:** Negative control for FGF-2 (10 nmol/L) binding to HSPGs in section of infiltrating carcinoma. The tissue section was treated with heparitinase (20 mIU/ml, EC 4.2.2.8) before the growth factor incubation step. **D:** Schematic representation of FR1-AP binding to the FGF-2/HSPG complex *in situ*. Both HSPG classes labeled “2a” and “2b” bind FGF-2, but only HSPG 2b promotes the assembly of the complete receptor complex. **E:** FR1-AP (30 nmol/L) binding to tissue section of infiltrating carcinoma previously incubated with FGF-2 (10 nmol/L). Bound FR1-AP is detected with anti-AP antibody and visualized with horseradish peroxidase. Note FR1-AP binding to carcinoma cells and (syndecan-1-bearing) plasma cells (PC) and a normal duct lacking FR1-AP binding. **F:** Negative control for FR1-AP binding to infiltrating carcinoma. The FGF-2 incubation step was omitted. **G–L:** Immunohistochemical detection of syndecan core proteins: **G:** Syndecan-1 localization in a normal breast lobule with mAb BB4. Note high expression in normal epithelial cells (E) and in plasma cells. **Inset** shows normal breast acini (AC) labeled with anti-muscle-specific actin (MSA) antibody to highlight myoepithelial cells (ME). **H:** Syndecan-1 localization in an infiltrating carcinoma. Note absence of staining in the infiltrating carcinoma cells, whereas normal acini serve as internal positive control of the staining reaction. **I:** Syndecan-1 localization in a different infiltrating carcinoma. Note higher expression in the infiltrating carcinoma cells compared to a normal duct. **J:** Detection of syndecan-4 in a normal breast lobule with mAb 8G3. Note high levels of expression in normal acinar predominantly in a cytoplasmic location. **Inset** shows normal breast AC labeled with anti-MSA antibody to highlight MEs. **K:** Syndecan-4 localization in an infiltrating carcinoma. Note absence of staining in the infiltrating carcinoma cells, whereas a normal duct serves as internal positive control. **L:** Syndecan-4 localization in a different infiltrating carcinoma. Note high expression in the infiltrating carcinoma cells. **M–O:** Co-localization of FR1-AP binding and syndecan staining in adjacent sections of an infiltrating ductal carcinoma. **M:** FR1-AP binding to tissue section of infiltrating carcinoma previously incubated with FGF-2 (see description for **E** for details). **N:** Localization of syndecan-1. **O:** Localization of syndecan-4. Original magnifications, $\times 400$.

duced by MCF-7 breast carcinoma cells by immunoblotting. Extracted HSPGs were analyzed by gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The blot was probed with an antibody (clone 3G10) that reacts with HS stubs remaining after heparitinase digestion and therefore recognizes all HSPGs independent of the identity of the core protein.³⁷ A number of HSPGs ranging over a wide molecular weight spectrum are detected (Figure 3, A and B, lane 1). The two bands of highest intensity migrate at ~36 kD and 80 kD, and are identified as syndecan-4 and syndecan-1, respectively, by staining with core protein-specific antibodies (Figure 3A). Another abundant HSPG with a molecular weight >205 kD is present, which is nonreactive with a polyclonal and a monoclonal anti-perlecan antibody or with antibody to the facultative HSPG CD44v3, using appropriate positive controls (not shown).

HSPGs extracted from MCF-7 breast carcinoma cells were fractionated according to their ability to form a stable ternary FGF-2/HSPG/FGFR-1 complex. HSPGs precipitating in the complex were compared with HSPGs found in the supernatant and in the crude HSPG extracts. This approach allows unbiased analysis of all HSPGs. Surprisingly, all major MCF-7 HSPGs are found in the receptor complex (Figure 3, lane 2) in relative abundance comparable to total MCF-7 HSPGs (Figure 3, lane 1). Syndecan-1 contributes the greatest amount of complexed HSPGs, followed by the large (>205 kD) unidentified HSPG and syndecan-4. Complex assembly is dependent on intact HS chains, as heparitinase digestion before fractionation eliminates HSPGs from the complex (Figure 3, lane 3). As expected, complex formation requires the presence of FGF-2 ligand because omission of FGF-2 from the incubation mixture abolishes HSPGs from the complex (Figure 3, lane 4). Resistance of the receptor complex to washes with 1 mol/L NaCl demonstrates the high affinity of the binding interaction. Syndecan-4 migrates as a doublet in these experiments; a migration pattern observed by others that has been attributed to incomplete GAG chain digestion.³⁹ Analysis of HSPGs present in the supernatants provides a complementary picture (Figure 3, lanes 5 to 7). This result does not prove that all HSPGs are equally physiologically relevant in promoting FGF-2 signaling, as compartmentalization of HSPGs within the cell membrane or the extracellular matrix may restrict access to the FGF RTKs. Also, caution is warranted when interpreting these observations because HSPGs expressed by the MCF-7 cell line likely do not represent the full complement of HSPGs displayed by carcinoma cells *in vivo*. Nevertheless, the fractionation experiments demonstrate that the HS chains on different core proteins are similarly capable of promoting FGF-2 binding to FGFR-1 in this widely used *in vitro* breast carcinoma model.

Breast Carcinoma Cell Surface HSPGs Syndecan-1 and Syndecan-4 Co-Localize with Receptor Complexes *in Situ*

Our *in vitro* biochemical data (Figure 3) are consistent with the hypothesis that all breast carcinoma cell-derived HSPGs are similarly capable of promoting binding of FGF-2 to its RTK FGFR-1. Among the cell surface HSPGs,

Figure 3. A: Characterization of HSPGs produced by the MCF-7 breast carcinoma cell line. HSPGs extracted from MCF-7 cells were treated with heparitinase and chondroitinase (see Materials and Methods) to degrade GAG chains and then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a 3.5 to 15% (w/v) gradient gel. **Lane 1:** 0.8×10^6 cell equivalents stained with the anti-HS stub (Δ HS) antibody 3G10. **Lane 2:** 1.2×10^6 cell equivalents stained with anti-syndecan-1 antibody BB4. **Lane 3:** 1.2×10^6 cell equivalents stained with anti-syndecan-4 antibody 8G3 (see Materials and Methods for details). **B:** Fractionation of breast carcinoma cell HSPGs. HSPGs extracted from MCF-7 breast carcinoma cells were separated according to their ability to promote binding of FGF-2 to FGFR-1. The crude HSPG preparation was incubated with FGF-2 (final concentration, 16 nmol/L) and FR1-AP, immobilized on anti-AP-agarose beads. HSPGs present in the complex (**lane 2**), in the supernatant (**lane 5**) and HSPGs present before this fractionation (**lane 1**) were analyzed on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis gradient gel after the samples were digested with heparitinase and chondroitinase to remove all GAG chains (see Materials and Methods for details). The membrane was blotted with antibody 3G10 that detects all HSPGs regardless of the nature of the core protein. Controls include digestion of the HSPG preparation before complex formation (**lanes 3 and 6**) and omission of FGF-2 from the binding reaction (**lanes 4 and 7**). Bands identified by an asterisk represent light and heavy chains of anti-AP antibody from the precipitated receptor complex. Loading: 0.5×10^6 cell equivalents in **lane 1** and 2.0×10^6 cell equivalents in **lanes 2 to 7**.

Figure 4. Semiquantitative measurement of syndecan-1 and syndecan-4 expression in breast carcinomas and adjacent normal breast gland by immunohistochemistry. The staining reaction was graded on a scale of 0 (no staining above background) to 4 (strong staining). Each line represents one patient. The scores for normal epithelial cells and for carcinoma cells were compared by paired *t*-test and the *P* values are indicated. **A:** Syndecan-1 staining. **B:** Syndecan-4 staining. **C:** Western analysis of T47D whole-cell extracts with mAb 8G3; the same antibody used for immunohistochemistry.

syndecan-1 and syndecan-4 are expressed abundantly and therefore contribute most to the assembly of the FGF receptor complex. Therefore, we decided to assess expression of these HSPGs in breast carcinomas by immunohistochemistry and to relate the findings to FR1-AP-binding activities.

Syndecan-1 is expressed moderately to strongly in normal breast duct epithelial cells and to a slightly lesser degree in breast lobule acinar epithelial cells (Figure 1, G and N). Myoepithelial cells also express syndecan-1, albeit in more variable amounts than luminal cells (Figure 1G and inset). Infiltrating breast carcinomas are characterized by variable syndecan-1 expression, with loss in some tumors (Figure 1H) and gain in others (Figure 1I and Figure 4A). Syndecan-4 is highly expressed in normal acinar epithelial cells and moderate amounts are detected in breast duct epithelial cells (Figure 1; J, K, and O). In myoepithelial cells, syndecan-4 is not conspicuous (Figure 1J and inset). In infiltrating carcinomas, syndecan-4 levels are heterogeneous, similar to syndecan-1. In the majority of carcinoma cases, a reduction of syndecan-4 is seen relative to their normal cell counterparts (Figure 1K), although several carcinomas maintain high levels of syndecan-4 expression (Figure 1, L and O, and Figure 4B). Overall, the decrease of syndecan-4 in carcinoma cells is statistically significant ($P = 0.003$). In normal epithelial cells as well as in carcinomas, syndecan-4 is found in the cytoplasm and at the membrane (Figure 1, J and O). The unexpected large amount of cytoplasmic syndecan-4 prompted us to confirm the specificity of the mouse monoclonal antibody on whole cell extracts of T47D breast carcinoma cells. Only a single

Figure 5. Total amount of HSPGs in normal *versus* malignant epithelial cells: **A:** HSPGs regardless of core protein identity were localized on paraffin-embedded sections of breast carcinoma tissues by fluorescence microscopy with monoclonal antibody 3G10. The unsaturated uronate epitope recognized by this antibody (Δ HS) is generated within the HS chain by treatment with heparitinase (see Materials and Methods for details). **B:** Omission of this enzyme digestion step served as negative control. Alexa-546-conjugated secondary antibody was used for visualization. Original magnifications, $\times 400$. Abbreviations: CA, carcinoma; ND, normal duct.

band of appropriate molecular size is detected, confirming the specificity of the antibody reagent (Figure 4C).

The increased HS-mediated binding activity of carcinoma cells compared to normal epithelial cells could be because of an increased amount of HSPGs or an altered structure of their HS chains. Clearly, syndecan-1 or syndecan-4 expression levels cannot account for the apparent lack of FGF-2- and FR1-AP-binding activities in normal cells, as they show high expression of both of these HSPGs (Figure 1; M, N, and O). Immunohistochemical detection of glypican-1, another cell-surface HSPG, reveals only very low levels in both normal and malignant cells (not shown), which is in keeping with the *in vitro* analysis. Still, these results do not rule out the possibility that carcinoma cells overexpress some other HSPG core protein that was not included in the immunohistochemical survey. Therefore, we decided to analyze the total HSPG content in benign and malignant epithelial cells by labeling tissue sections with monoclonal antibody 3G10 that decorates HS stubs that remain after heparitinase digestion. Staining with this antibody would be predicted to correlate with the number of HS attachment sites, thereby providing an estimate of the total HSPG content in a tissue regardless of the core protein. Staining intensity using this antibody does not differ significantly between normal and malignant cells (Figure 5).

Among the breast carcinoma cases, syndecan-1 expression correlates significantly with FR1-AP binding, suggesting that in keeping with the *in vitro* results, syndecan-1 plays a major role in promoting FGF receptor complex assembly (Figure 6A). The relationship between HSPG core protein levels and FR1-AP binding is further solidified when syndecan-4 expression is taken into ac-

Figure 6. Correlation between FR1-AP binding and syndecan core protein expression levels. FR1-AP binding and immunohistochemical determination of syndecan expression were graded as described for Figures 2 and 4. Linear regression analysis was performed and correlation coefficient and *P* values are indicated. **A:** Correlation between FR1-AP binding and syndecan-1 expression. **B:** Correlation between FR1-AP binding and the additive immunohistochemical score of syndecan-1 and syndecan-4 expression.

count. FR1-AP binding closely correlates with an additive immunohistochemical score of syndecan-1 and syndecan-4 expression ($r = 0.96$; $P < 0.0001$) (Figure 6B). In tissue sections, FR1-AP-binding activity and expression of syndecan core proteins co-localize (Figure 1; M, N, and O) as FR1-AP binds to carcinoma cell membranes and cytoplasm, corresponding to the cumulative distribution pattern of syndecan-1 and syndecan-4. This observation is consistent with our results obtained *in vitro* using MCF-7 cells, and indicates that: breast carcinoma cell-associated HSPGs are decorated with HS chains similarly capable of promoting the FGF-2/FGFR-1 complex and response to HS-binding growth factors including FGF-2 may be regulated by total HSPG core protein levels.

Discussion

HSPGs are not only required for FGF binding to its tyrosine kinase receptors, but are dynamic regulators of FGF signaling. For example, dramatic changes in HSPG composition occur during early central nervous system development with substantial effects on signaling of different FGFs.¹⁹ HSPG alterations have also been ob-

served during malignant transformation. Pancreatic carcinoma cells overexpress the HSPG glypican-1 that is required for FGF-2 signaling in this tumor type.³¹ However, a more complete knowledge of HSPG function is required to better understand FGF signaling in breast cancer. In most studies to date, HSPGs have been extracted from cultured cells and activities of individual HSPGs have been studied in isolation. These approaches have yielded much important information, but have some critical disadvantages such as cells in culture and in particular immortalized cell lines may have altered HSPG profiles compared to cells *in vivo*. Also, focusing on one particular HSPG species unavoidably introduces bias. Alternatively, HSPGs extracted from tissues could be used for functional analysis. However, these HSPGs would be derived from multiple cell types and unavoidably, any location-specific information would be lost. To circumvent these pitfalls, we have chosen a comprehensive analysis of functional aspects of HSPGs *in situ* and *in vitro*.

The FGF-2/HSPG/FGFR-1 complex was reconstituted in a stepwise fashion, to examine the HSPG function in intact tissues. Several limitations of this approach should be kept in mind. The assay measures receptor binding and not activation. A discordance of ligand binding and intracellular signaling has been observed under certain conditions *in vitro*.⁴⁰ Also, recent structural analysis indicates that the complete FGF signaling complex consists of a FGF/FGFR/HSPG hexamer in a 2:2:2 ratio.¹⁶ It is not known whether the complex observed *in situ* fully represents this stoichiometry. Nevertheless, the assay measures the ability of HS chains to stabilize the interaction between FGF ligand and FGFR, a crucial requirement for signaling. Also, the technique is powerful because it allows us to relate topographic and functional information for this important class of molecules. Compared to normal breast epithelial cells, we observe an increased ability to promote the binding interaction between FGF-2 ligand and the FGFR-1 RTK in breast carcinoma HSPGs. Likely, this elevated binding activity contributes to the responsiveness of breast carcinoma cells to FGF-2. The relevance of HSPG-mediated ligand binding may extend to other HS-binding mitogens including hepatocyte growth factor/scatter factor, and the heregulins.

Receptor reconstitution *in situ* allows the association of binding activities with cell types, but this assay is not designed to assign binding activities to individual HSPG species. Emerging evidence indicates that HS GAG chains display specificity regarding their interactions with different members of the FGF family and different FGFRs.^{19,20,25,26} However, it is unclear whether different HSPG core proteins within a given cell type can be decorated with HS chains with divergent functions in FGF signaling. Using MCF-7 cells as model, we find that all HSPGs produced by this cell type (predominantly syndecan-1, syndecan-4, and an unidentified HSPG of ~250 kd) are decorated with HS GAG chains similarly capable of promoting FGF-2/FGFR-1 complex assembly. Our observations are in striking agreement with data generated by Rahmoune and co-workers.³² Using a Biacore sensor device, these investigators identified a single FGF-2-

binding activity within breast carcinoma cell (including MCF-7) HSPGs that was characterized by a slow association rate and a low affinity, but with an activating role in FGF-2 signaling. Conversely, two binding sites were identified within HS GAGs obtained from mammary fibroblasts and from myoepithelial-like cells; one with an activating and one with an inhibitory effect on FGF-2 signaling. However, a HS species with an inhibitory effect on FGF-2 signaling has also been observed in MDA-MB-231 breast carcinoma cells.¹³ These authors did not associate binding activities with HSPG core proteins. Another group of investigators recently described overexpression of the HSPG glypican-1 in breast carcinomas, an alteration not observed in our samples.⁴¹ Differences in methodology may explain the discrepancy.

If breast carcinoma cell HSPGs are decorated with HS GAGs similarly capable of promoting FGF receptor complex assembly, one would expect a modulation of this signaling system to occur at the level of core protein expression. Indeed, breast carcinoma cases not only show dramatic differences in expression of the HSPG core proteins syndecan-1 and syndecan-4, but the quantity of the FGF-2/HSPG/FGFR-1 complex closely follows total levels of these HSPGs. The wide spectrum of syndecan levels in carcinomas raises the question of how expression of these HSPGs is regulated. The syndecan-1 gene contains a regulatory element in its 5'-untranslated region termed FIRE that is induced specifically by members of the FGF family.⁴² This opens up the possibility of an autocrine/paracrine-positive feedback loop. Syndecan-1 expression is also modulated by extracellular matrix components.⁴³ This latter regulatory mechanism may be important in breast carcinomas, which can show a highly diverse stromal morphology ranging from richly cellular (desmoplastic) to sclerotic (scirrhous). The most striking difference between normal and malignant breast epithelial cells with respect to HSPG core proteins seen in this study is a significant reduction in the amount of syndecan-4. This proteoglycan has several proposed roles apart from participating in growth factor signaling. Syndecan-4 plays a crucial role in focal adhesion complex assembly and in cell migration.⁴⁴ As syndecan-4 regulates migration, a reduction in its level during malignant transformation may contribute to an invasive phenotype. This notion is supported by very recent work describing delayed wound healing in mice deficient in syndecan-4.⁴⁵

Syndecan-1 and syndecan-4 levels may explain differences in FGF receptor complex abundance between the breast carcinoma cases, but they fail to account for the differences in receptor complex formation between normal and malignant cells. Normal breast ductal and acinar epithelial cells express moderate to high levels of syndecan-1 and large amounts of syndecan-4, while essentially lacking the ability to form FGFR complexes *in situ*. Staining with an antibody that detects HSPGs regardless of the nature of the core protein excludes the possibility that increased expression levels of other HSPGs are responsible for the elevated HSPG-binding activities in carcinoma cells. Clearly, factors other than core protein levels must play a role in determining binding activities. Most likely, the differences in binding

behavior are because of differences in HS GAG chain structure. Data generated by other investigators support this notion. The malignant transformation of colonic adenoma cells is accompanied by an increase in HS GAG sulfation in the 6-O position of glucosamine.⁴⁶ Similarly, a twofold increase in 6-O-sulfated HS was seen in breast carcinoma cells lines compared to two nonmalignant counterparts.⁴⁷ Interestingly, 6-O sulfate on glucosamine is the main structural feature within HS GAGs required to promote binding of FGF-2 to FGFR-1 and to stimulate signaling.^{24,25} Secondly, degradative modifications of HS GAGs at the carcinoma cell surface may also be important. Kato and co-workers⁴⁸ demonstrated that in postmastectomy wound fluids, syndecan-1 is converted from an inhibitor of FGF-2 activity to an activator by the action of heparanase enzymes. Interestingly, a very recent report describes a correlation between syndecan-1 levels and mammalian heparanase expression and poor outcome in breast carcinoma patients.⁴⁹

Future work will examine at what stage during the malignant progression of breast epithelium HSPGs are changing to stimulators of FGF-2 action. *In situ* assays will be crucial tools in mapping HS motifs in localized premalignant and emerging malignant lesions. Other investigators are developing phage display-derived antibodies to characterize differences in HS GAGs in intact tissues.⁵⁰ Our receptor reconstitution assays may allow a more direct association of location and function. Also, it will be important to determine exactly what alterations in HS GAG structure are occurring during malignant transformation. The recent development of techniques allowing sequence analysis of HS fragments brings this goal within reach.^{23,51} A better understanding of structure/function relationships will aid in the rational design of pharmacological agents targeted at disrupting unwanted FGF activity.

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