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Differential Binding of Fibroblast Growth Factor-2 and -7 to Basement Membrane Heparan Sulfate

Comparison of Normal and Abnormal Human Tissues

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Fibroblast growth factors (FGFs) play multiple roles during development and in adult tissues as paracrine regulators of growth and differentiation. FGFs signal through transmembrane receptor tyrosine kinases, but heparan sulfate is also required for signaling by members of the FGF family. In addition, heparan sulfate may be involved in determining tissue distribution of FGFs. Using biotinylated FGF-2 and FGF-7 (KGF) as probes, we have identified specific interactions between FGFs and heparan sulfates in human tissues. Both FGF species bind to tissue mast cells and to epithelial cell membranes. Binding to basement membrane heparan sulfate is tissue source dependent and specific. Although FGF-2 strongly binds to basement membrane heparan sulfate in skin and most other tissue sites examined, FGF-7 fails to bind to basement membrane heparan sulfate in most locations. However, in subendothelial matrix in blood vessels and in the basement membrane of a papillary renal cell carcinoma, strong FGF-7 binding is seen. In summary, distinct and specific affinities of heparan sulfates for different FGFs were identified that may affect growth factor activation and local distribution. Heparan sulfate may have a gatekeeper function to either restrict or permit diffusion of heparin-binding growth factors across the basement membrane. (Am J Pathol 1997, 150:1443–1455)

Numerous functions have been attributed to the fibroblast growth factors (FGFs), including stimulation of mitogenesis, induction or inhibition of differentiation, and survival of central nervous system neurons, as well as a role in physiological and pathological angiogenesis (reviewed in Refs. 1 and 2). Currently, nine members of the FGF family of cytokines have been described and have been designated as FGF-1 through -9. FGFs act on a wide range of target cells, and indeed most cell types respond to at least one of these growth factors.

The FGFs signal through transmembrane receptor tyrosine kinases (reviewed in Ref. 3). The extracellular domain of the FGF receptor is subject to splice variation leading to forms with two or three immunoglobulin-like domains. The ligand-binding site is thought to reside in the second half of the most proximal loop (loop III). A heparan sulfate binding site resides in the distal region of loop II.[4] The intracellular domain carries a split tyrosine kinase domain and six potential phosphorylation sites on tyrosine residues.[5]

Ligand-induced receptor activation is believed to involve receptor dimerization and trans-phosphorylation.[6] The signal transduction cascade that ultimately leads to transcription factor activation within the nucleus is mediated through intracellular molecules such as grb-2, shc, or sos that form a multimeric complex with the receptor, resulting in activation of ras and the MAP kinase pathway. A second signaling pathway involving phospholipase-C-γ and arachidonic acid metabolites regulating intracellular calcium flux has also been identified (reviewed in Ref. 7).

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Four genes are known to date to encode FGF receptors (FGFR-1 through -4; reviewed in Ref. 3). There is a great degree of overlap in the binding interaction between ligands and receptors. For example, FGF-1 and FGF-2 bind well to FGFR-1, -2, -3, and -4. This apparent overlap in binding interactions raises the question of how specificity in signaling is maintained.

One way in which binding is restricted is by the generation of alternative receptor splice variants.[8] Post-transcriptional mRNA modification has been observed in FGFR-1, -2, and -3. Alternative splicing has been shown to affect receptor affinities for different members of the FGF family. For example, the IIIb variant of FGFR-2 (KGF receptor) favors binding of FGF-7 and FGF-1 over that of FGF-2, whereas the IIIc isoform binds FGF-1 and -2 equally well but not FGF-7.

The prototypes within the FGF family, FGF-1 (also known as acidic FGF) and FGF-2 (basic FGF), were originally operationally termed heparin-binding growth factors for their ability to bind to heparin that facilitated their purification. This attribute is shared by the other FGFs. Recent experimental evidence[9,10] clearly demonstrates that FGFs not only bind heparin but that heparin or heparan sulfate also is required for binding of FGFs to the receptor tyrosine kinase and the generation of an intracellular signal. An augmenting effect of heparan sulfates on signaling has so far been demonstrated for FGF-1, -2, -4, -5, -8, and -9.

Heparan sulfate is a glycosaminoglycan that is characterized by repetitive disaccharide units consisting of glucuronic acid and *N*-acetyl-glucosamine and is synthesized while linked to a proteoglycan core protein (reviewed in Ref. 11). The sugar backbone is subsequently modified by a battery of enzymes that facilitate epimerization reactions (glucuronic acid to iduronic acid), and the introduction of sulfate groups in various positions, generating an affinity for FGF. In the case of heparan sulfate, these secondary modifications occur in a discontinuous and nonrandom fashion; ie, highly modified areas will alternate with less modified regions. This process is responsible for heterogeneity not only within a single glycosaminoglycan chain but also between different chains. It is unknown how these events are orchestrated. In contrast, in the case of heparin, which is a specialized mast cell heparan sulfate, the isomerization and substitution reactions are carried out almost to completion, resulting in a homogeneous chain with high affinity for FGFs.

The goal of this study was to examine binding of FGF-2 and FGF-7 to heparan sulfates in human tissues. These two growth factors were chosen for several reasons. First, they have different target cell specificity with FGF-2 mainly acting on mesenchymal and neuroectodermal cells and FGF-7[12] being the only example next to FGF-3[13] within this group of growth factors with selectivity for epithelial cells. Second, reports in the literature point to different effects of heparan sulfates on these two growth factors. Whereas heparan sulfate and heparin generally enhance the activity of FGF-2, they appear to suppress FGF-7 action.[14] This raises the question of whether or not different FGFs interact with distinct heparan sulfate proteoglycans in tissues.

Indeed, this study demonstrates that FGF-2 binds strongly to heparan sulfate in the epidermal basement membrane, whereas FGF-7 fails to bind to the same structure. In stark contrast to skin, FGF-7 binds avidly to basement membrane in several other tissues including a renal cell carcinoma. As FGF-7 fails to bind to normal renal tubular basement membrane, this demonstrates a switch in the affinity of heparan sulfate proteoglycans during malignant transformation. In conclusion, the interaction between FGFs and heparan sulfate is a specific one, and the tissue-specific occurrence of certain heparan sulfate sequences may have a profound effect on the distribution and activation of different FGFs.

Materials and Methods

Biotinylation of Growth Factors

Human recombinant FGF-2 was kindly provided by Dr. Brad Olwin, Purdue University, IN. Human recombinant FGF-7 was a gift from Amgen Corp., Thousand Oaks, CA. The following conditions were found to be optimal for the biotinylation of both FGF-2 and FGF-7. A 44-μg amount of FGF was bound to 200 μl of prewashed heparin-agarose beads (Sigma Chemical Co., St. Louis, MO) in 0.2 mol/L sodium bicarbonate buffer at pH 8.1. Sulfo-NHS-biotin (Pierce, Rockford, IL) was added to a final concentration of 20 mmol/L and incubated at room temperature for 5 minutes. The reaction was terminated by washing the beads six times with wash buffer (20 mmol/L Hepes, pH 7.4, 400 mmol/L NaCl for FGF-2 or 200 mmol/L NaCl for KGF). Biotinylated growth factor was eluted with 2x 200 μl of elution buffer (20 mmol/L Hepes, pH 7.4, 0.2% bovine serum albumin, 3 mol/L NaCl for FGF-2 or 1.5 mol/L for KGF). Aliquots were snap-frozen and stored at −70°C.

The efficacy of biotinylation was analyzed by detecting the FGFs on Western transfers using either FGF-specific or biotin-specific antibodies. Nitrocel-

lulose membrane was obtained from Biorad (Hercules, CA), anti-FGF-2 antibody (DE-6) was a gift from DuPont (Wilmington, DE), and polyclonal goat anti-FGF-7 antibody and anti-biotin antibody were purchased from R&D systems (Minneapolis, MN) and Sigma, respectively.

Bound antibodies were visualized with horseradish-peroxidase-conjugated anti-mouse and anti-goat antibodies (Jackson Immunoresearch, West Grove PA) using the ECL detection system (Amersham, Arlington Heights, IL).

Tissue Culture and Mitogenesis Experiments

Swiss 3T3 fibroblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and 100 U/ml penicillin and 100 μg/ml streptomycin. Mitogenesis experiments were performed to compare native and biotinylated FGF-2 using 3T3 cells. A total of 30,000 cells were plated per well in 24-well plates (Falcon, Becton-Dickinson, Lincoln Park, NJ) and were allowed to adhere for 24 hours. The cells were then serum starved for 24 hours by replacing serum-containing medium with Dulbecco's modified Eagle's medium plus 0.1% bovine serum albumin, after rinsing the cells twice with the same medium. Serial dilutions of growth factors were then added in serum-free medium for 24 hours. The concentrations for biotinylated growth factors were calculated under the assumption of 100% recovery during the biotinylation reaction. Aliquots of biotinylated FGF-2 were incubated with streptavidin-agarose beads to extract biotinylated growth factor. During the last 6 hours of FGF-2 stimulation, 2 μCi per well of tritiated thymidine was added. The culture medium was then aspirated and the cells were fixed in 5% trichloroacetic acid for 15 minutes. The wells were then washed with 70% ethanol twice. The fixed cells were lysed in 0.5 ml of 0.1 N NaOH, and the lysate was transferred into scintillation vials. A 10-ml volume of scintillation liquid was added (Bio-Safe II, Research Products International Corp., Mt. Prospect, IL) and radioactivity was determined with an LS 5800 scintillation counter (Beckman Instruments, Somerset, NJ).

Tissues and in Situ *FGF Binding*

Tissue was obtained fresh from the operating room, embedded in OCT compound, snap-frozen, and stored at −70°C. The 5-μm-thick frozen sections were prepared and fixed in 4% paraformaldehyde (EMS, Ft. Washington, PA). The sections were treated with sodium borohydride (0.05%; prepared in 4°C double-distilled H_2O and then placed at room temperature) for 5 and 10 minutes and with glycine (0.1 mol/L) in phosphate-buffered saline (PBS) overnight at 4°C to reduce autofluorescence. After blocking with Tris-buffered saline plus 1% bovine serum albumin (room temperature for 30 minutes), sections were incubated with biotinylated FGFs (10 nmol/L FGF-2, 200 nmol/L FGF-7) for 1 hour at room temperature. The concentration of FGF-7 was chosen higher because the overall affinity of FGF-7 for heparan sulfate is lower than that of FGF-2. This difference is reflected by the different salt concentrations required to elute the FGFs off a heparin column (0.5 to 0.6 mol/L for FGF-7 *versus* approximately 2 mol/L for FGF-2[15]). Sections were washed after this and after subsequent incubations four times with Tris-buffered saline. Bound growth factor was detected with monoclonal mouse anti-biotin (Sigma) and rhodamine-conjugated donkey anti-mouse (Jackson ImmunoResearch) antibodies. Sections were examined using a Nikon Microphot FX microscope equipped for epifluorescence. Images were acquired with a Photometrics (Tucson, AZ) CCD camera and Image-Pro-Plus analysis software (Media Cybernetics, Silver Springs, MD). Controls were included with all binding experiments to assure specificity and saturability of binding and to positively determine the identity of the binding sites. Excess unlabeled FGF was added to some slides at a concentration of 1 μmol/L for FGF-2 and 10 μmol/L for FGF-7 to demonstrate saturability of the binding sites. Heparin (Sigma) was added in competition experiments at a concentration of 1 mg/ml. To identify the binding sites as heparan sulfate, tissues were predigested with heparitinase (heparinase III; ICN, Woburn, MA) at a final concentration of 12 mIU/ml in PBS plus 0.1% bovine serum albumin. The digestion was carried out at 37°C for 2 hours. After the first hour, the entire enzyme amount was replenished. To show that receptor tyrosine kinase did not contribute to detected binding, some slides were washed with 2 mol/L NaCl solution in phosphate buffer at pH 7.4.

Some of the binding experiments were carried out with native growth factor, detected with specific anti-FGF antibodies to rule out the possibility that the biotinylation reaction had changed the FGF binding pattern. The concentrations of native FGF-2 and FGF-7 were the same as those of the biotinylated preparations, ie, 10 nmol/L and 200 nmol/L, respectively. Mouse monoclonal anti-FGF-2 antibody (DE-6) was added at 10 μg/ml and goat anti-FGF-7 antibody (R&D Systems) at 100 μg/ml. Rhodamine-labeled secondary antibodies (Jackson ImmunoResearch) were used for detection.

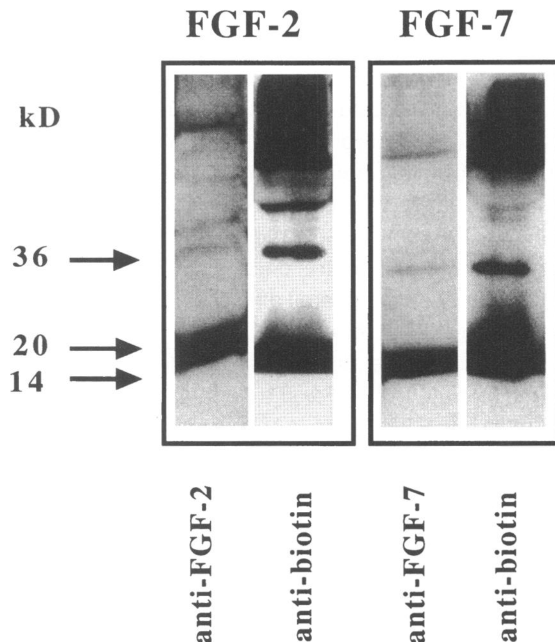

Figure 1. *SDS-polyacrylamide gel electrophoresis of biotinylated FGF-2 and FGF-7. The membranes were probed with anti-FGF-2 or anti-FGF-7 antibodies and anti-biotin antibodies. Molecular weights derived from standards are indicated on the left.*

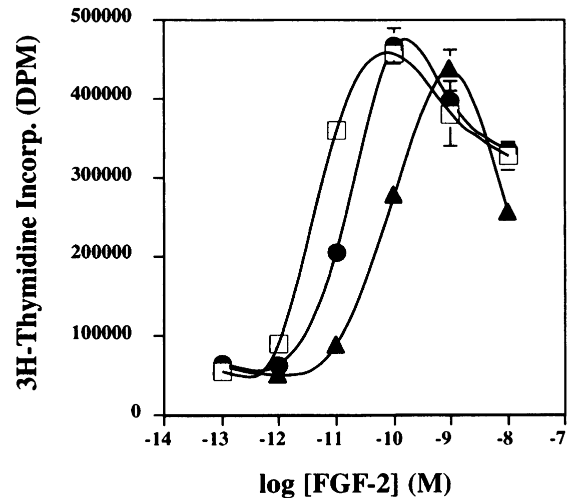

Figure 2. *Mitogenesis assay with biotinylated FGF-2. Tritiated thymidine incorporation was measured using Swiss 3T3 cells. Shown are dose-response curves comparing native (□) with biotinylated growth factor (●) and biotinylated FGF-2 after depletion with immobilized streptavidin (▲). Error bars indicate standard error. The curve fits represent interpolations.*

Results

Biotinylation of FGF-2 and FGF-7

Biotinylated FGFs were generated as probes to determine the binding specificity of heparan sulfates within tissues. To protect the heparin-binding site on the FGF molecules, the growth factors were bound to immobilized heparin during the labeling reaction. The biotinylated growth factor was then removed from the heparin-agarose beads by washing in high salt. To analyze the efficacy of the biotinylation reaction, the eluates were then subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and the membranes were probed with anti-FGF-2, anti-FGF-7, and anti-biotin antibodies (Figure 1). A strong signal is detected with the anti-biotin antibody indicating a high labeling intensity for both growth factors. The FGF-2 and FGF-7 monomers are seen as strong bands at approximately 18 kd. In addition, there are several bands of higher molecular weights, which most likely represent growth factor dimers and multimers. The multimers appear to be biotin labeled to a higher degree than the monomers.

As a second means of quantifying the biotinylation, the FGF preparation was used in an activity assay before or after depletion of the biotinylated form. For this purpose, mitogenesis was measured using a [^{3}H]thymidine incorporation assay with Swiss 3T3 fibroblasts. Using native growth factor as a standard, serial dilutions of biotinylated growth factor were tested before and after depletion with immobilized streptavidin. The dose-response curves for native and biotinylated FGF-2 are almost superimposed, indicating that biotinylated FGF-2 is biologically active (Figure 2). Depletion with streptavidin-agarose leads to a shift of the dose-response curve by approximately one order of magnitude. This result indicates that approximately 90% of the FGF-2 was labeled.

Specific in Situ *Detection of FGF-Binding Heparan Sulfates*

Biotinylated FGFs were used to determine the specificity of growth factor binding to heparan sulfates in human tissues. For this purpose, frozen sections of fresh tissues were incubated with labeled growth factor, and binding was detected by immunofluorescence.

The prototype of the tissues examined was skin, where FGFs likely play important roles in epidermal differentiation, wound healing, and hair follicle development. Binding of biotinylated FGF-2 is detected within the epidermis on keratinocyte membranes ranging from the stratum basale to the upper stratum spinosum (Figure 3A). Strong staining of the basement membrane is noted at the dermo-epidermal junction. Within the dermis, FGF-2 binds to small blood vessels in a linear pattern, most likely also representing basement membranes. In addition, a very intense decora-

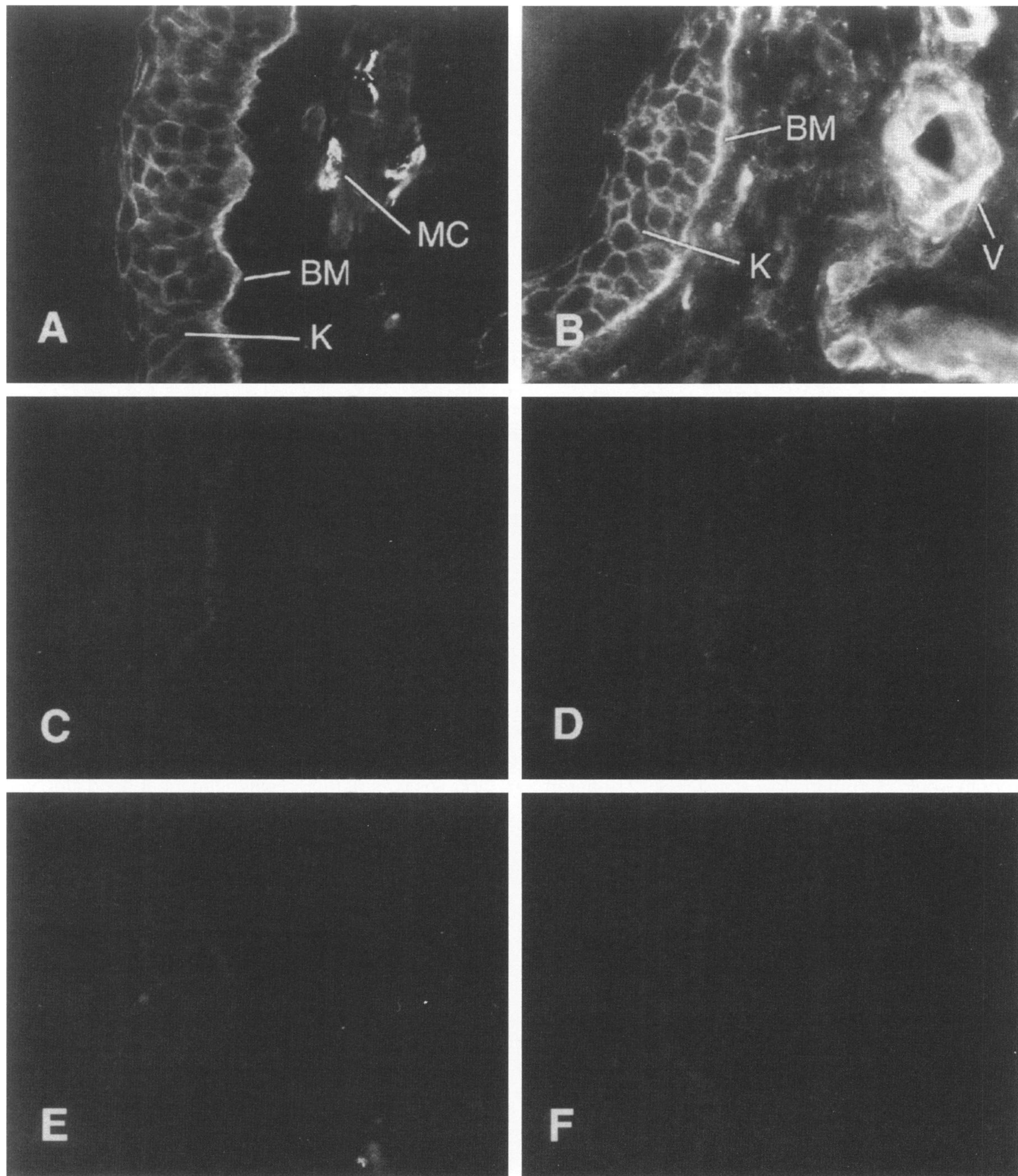

Figure 3. In situ *binding of FGF-2 to heparan sulfate proteoglycans in skin. Frozen sections of skin were exposed to biotinylated FGF-2 and bound growth factor was detected by immunofluorescence in* A *and* C *through* F. A: *Biotinylated FGF-2 alone.* C: *Competition with 1 μmol/L unlabeled FGF-2 during the binding reaction.* D: *Competition with 1 mg/ml heparin.* E: *Predigestion of tissue with heparitinase.* F: *Wash with 2 mol/L NaCl after binding of FGF-2.* B: *Total tissue heparan sulfate detected with monoclonal antibody 3G10. BM, basement membrane; MC, mast cell; K, keratinocytes; V, blood vessels. Magnification, ×400.*

tion of individual cells within the dermis was observed, which occurs frequently in association with the microvasculature. These cells are of fusiform or polygonal morphology and were identified as mast cells by toluidene blue and Giemsa stains. FGF-2 is observed bound to mast cell granules, and degranulation is frequently noted within a variety of tissues resulting in extracellular granular FGF-2 binding affinities. The binding partner in mast cell granules is most likely heparin, which is supported by the observation that an excess of externally added heparin inhibits binding (see below).

The fact that the observed FGF binding pattern indeed represents an interaction with heparan sulfate is supported by several lines of argument. First, staining with anti-heparan sulfate antibody 3G10 after heparitinase digestion of the tissue reveals a pattern very similar to that seen with biotinylated FGF-2 (Figure 3B). This antibody recognizes unsaturated uronate groups that are uniformly present on the heparan sulfate stubs that remain after heparitinase digestion regardless of the core protein carrying it. Staining with this antibody therefore represents the total of all tissue heparan sulfate proteoglycans. These results are in keeping with the observations by David and co-workers.[16] Second, adding unlabeled FGF-2 in excess abolishes FGF-2 binding (Figure 3C), demonstrating saturability of the binding sites. Finally, heparan sulfate is identified as the binding partner of FGF-2 by competitive inhibition with heparin (Figure 3D) and by abolition of binding by heparitinase digestion of the tissue before the growth factor incubation step (Figure 3E). Notably, heparitinase (heparinase III) does not significantly reduce mast cell staining, presumably because the enzyme is specific for heparan sulfate rather than heparin. To rule out the possibility that the observed growth factor binding is due to interaction with receptor tyrosine kinases, the sections were subjected to a 2 mol/L salt wash at neutral pH after FGF-2 binding. This ionic strength would disrupt the low affinity FGF-2 heparan sulfate binding interactions but would have no effect on FGF-2 bound to FGF receptor. Indeed, FGF-2 binding is greatly reduced by the high salt concentration (Figure 3F).

FGF-2 and FGF-7 Bind to Distinct Heparan Sulfates in Skin

To determine whether different members of the FGF family have specific affinities for distinct tissue heparan sulfates, binding of FGF-2 and FGF-7 to tissue heparan sulfate proteoglycans were compared. In dramatic contrast to FGF-2 (Figure 4A), FGF-7 does not bind to the basement membrane at the dermoepidermal junction, whereas binding to keratinocyte membranes and to mast cells within the dermis is equivalent to that of FGF-2 (Figure 4B). FGF-7 staining of keratinocyte cell surfaces is present from the basal layer all the way to the stratum corneum. This is also in contrast to the binding of FGF-2, which is more restricted and is not seen above the granular layer. The differential ability of FGF-2 and FGF-7 to bind to basement membrane is even more accentuated in the hair follicle (Figure 4, C and D). Again, FGF-2 binds strongly to the basement membrane surrounding the cross-sectioned hair follicle, whereas FGF-7 fails to attach to basement membrane heparan sulfate proteoglycans. However, FGF-7 binds to cells of the root sheaths and to the cuticle, recapitulating the binding pattern seen in the epidermis.

The binding of FGF-7 was shown to be specific and to be due to interaction with heparan sulfate by using heparitinase and 1 mol/L salt controls (data not shown). Importantly, native FGFs detected with anti-FGF-2 and anti-FGF-7 antibodies displayed a binding pattern identical to that seen with the biotinylated forms (Figure 4, E and F). This result rules out the possibility that the chemical modification of the growth factors during biotinylation leads to a change in binding behavior. This control is important because the biotinylation of FGF-7 was accompanied by a partial loss of mitogenic activity ranging from 90 to 99%. Evidence generated by other investigators also makes it appear unlikely that this loss of mitogenic activity could be caused by an alteration of the heparin/heparan sulfate binding site. Reich-Slotky and co-workers[14] have shown that the heparan sulfate binding site in FGF-7 has a negative regulatory role. Therefore, it would be predicted that the destruction of this domain enhances mitogenesis. No specific staining with anti-FGF-2 and anti-FGF-7 antibodies was detected when the incubation step with FGFs was omitted, indicating that the concentration of endogenous FGF is too low for detection (data not shown).

Distinct FGF-2 and FGF-7 Binding to Heparan Sulfates in Normal Tissues

To investigate whether heparan sulfates that display differential affinities for FGFs exist in other sites, a variety of tissues were screened using the same binding assay (data shown in Table 1). Interestingly, the inability of FGF-7 to bind to basement membrane is not universal. Notably, both FGF-2 and, to a slightly lesser degree, FGF-7 bind to basement membrane heparan sulfate proteoglycans surrounding endometrial glands within the uterus and colonic crypts. In thyroid gland, follicular basement membranes stain equally well with FGF-2 and FGF-7. Subendothelial basement membrane of arteries was also found to bind both FGF-2 and, to a slightly lesser degree, FGF-7 (Figure 5). Apparently, the ability of FGF-7 to interact with basement membrane heparan sulfate proteoglycans is tissue type specific.

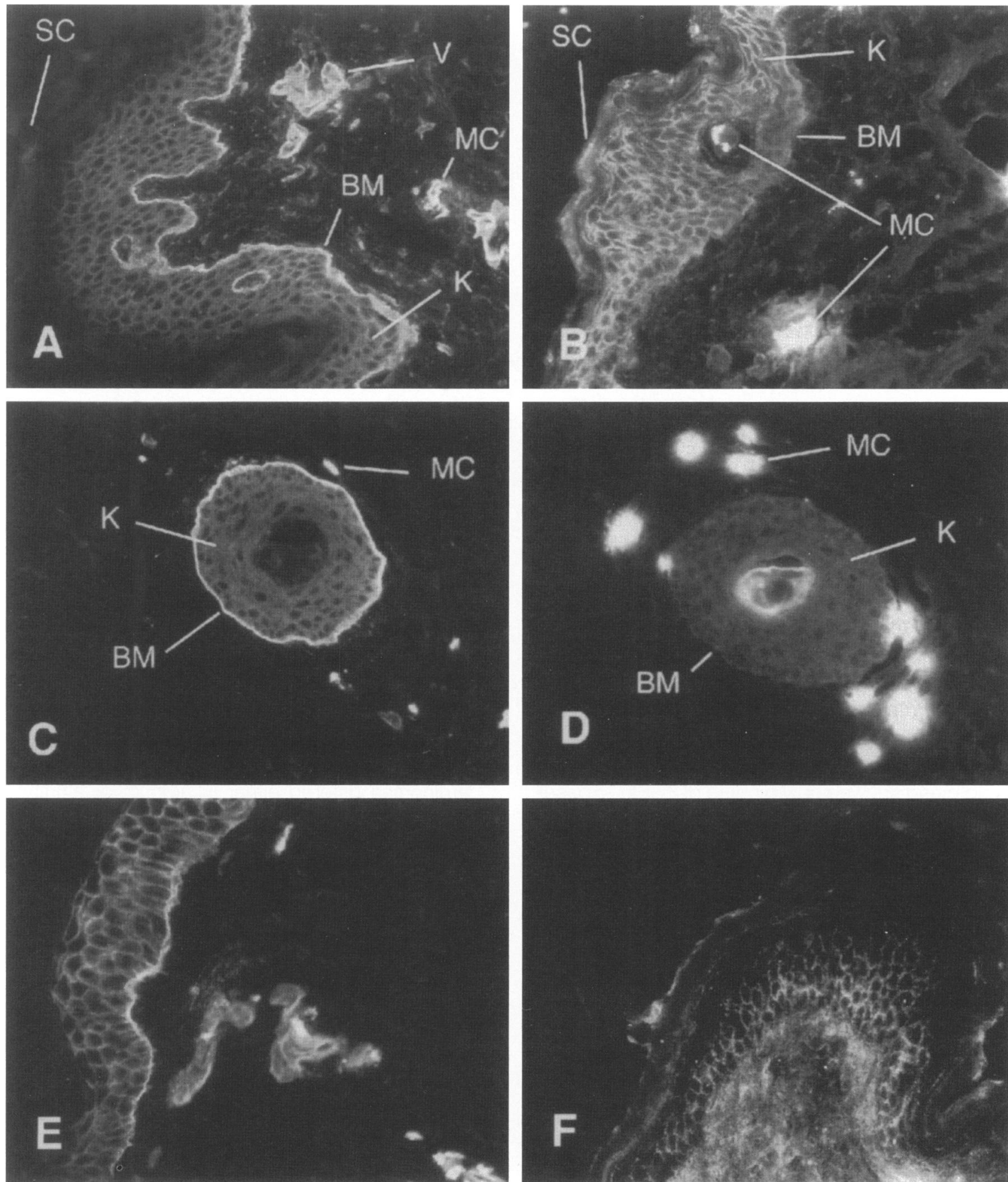

Figure 4. *FGF-2 and FGF-7 bind to distinct heparan sulfate proteoglycans in skin. Shown is binding to superficial skin* (**A**, **B**, **E**, *and* **F**) *and to a cross-sectioned hair follicle* (**C** *and* **D**), *using biotinylated FGF-2* (**A** *and* **C**) *and biotinylated FGF-7* (**B** *and* **D**) *as probes. Binding of native growth factor detected with antibodies is shown in* **E** (*FGF-2*) *and* **F** (*FGF-7*). *BM, basement membrane; MC, mast cells; V, blood vessel; K, keratinocytes; SC, stratum corneum. Magnification,* ×*200.*

Distinct FGF-2 and FGF-7 Binding to Heparan Sulfates in Abnormal Tissues

A survey of binding patterns of FGF-2 and FGF-7 in a diverse series of pathological tissues yielded very interesting results (Table 2). In a tubulo-papillary renal cell carcinoma, binding of FGF-7 to basement membrane structures lining the stromal cores greatly exceeded that of FGF-2 (Figure 6, A and B) contrasting with the pattern seen in skin (Figure 4, A and B). The binding sites were identified as heparan sulfate because heparitinase digestion abolished staining

Table 1. *Summary of Binding Experiments with Normal Human Tissues Using Biotinylated FGF-2 and FGF-7 as Probes*

Organ	Structure	FGF-2	FGF-7
Skin	Keratinocytes	++	++
	Dermo-epidermal BM	+++	−
	Dermal vessel BM	++	−
	Mast cells	+++	+++
Endometrium	Glandular epithelial cells	+/−	+/−
	Glandular BM	++	+
	Stroma	+	−
Colon	Crypt epithelial cells	−	−
	Crypt BM	++	+
Thyroid gland	Follicular cells	−	−
	Follicular BM	++	++
Kidney	Glomerular BM	−	−
	Tubular BM	++	−
Mammary gland	Acinar and ductal epithelial cells	+	+
	Acinar and ductal BM	++	−
	Microvasculature	++	−
Blood vessels	BM (subendothelial matrix)	++	++
Lung	BM (bronchial epithelium)	++	−

Staining intensity was scored as follows: +++, strong; ++, moderate; +, weak; −, negative. BM, basement membrane.

(Figure 6, C and D). As only FGF-2 but not FGF-7 is able to bind to basement membrane of normal renal tubules (Table 1), the alleged site of origin of renal cell carcinomas, this is the first observation of a change in the expression of heparan sulfate proteoglycans with different specific affinities for FGFs accompanying malignant transformation.

Interestingly, this phenomenon was not seen in all papillary neoplasms. A papillary carcinoma of the thyroid gland showed only binding of FGF-2 to basement membranes but not FGF-7 (Table 2). This result stands in contrast to benign thyroid tissue, where basement membrane binding of both FGF-2 and FGF-7 were observed (Table 1). Apparently, loss as well as gain of the ability to bind FGF-7 can occur during the acquisition of the malignant phenotype.

Comparing normal breast tissue with infiltrating ductal carcinomas, it was noted that FGF-2 bound well to extracellular matrix around microvessels in close proximity to normal breast lobules, whereas no or very little binding was observed within the carcinomas. This is surprising because the development of carcinomas is accompanied by angiogenesis and the density of microvessels has been shown to correlate with the presence of metastatic disease.[17] Apparently, the subendothelial heparan sulfate proteoglycans in newly formed blood vessels within the carcinomas differed either in amount or affinity for FGF-2.

Discussion

The purpose of this study was to identify heparan-sulfate-associated FGF binding in tissues and to determine whether binding activities exist that are spe-

Table 2. *Summary of Binding Experiments with Abnormal Human Tissues Using Biotinylated FGF-2 and FGF-7 as Probes*

Organ/disease	Structure	FGF-2	FGF-7
Kidney			
Global glomerulosclerosis	Glomerulus	−	−
Tubulo-papillary renal cell carcinoma	BM (papillary cores)	++	+++
Clear cell renal cell carcinoma	Carcinoma cells	+	−
Thyroid gland			
Papillary carcinoma	BM (papillary cores)	++	−
Lung			
Squamous cell carcinoma	BM (surrounding some tumor islands)	+	+
Uterus			
Leiomyoma	Tumor (smooth muscle)	−	−
Mammary gland			
Infiltrating ductal carcinoma	Carcinoma cells	+	+
	Microvasculature	−	−

Staining intensity was as follows: +++, strong; ++, moderate; +, weak; −, negative. BM, basement membrane.

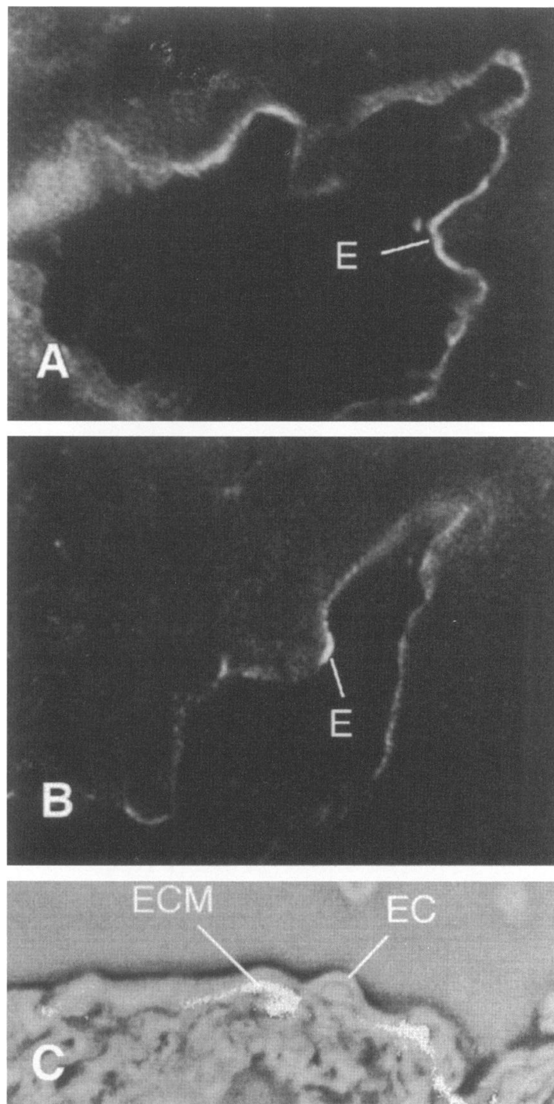

Figure 5. *Binding pattern of FGF-2 and FGF-7 to subendothelial matrix of a myometrial artery.* **A**: *Binding of biotinylated FGF-2.* **B**: *Binding of biotinylated FGF-7.* **C**: *Composite phase contrast and FGF-2 fluorescence image showing the subendothelial location of the binding sites. E, endothelium; EC, endothelial cell; ECM, subendothelial extracellular matrix. Magnification,* ×*400* (**A** *and* **B**) *and* ×*2000* (**C**).

cific for different FGF species. Although some heparan sulfates bind both FGF-2 and FGF-7, dramatic differences in the affinity of basement membrane heparan sulfate for these two FGFs were identified. This selectivity raises important questions about the regulation of heparan sulfate synthesis and its role in modulating FGF activity.

The control of cell behavior by the FGFs is regulated by the availability of three components: the FGF itself, its receptor tyrosine kinase, and heparan sulfate proteoglycan.

The localization of the endogenous FGFs has been addressed by several reports in the literature. In two studies of skin, FGF-2 is localized to the epidermal basal cell layer.[18,19] It is also localized to epithelial cells of the hair follicles and sweat and sebaceous glands and to blood vessels and fibroblasts within the dermis. In contrast, others found no specific staining of epidermal keratinocytes.[20] Interestingly, dermal mast cells have been proposed to be a major site of FGF-2 storage and production by some investigators.[21] In the vascular system, FGF-2 immunoreactivity was found within the subendothelial basement membrane of blood vessels ranging from mid-sized arteries to the capillary bed.[20] Capillary endothelial cells were also found to react with anti-FGF-2 antibodies. In addition, FGF-2 was localized to colon (epithelial cells, lamina propria, smooth muscle, microvasculature), liver (hepatocytes), renal tubular epithelium (rather than glomeruli), ovary, adrenal cortex, anterior pituitary, and placenta (villous connective tissue). Cardiac myocytes and skeletal muscle also strongly expressed FGF-2 protein.

FGFR-1, a major receptor tyrosine kinase for FGF-2 is ubiquitously present in microvessels, and expression is most pronounced in post-capillary venules.[19] FGFR-1 is equally identified in a limited number of epithelia that include palatine tonsil, ectocervix, bronchus (basal cells), and thymus.

FGF-7 is a paracrine growth factor that is produced by stromal cells and acts on epithelial cells.[12] The epidermis and hair follicle are considered the predominant targets for FGF-7, but this growth factor has also been implicated as a second messenger in steroid hormone action in uterus[22] and seminal vesicle[23] and has been shown to be a mitogen for breast, pancreas, and lung epithelium *in vivo*.[24] FGF-7 appears to regulate terminal differentiation of keratinocytes[25] and is involved in hair follicle growth and differentiation.[26–28] A function in healing of injured skin has also been suggested.[29,30] Reports on the tissue distribution of FGF-7 are more spurious due to the lack of suitable antibodies. FGF-7 (KGF) mRNA transcripts were identified by *in situ* hybridization in follicular dermal papillae in rats.[28] The same group of investigators identified KGF receptor mRNA (IIIb splice variant of FGFR-2) in basilar epidermis and in follicular keratinocytes. Aaronson and colleagues followed a different strategy to determine KGFR expression[31] by using a KGF-immunoglobulin chimera for ligand-mediated *in situ* detection of the KGFR. They found KGFR within the stratum spinosum and to a lesser extent the stratum basale. The bulb region of the hair follicle bound the ligand strongly, whereas the upper regions of the hair follicle failed to do so. *In situ* hybridization results correlated well with these findings.

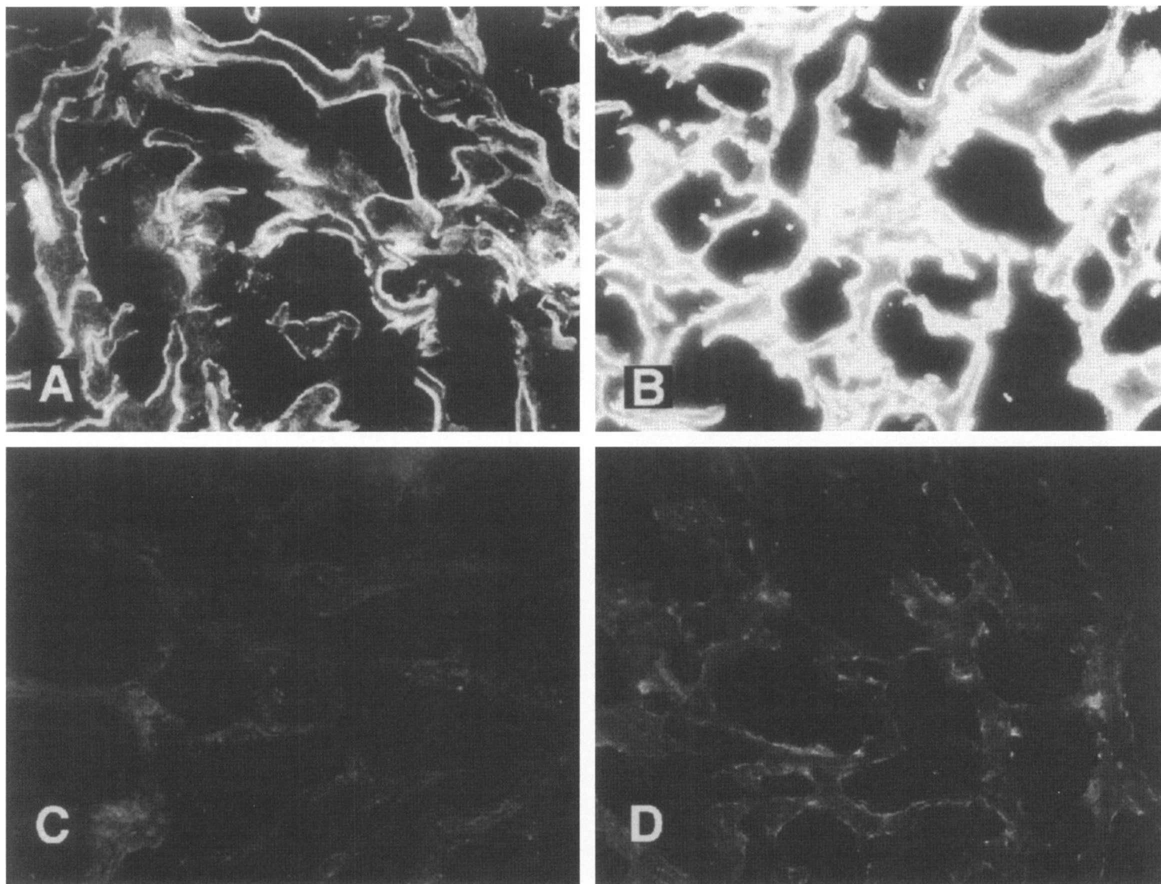

Figure 6. *FGF-7 binds avidly to basement membrane heparan sulfate proteoglycans in a papillary renal cell carcinoma:* **A**: *Binding of biotinylated FGF-2.* **B**: *Binding of biotinylated FGF-7.* **C**: *Heparitinase-digested control for FGF-2.* **D**: *Heparitinase-digested control for FGF-7. Magnification, ×400.*

Heparan sulfate proteoglycans have been shown to play an important role in FGF signaling using different experimental approaches. Treatment of cells in culture with sodium chlorate leads to a competitive inhibition of the sulfation of glycosaminoglycans and abolishes FGF effects on cells. Similarly, FGF has no effect on a heparan-sulfate-deficient mutant cell line, unless exogenous heparan sulfate or heparin is supplied (reviewed in Ref. 32). Binding of heparan sulfates to FGFs is required but not necessarily sufficient to promote signaling. For example, heparin selectively desulfated in the 6-*O* position retains its ability to bind FGF-2 but does not mediate binding to the receptor.[33] Thus, this heparin derivative is a competitive inhibitor of FGF-2 signaling. Different FGFs have distinct requirements for heparan sulfates.[33] Furthermore, the activity of heparan sulfate as either promoter or inhibitor of FGF is different for different FGF family members. Although the 6-*O*-desulfated heparin acts as a competitive inhibitor of FGF-2-induced mitogenesis of 3T3 fibroblasts, this heparin derivative has no effect on FGF-1 and enhances signaling by FGF-4.

A heparan sulfate binding site on the FGFR[4] is also crucial in the formation of a ligand-receptor complex. Loss of function of this receptor domain by site-directed mutagenesis or treatment with a peptide as competitive inhibitor resulted in a dramatic decrease in activity. Similar to the FGF ligands, the heparan sulfate binding site on the receptors appears to display structural selectivity as peptide competitors modeled after the heparan sulfate binding sites of different FGFRs specifically interfere with cell heparan sulfate/FGF interactions.[34] Although it has been postulated that heparan sulfate may induce a conformational change that increases the affinity of FGF for the FGF receptor,[35] it is likely that it acts by binding the FGF and receptor and thus mediating the formation of a stable ternary complex consisting of FGF, one or two FGF receptors, and the heparan sulfate chain.[36–38]

Heparan sulfate proteoglycans can be separated into cell surface and extracellular forms. The cell surface heparan sulfate proteoglycans include transmembrane proteoglycans such as the syndecan family and forms that are anchored in the cell membrane with a glyosyl-phosphoinositol-linked lipid tail (glypicans). Members of all of these cell surface groups have been shown to bind FGFs. Binding of FGF-2 has been demonstrated to syndecan-1,[39] -2,[40] -3,[41] and -4[40] and to the V3 isoform of CD44, a "part-time" proteoglycan with various other functions.[42] Perlecan is a prototypical extracellular heparan sulfate proteoglycan that is abundantly present in basement membrane. Perlecan has been proposed to be highly effective in its ability to mediate binding to the receptor tyrosine kinase. Yayon and colleagues[43] identified perlecan as the proteoglycan with the best ability to promote FGF-2 binding using an *in vitro* binding assay with immobilized FGFR-1. Similarly, Nurcombe's group also characterized a developmentally regulated heparan sulfate proteoglycan expressed in embryonal mouse neuroepithelium as a perlecan-like molecule.[44,45] In this model, the affinity of heparan sulfate for FGF-1 and FGF-2 is dramatically altered within a time frame of only 2 days, providing powerful evidence that heparan sulfates with selective effects on different FGFs also occur naturally and act as regulators of FGF. Thus, the differential binding of FGF-2 and FGF-7 to basement membranes described in this paper is a striking finding.

In summary, heparan sulfates play an important role as positive and negative regulators of FGF signaling and display specificity for different members of this growth factor family. Basement membrane heparan sulfate proteoglycans such as perlecan could serve several functions in FGF signaling. First, they may directly promote binding of FGF to adjacent epithelial or endothelial cells. Second, they may function as a storage site for FGFs, immobilizing FGF close to the location of the target cells, and last, basement membrane heparan sulfate proteoglycans may act as gatekeepers to limit access of heparin-binding growth factors to subjacent target cells. Enzymatic extracellular matrix degradation may be required before FGF complexed to active perlecan glycosaminoglycan sequences can exert its effect on target cells. This scenario has been demonstrated in tissue inflammation, where proteinases or heparanases produced by lymphocytes and macrophage-derived foam cells lead to the release of active FGF/heparan sulfate complexes.[46–48] Similar events can be triggered by invading malignant melanoma cells.[49,50] As shown in this report, mast cell heparin is abundantly present in tissues and may play an important role as a cofactor in mobilizing and activating FGFs. The importance of mast cells in angiogenesis models has indeed been known for many years.[51]

A gatekeeper role of basement membrane heparan sulfate proteoglycans is in keeping with differential binding of FGF-2 and FGF-7 reported in this paper. FGF-7 is a stromal-cell-derived paracrine growth factor produced by fibroblasts in dermis and hair follicle papillae that has to cross the basement membrane to reach its target cells, keratinocytes in the epidermis and the hair follicle.[26,27] Therefore, the inability of FGF-7 to bind to epidermal basement membrane makes sense physiologically. Conversely, capillary endothelial cells should be protected from the effects of FGF-2 to prevent unwanted neovascularization. Indeed, FGF-2 strongly binds to subendothelial basement membranes in normal tissues. Interestingly, in malignant tumors, such as infiltrating ductal carcinoma of the breast, where angiogenesis is prominent and possibly of prognostic importance,[17] no binding of FGF-2 to subendothelial basement membranes is seen. Alternative explanations, such as insufficient time to establish a recognizable extracellular matrix, also have to be considered in this situation.

Understanding the role and functions of tissue heparan sulfate proteoglycans will provide important insights into FGF signaling in physiological and pathological conditions. This knowledge is also a prerequisite for designing therapeutic approaches that are aimed at either inhibiting unwanted FGF activity or at stimulating insufficient activity.

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