Regulation of Fibroblast Growth Factor-2 Activity by Human Ovarian Tumor Endothelium

Melissa K. Whitworth,1 Alison C. Backen,1 Andrew R. Clamp,1 Godfrey Wilson,2 Rhona McVey,2 Andreas Friedl,5 Alan C. Rapraeger,5 Guido David,6 Alan McGown,3 Richard J. Slade,4 John T. Gallagher,1 and Gordon C. Jayson1

Abstract Fibroblast growth factor-2 (FGF-2) is a potent angiogenic cytokine that is dependent on heparan sulfate for its biological activity. We have investigated the relationship among heparan sulfate, FGF-2, and the signal-transducing receptors in human, advanced-stage, serous ovarian adenocarcinoma. Using a unique molecular probe, FR1c-Ap, which consisted of a soluble FGF receptor 1 isoform IIIc covalently linked to an alkaline phosphatase moiety, the distribution of heparan sulfate that had the ability to support the formation of a heparan sulfate/FGF-2/FGFR1 isoform IIIc alkaline phosphatase heparan sulfate construct complex was determined. This may be taken as a surrogate marker for the distribution of biologically active heparan sulfate and was distributed predominantly in endothelial cells and stroma but was absent from adenocarcinoma cells. In situ hybridization revealed the expression of FGFR1 mRNA in the endothelium and reverse transcription-PCR confirmed the presence of FGFR1 isoform IIIc but not isoform IIIb. The presence of FGF-2 around tumor endothelium was detected through immunohistochemistry. Double-staining techniques showed that heparan sulfate was found predominantly at the basal aspect of the endothelium and suggested that syndecan-3 might function as one of the proteoglycans involved in FGF-2 signaling in the endothelium. The data suggest that the entire extracellular signaling apparatus, consisting of FGF-2, biologically active heparan sulfate, and FGFRs capable of responding to FGF-2, is present in ovarian cancer endothelium, thereby highlighting the cytokine and its cognate receptor as potential targets for the antiangiogenic treatment of this disease.

Ovarian cancer is the commonest cause of gynecologic cancer mortality accounting for 25,000 deaths in the United States each year. Despite improvements in surgery and chemotherapy, advanced disease remains largely incurable and new treatment approaches are required. Recent data have shown that the inhibition of angiogenesis, the process of new blood vessel formation, has been associated with improved progression-free (1) and overall survival in some cancers (2). Although we have reported antitumor activity in patients with ovarian cancer treated with an antiangiogenic monoclonal antibody (3), most research in this area has targeted vascular endothelial growth factor, yet many other angiogenic cytokines are involved (4, 5).

One of the most potent angiogenic cytokines is fibroblast growth factor-2 (FGF-2), the prototype of a 23-member family of polypeptide growth factors that have been implicated in several different cancer phenotypes (6) but which in particular are involved in angiogenesis (7–10). FGFs signal through transmembrane receptor tyrosine kinases of which five types have been identified [FGF receptors (FGFR) 1-5; ref. 11].

Previous studies have investigated the response to, and expression of, FGF-2 and FGFRs in a variety of ovarian cancer cell lines. Several cell lines respond to FGF-2 with an increase in cell proliferation and FGF-2 mRNA expression (12). Levels of FGF-2 mRNA are also significantly higher in the ovarian tumors in patients with stage III and IV ovarian cancer when compared with normal ovaries (P < 0.05), suggesting that FGF-2 may be an appropriate cytokine to target in ovarian cancer (13).

Heparan sulfate is a linear glycosaminoglycan polysaccharide that consists of alternating hexuronic acid and N-substituted glucosamine residues and is a potent regulator of FGF signaling. Heparan sulfate proteoglycan (HSPG) alterations have also been observed in development and during malignant transformation (14), and detailed studies have shown that heparan sulfate undergoes specific structural changes during the transformation from human colon adenoma to carcinoma in vitro (15) leading to changes in the capacity of cells to respond to FGF-2 (16).
Both FGF-2 and its signaling receptors are characterized by their affinity and biological dependence on heparan sulfate (17–19). In view of this, a unique antiangiogenic strategy could be envisaged in which the antagonism of FGF-2 activity is affected through disruption of heparan sulfate function. However, there are no data on the relevance of this mechanism to human ovarian cancer. Here, we have used a unique molecular probe FR1c-Ap, which consists of the FGFRI IIC extracellular domain expressed as a chimera with human placental alkaline phosphatase to investigate this mechanism in situ. HSPGs have been shown to have differential abilities to support the binding of FGF-2/FR1c-AP constructs (20). Recently, these molecular probes have been used to suggest that glioma heparan sulfate can activate FGF-2 (21) and that breast carcinoma HSPGs may have a significantly greater ability to promote FR1c-AP binding than normal breast gland epithelial cells, suggesting that alterations in heparan sulfate structure occur during malignant transformation (22).

In the present study, several techniques have been combined to investigate the relationship among FGF-2, heparan sulfate, and the signal-transducing receptors in human epithelial ovarian cancer. The data suggest that the complete pericellular signaling apparatus is present in the endothelium and highlight FGF-2 as a potential target for antiangiogenic therapy in ovarian cancer.

Materials and Methods

Tissues

We conducted all immunohistochemical studies in histologically verified samples of serous epithelial ovarian cancer taken from 10 patients. Fresh material was immediately frozen in liquid nitrogen and 7-µm sections mounted on 3-amino-propyl-triethoxysilane-coated slides (Shandon Scientific Ltd., Runcorn, United Kingdom). All tissues were reviewed by a gynecologic histopathologist. The project was approved by South Manchester Local Ethics Committee and all patients gave informed consent.

Immunohistochemistry

In all immunohistochemical studies, we used normal tonsil as a control tissue as stratified squamous epithelium had been used in the development of the FR1c-AP reagent (20, 23). Heparan sulfate proteoglycan detection. The application of the 3G10 and 10E4 antibodies has been described previously (24). Sections were treated for 4 hours at 37°C with a mixture of 50% heparinase I and II [Grampian Enzymes, Orkney, United Kingdom; 5 mM in 0.5 mmol/L CaAc, 50 mmol/L NaAc, 0.1 mg/mL heparinase I and II [Grampian Enzymes, Orkney, United Kingdom; 1:200 dilution] followed by a hematoxylin counterstain.

Extensive controls were done at each step to optimize staining.

Frozen sections were cut and fixed in 4% paraformaldehyde in PBS for 10 minutes. To ensure that fixation was not acting to remove FGF-2, control sections were preincubated with FGF-2 before immersion in 4% paraformaldehyde. This exogenous FGF-2 was easily detectable. Endogenous tissue peroxidase was quenched by placing slides in PBS for 5 minutes and then plunging into 0.3% hydrogen peroxide/20% methanol in PBS for 10 minutes. Due to the detection of endogenous bionin expression in initial experiments, sections were incubated with two drops of avidin-blocking solution and two drops of biotin-blocking solution for 15 minutes each. Non-specific binding was blocked by incubating for 30 minutes with 20% normal rabbit serum. After section preparation, primary mouse anti-human FGF-2 monoclonal antibody (Serotec, Oxford, United Kingdom) or negative control (non-specific IgG) diluted in 10% normal rabbit serum was applied and the sections were incubated overnight at 4°C.

Sections were then incubated for 30 minutes at room temperature with the secondary biotinylated rabbit anti-mouse antibody (RAMBO) followed by a further 30-minute incubation, at room temperature, with 100 mL freshly prepared StreptABCComplex/horseradish peroxidase kit (DAKO). Visualization was done by incubation with 3,3′,5-diaminobenzidine for 5 minutes followed by extensive rinsing in double-distilled H2O. Between each step, slides were washed twice in PBS containing 0.05% Tween 20.

Using the above protocol, all negative controls (where different amplification steps were omitted in different control experiments) were negative and using a dilution series, endogenous FGF-2 was detected, with the strongest, specific color reaction being achieved with 1:125 anti-FGF-2 and 1:400 RAMBO.

FGFR complex reconstitution in situ

Fixation was done as described previously and sections were incubated with 20% normal swine serum in PBS (DAKO). As 6 of the 10 specimens expressed endogenous AP (as seen previously; refs. 25, 26), sections were incubated for 24 hours with rabbit anti-–placental alkaline phosphatase (rabbit anti–placental alkaline phosphatase; 1:10 dilution; Serotec). Sections were incubated then with FGF-2 (30 nmol/L; R&D Systems, Abingdon, United Kingdom) and detected with anti-FGF-2 monoclonal antibody or with FR1c-AP (100 nmol/L; ref. 23). Bound FR1c-AP was detected using a monoclonal anti–placental alkaline phosphatase antibody (Sigma-Aldrich, Poole, United Kingdom). Signals were visualized using horseradish peroxidase–conjugated rabbit anti-mouse secondary antibody.

Double staining

A double-stain technique was done using the EnVision Doublestain kit (DAKO) using a modification of the manufacturer’s protocol. Frozen sections were cut, acetone fixed (10 minutes), dried, and then immersed in PBS. Non-specific binding was prevented by incubation for 30 minutes at room temperature with 20% normal swine serum, and endogenous peroxidase was also blocked with peroxide. Sections were then incubated with the first primary antibody, 10E4, diluted 1:200 in 10% normal swine serum, for 60 minutes followed by a 30-minute incubation with horseradish peroxidase–conjugated rabbit anti-mouse secondary antibody diluted 1:200. The first epitope detection was then developed by incubating with 3,3′,5-diaminobenzidine for 5 minutes followed by extensive rinsing in double-distilled H2O. Two drops of double-stain block were then applied for 10 minutes. The second primary antibody mouse anti-human CD31 (1:250 dilution) was then applied for 30 minutes followed by a 30-minute incubation with AP-labeled polymer. The second antibody was developed by incubating for 5 minutes with Fast Red chromogen solution. After rinsing in double-distilled H2O and counterstaining with hematoxylin, sections were mounted using DAKO Faramount aqueous mount.

The double-stain dilution series was then repeated on serial sections employing murine anti-syndecan-3 monoclonal antibody (1C7 1:60 dilution) in place of 10E4. A minimum of three repeats per specimen were done.
FGFR1 in situ hybridization

Riboprobe preparation. The National Center for Biotechnology Information nucleotide-nucleotide blast program was used to identify a unique 150-bp region of FGFR1. Primers A and B were designed to allow this region of FGFR1 to be amplified by reverse transcription-PCR (RT-PCR) from normal human ovary RNA (Ambion, Inc., Austin, TX) and cloned into the transcription vector pSPT19 (Roche, Basel, Switzerland): primer A 5'-AACCGAGCCGCTGGAGGAG-3' and primer B 5'-AAAACGCAGCGCGAAGGATC-3' (Roche). RNase-free conditions were used throughout. The Digoxigenin RNA Labeling kit (Roche) was used to label the riboprobe with digoxigenin. Both sense and antisense probes were prepared, and quantification of probe labeling was carried out using the digoxigenin nucleic acid detection kit and test strips (Roche).

In situ hybridization method. Four-micron sections of paraffin-embedded tissues were mounted on charged Superfrost Plus microscope slides (BDH, Poole, United Kingdom). The sections were dewaxed in xylene, rehydrated in ethanol, soaked for 20 minutes in 0.2 mol/L HCl, and rinsed briefly in 2× SSC. Slides were equilibrated in 0.05 mol/L Tris-HCl (pH 7.4) before treating with 5 μg/mL proteinase K in 0.05 mol/L Tris-HCl (pH 7.4) at 37°C for 30 minutes. Washes were carried out in 0.2% glycine in PBS and then PBS alone before immersing in cold 0.4% paraformaldehyde in PBS for 20 minutes. GeneFrames (Thermo Electron Corp., Hemel Hempstead, United Kingdom) were applied to each slide to create the in situ reaction chamber around the tissue section. Riboprobe (600 ng-1,800 ng/μL) was added to 125 μL hybridization solution containing 5 μL herring sperm (Sigma, Gillingham, United Kingdom). The hybridization mix was denatured before use, and hybridization was carried out at 30°C overnight. The slides were washed in 4× SSC for 30 minutes at room temperature, 2× SSC for 10 minutes at 37°C, 0.2× SSC for 1 hour at 70°C, and TBS for 5 minutes at room temperature. The slides were then treated with 10% normal goat serum (DAKO) in TBS for 1 hour at room temperature. The anti-digoxigenin alkaline phosphatase antibody (Roche; 1:1,500 dilution) was added to 1% goat serum in TBS, applied onto the sections, and incubated overnight at room temperature. The slides were washed twice in TBS and equilibrated in 0.1 mol/L Tris (pH 9.5), 0.1 mol/L NaCl, and 50 mmol/L MgCl2. Nitroblue tetrazolium (4.5 μL/μL; Roche), 3.5 μL/μL 5-bromo-4-chloro-3-indolyl phosphate (Roche), and 25 μL levamisole (DAKO) in 0.1 mol/L Tris base, 0.1 mol/L NaCl, and 50 mmol/L MgCl2-6 H2O (pH 9.5) were added to the slides and incubated at room temperature until the color reaction developed. The sections were fixed in an aqueous mountant (DAKO) and examined with an AxioSkop microscope (Zeiss, Welwyn Garden City, United Kingdom) using a ×40 plan neofluar 1.35 numerical aperture oil immersion objective lens. Image acquisition was carried out using a Progress C14 camera (Jenoptik, Jena, Germany) via Adobe PhotoShop 7.

FGFR1 receptor analysis

RNA was isolated from several frozen specimens of serous adenocarcinoma of the ovary using the RNeasy kit (Qiagen, Crawley, United Kingdom). The presence or absence of FGFR1 isoforms IIIb and IIIc was investigated using the RT-PCR technique described in ref. 28.

Slide evaluation

Results were graded on an intensity scale from – (no staining beyond background) to +++ (strong staining). The predominant signal intensities seen in the majority of epithelial, endothelial, and stromal cells are summarized and illustrated in each tumor section. Analysis was done in a blinded fashion by two independent investigators. Using a Wilcoxon matched-pairs signed rank test, we showed that there was no significant interobserver variation.

Results

We investigated samples of primary serous epithelial ovarian cancer taken from 10 patients. One tumor was well differentiated, four moderately differentiated, and five poorly differentiated. Two of the poorly differentiated samples originated from patients with International Federation of Gynecology and Obstetrics stage IV disease, whereas the other eight patients had International Federation of Gynecology and Obstetrics stage IIIc disease. There were no stage-related or differentiation-related differences between these samples with respect to the following analyses.

Heparan sulfate analysis. Three antibodies were used to provide a map of heparan sulfate (3G10 and 10E4) and syndecan-3 (1C7) within each tumor specimen. Syndecan-3 was of particular interest, as preliminary data had suggested this was present in the endothelium (24). 3G10, which recognizes the unsaturated uronate groups that remain after heparinase digestion regardless of core protein, showed total tissue heparan sulfate distribution. Analysis revealed mild staining of carcinoma cells and stroma (cells and/or basement membrane) but strong staining of endothelial cells (Fig. 1A; Table 1). No 3G10 staining was seen without heparinase digestion (data not shown). There was some heterogeneity of staining within each cell type. For example, 75% to 100% of stromal cells stained in the majority of specimens (Table 1). Sulfated heparan sulfate chain expression, assessed through 10E4 binding, showed stromal and endothelial cell expression, with ~75% of each cell type staining. There was minimal binding to adenocarcinoma cells.

Endogenous fibroblast growth factor-2 protein localization. FGF-2 was expressed at low levels and the signal could not be visualized using a monoclonal peroxidase-conjugated rabbit anti-mouse secondary antibody (Fig. 1B). However, endogenous FGF-2 was successfully visualized using a three-layer technique (see Materials and Methods). FGF-2 was present in ~80% of adenocarcinoma cells and 90% of stromal cells but at low levels in epithelial and stromal cells with moderate expression in endothelial cells (Fig. 1C).

Specific in situ detection of fibroblast growth factor-2–binding heparan sulfates. The ability of endogenous heparan sulfate to bind FGF-2 was investigated by adding exogenous cytokine and detecting its presence with anti-FGF-2 antibodies. FGF-2-binding potential was detected within the stroma, in carcinoma cells and around stromal blood vessels and endothelial cells. The strongest staining was noted in endothelial cells and the subendothelial basement membrane and endothelial cells of larger capillaries (Fig. 1D). Interestingly, the distribution of staining in tissues treated with FGF-2 and then probed with anti-FGF-2 antibodies exactly matched the staining distribution of staining in tissues treated with FGF-2 and then detecting its presence with anti-FGF-2 antibodies. FGF-2 was expressed at low levels and the signal could not be visualized using a monoclonal peroxidase-conjugated rabbit anti-mouse secondary antibody (Fig. 1B). However, endogenous FGF-2 was successfully visualized using a three-layer technique (see Materials and Methods). FGF-2 was present in ~80% of adenocarcinoma cells and 90% of stromal cells but at low levels in epithelial and stromal cells with moderate expression in endothelial cells (Fig. 1C).
serous adenocarcinoma of the ovary and that heparan sulfate has the ability to bind FGF-2, the critical question is whether ovarian cancer heparan sulfate recognizes both FGF-2 and the receptor, thereby promoting and/or stabilizing their assembly into an active conformation (29). Although issues of receptor dimerization could not be assessed, the molecular probe FR1c-AP was used to identify heparan sulfate that was able to form and/or support a complex between receptor, FGF-2, and heparan sulfate, a process termed ligand and carbohydrate engagement. In effect, the probe was used as a surrogate marker for biologically active heparan sulfate.

Fig. 1. Expression of the extracellular signaling apparatus of FGF-2 in human serous adenocarcinoma of the ovary, including the in situ binding of FGF-2 and FR1c-AP-soluble receptor alkaline phosphatase fusion protein. A, detection of total HSPGs with antibody 3G10 on heparinase-digested section. HSPGs are widely expressed with moderate membrane staining of carcinoma cells and stroma and strong staining of endothelial cells. Magnification, ×40. B, endogenous FGF-2 could not be detected using an anti-FGF-2 primary antibody with a peroxidase-conjugated secondary antibody. FGF-2 was found to be expressed at low levels in epithelial and stromal cells with moderate expression in endothelial cells. Magnification, ×40. C, endogenous FGF-2 was successfully detected using an anti-FGF-2 primary antibody with a biotinylated secondary antibody. FGF-2 was bound to be expressed at low levels in epithelial and stromal cells with moderate expression in endothelial cells. Magnification, ×40. D, binding of FGF-2 (30 nmol/L) to human serous adenocarcinoma of the ovary. Bound FGF-2 is detected with anti-FGF-2 antibody. This identifies total FGF-2 binding and resulted in moderate to strong staining of endothelial cells, mild staining in epithelial cells, and moderate staining in stromal cells. E, binding of FR1c-AP (100 nmol/L) to normal human stratified squamous epithelium of tonsil. Tissue sections were preincubated with FGF-2 before FR1c-AP exposure. Section shows moderate staining of basement membrane and stromal cells and strong staining of endothelial cells. F, binding of FR1c-AP (100 nmol/L) to serous adenocarcinoma preincubated with FGF-2 (30 nmol/L) without heparinase treatment. Bound FR1c-AP is detected with anti-AP antibody and visualized with horseradish peroxidase. This resulted in weak diffuse staining of stromal cells, no staining of epithelial cells, but moderate to strong staining of endothelial cells. G, in situ hybridization for FGFR1 antisense riboprobe produces the positive experimental result and shows strong FGFR1 RNA expression in endothelial and stromal cells and moderate RNA expression in carcinoma cells. H, in situ hybridization for FGFR1 sense riboprobe provides the negative experimental control. Small (I) and large (J) capillary double stained for heparan sulfate (10E4 antibody; brown) and CD31 (red). Magnification, ×60. Large (K) and small (L) capillary double stained for syndecan-3 (1C7; brown) and CD31 (red).

FR1c-AP staining in normal human tonsil, as this probe was developed using stratified squamous epithelia (ref. 20; Fig. 1E). This tissue was subsequently used as a positive control for further experiments. In the absence of FGF-2 or if tissues were pretreated with heparinases, FR1c-AP failed to bind to heparan sulfate in human serous adenocarcinoma of the ovary (data not shown). In the presence of FGF-2, FR1c-AP bound FGF-2-heparan sulfate in a pattern restricted to stromal and endothelial cells (Fig. 1F). In general, the binding to fibroblasts and smooth muscle cells of the stroma was seen in 75% of cells where it was weak. Interestingly, 80% of adenocarcinoma cells did not bind FR1c-AP and those that did were stained with mild intensity. Importantly, the distribution of FR1c-AP binding was
human cancer biology

Table 1. Relative staining intensities for different cell lineages in the ovarian cancer specimens

<table>
<thead>
<tr>
<th>Adenocarcinoma cells</th>
<th>Stromal cells</th>
<th>Endothelial cells</th>
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</thead>
<tbody>
<tr>
<td>Total heparan sulfate</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sulfated heparan sulfate</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Syndecan-3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Endogenous FGF-2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FR1c (in situ hybridization)</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>FR1c-AP</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

NOTE: Comparisons were made between different cell lineages within individual tumor sections investigated for the expression of a particular factor.

significantly different from that of total heparan sulfate (3G10), sulfated heparan sulfate (10E4), and the widespread binding of exogenous FGF-2, suggesting that only a subset of heparan sulfate was capable of assembling a trimolecular signaling complex.

Within ovarian serous adenocarcinoma, the subset of FGF-2-binding heparan sulfate that is capable of activating FGF-2 is expressed predominantly by endothelial cells as well as the stroma. The location of FGF-2-activating heparan sulfate on endothelial cells is of particular interest when considering the role of heparan sulfate/FGF-2 in angiogenesis in serous adenocarcinoma of the ovary.

*Fibroblast growth factor receptor 1 RNA expression in situ hybridization.* FGF1 mRNA expression was investigated using *in situ* hybridization. Specific antisense and sense probes were generated and hybridized to several sections of serous ovarian adenocarcinoma. The antisense riboprobe produces purple/blue 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium product (Fig. 1G), suggesting that the FGF1 mRNA is present at high levels in the cytoplasm of endothelial and stromal cells and moderately in carcinoma cells. The antiparallel sense probe acts as the negative control (Fig. 1H).

FGF1 is generated as the IIIa, IIIb, or IIIc isoforms. The IIIa variant contains only two immunoglobulin domains and is a soluble nonsignaling protein, whereas IIIb and IIIc vary in the exons used to code for the second half of the third immunoglobulin loop creating receptors that are expressed in the epithelium and mesenchyme, respectively, and which differ in their ligand-binding potential with respect to FGF-2, FGF-4, FGF-7, and FGF-10 (30). These changes seem to have clinical significance. In prostate cancer cells, progression is associated with a switch from IIIb to IIIc, which is accompanied by increased responsiveness to FGF-2 (31). To differentiate between the IIIb and IIIc isoforms of FGF1, the amplified RT-PCR product (209 bp) of tumor tissue mRNA was treated with appropriate restriction enzymes and examined by electrophoresis. BsaAI (Fig. 2, lane 2) cleaved the RT-PCR product to yield a molecular weight product consistent with FR1c. Degradation of RT-PCR product with *Hae*III again produced a small molecular weight shift consistent only with the IIIc isoform (Fig. 2, lane 3). Products of the IIIb isoform would not be digested by these enzymes, implying that all cell types in the tumor (endothelial, stromal, and malignant) expressed the IIIc form of the receptor (32).

**Double staining.** In Table 1, we have summarized the relative intensities of stains for each of the cell lineages when compared within each tumor specimen. The data represent the most frequently observed relative staining patterns and highlight the endothelium as the site where the extracellular signaling apparatus is most intensively expressed. We wanted to investigate this issue further, and Fig. 1I and J show double-stain results in which the heparan sulfate (brown) is seen at the basal aspect of the endothelial cells, identified through staining for CD31 (red). As we had previously detected syndecan-3, conventionally believed to be a neuronal proteoglycan, in ovarian cancer (24), we used the same double-staining technique to assess its expression in tumor blood vessels. We found that syndecan-3 (brown in Fig. 1K and L) is expressed in the same distribution as the endothelial heparan sulfate in both large (Fig. 1K) and small (Fig. 1L) capillaries.

**Discussion**

FGF-2 is a potent angiogenic cytokine, but its contribution to ovarian cancer pathogenesis is unclear. The work described here focuses on the distribution of total heparan sulfate, FGF-2-binding heparan sulfate, and the presence and cellular distribution of heparan sulfate that has the ability to engage or stabilize FGF-2 and the signaling receptor simultaneously, suggesting that this heparan sulfate has the potential to mediate or promote FGF-2 cell signaling. *In situ* hybridization showed the distribution of FGR1 mRNA, and RT-PCR showed the presence of FR1c.
The relatively widespread presence of the minimum binding of FGF-2 and the more restricted distribution of the dimerization, it is likely that binding of the receptor construct forms a trimolecular complex with FGF-2 and heparan sulfate. To gather data about the functional role of heparan sulfate, we used the FR1c-AP molecular probe that forms a trimeric complex with FGF-2 and heparan sulfate. Although FR1c-AP does not address issues of receptor dimerization, it is likely that binding of the receptor construct reflects the presence of a mitogenically active FGF-2-heparan sulfate complex. The difference between the widespread binding of FGF-2 and the more restricted distribution of the FR1c-AP-FGF-2-heparan sulfate complexes is probably due to the relatively widespread presence of the minimum binding sequence for FGF-2 (N-sulfated glucosamine/iduronic acid 2-O-sulfate/N-sulfated glucosamine/iduronic acid/N-sulfated glucosamine ref. 38), whereas the FGF-2-activating sequence, which requires 6-O-sulfate groups (39), has a more restricted distribution. An alternative possibility, suggested by recent data, is that the heparan sulfate sequence required for receptor-cytokine complex assembly is only rarely found and is distinct from that required to bind the cytokine or receptor independently (29).

Our data suggest that the entire FGF-2 cell surface signaling apparatus is present around the ovarian cancer endothelium. The question is whether one component of the apparatus is more critical than another. Using the three-layer immunohistochemistry approach, we showed that endogenous FGF-2 is widely distributed, although the highest concentration was found around the endothelium. We examined the distribution of FGFR1 using in situ hybridization and RT-PCR and found that FR1c was present in the stroma and endothelium, consistent with other data (28, 35), indicating that growth factor and receptor isoform distribution are not critical. In contrast, the fact that there was a significant increase in expression of total heparan sulfate, sulfated heparan sulfate, and FR1c-AP binding around the endothelium when compared with other cell lineages in the tumor suggests that heparan sulfate may be the critical regulator of FGF-2 activity. The RT-PCR data are particularly important, as they show that the receptor expressed in the ovarian cancer tissue is the same as that in the molecular probe (i.e., FR1c). Thus, it is reasonable to infer that the findings obtained with FR1c-AP are physiologically relevant.

Our data strongly suggest that tumor-associated heparan sulfate, especially that in close proximity to the vasculature, is able to promote FGF-2 signaling and in this way contribute to the progressive angiogenesis of the expanding tumor. Ovarian cancer-associated vasculature produces all three elements of the FGF signaling system, indicating that heparan sulfate-driven FGF-2 angiogenesis is a potential target for drug intervention. As FGFRs have been shown in adenocarcinoma cells (Fig. 1A and G), the data also suggest that FGF inhibitors might also have antitumor activity.

No relationship was observed among microvessel density, size of vessels, and codistribution of the signaling complex, implying that these complexes might be more generally expressed in the vasculature. On the other hand, vessels present in normal tissues do not bind FR1c-AP, suggesting that vessels in tumors may undergo a regional change in favor of FGF-2 (20).

In a previous study, we had detected syndecan-3 and to a lesser extent syndecan-1 around ovarian cancer endothelium (24). Here, we have shown that in relation to CD31-expressing endothelial cells, both heparan sulfate and syndecan-3, are expressed at the basal parts of the endothelial cells consistent with other data (40, 41). Although the data do not prove that syndecan-3 bears the relevant heparan sulfate, it suggests that further investigation of this proteoglycan is warranted. Most antiangiogenic drugs developed to date have focused on the inhibition of vascular endothelial growth factor. In the light of these experiments, it may be appropriate to investigate the effects of inhibitors of FGF-2 in ovarian cancer, either by direct inhibition (anti-FGF-2 antibodies or receptor kinase inhibitors) or through antagonists of heparan sulfate. However, because heparan sulfate modulates both vascular endothelial growth factor and FGF-2 signaling, properly designed heparan sulfate mimetics might be capable of disrupting several crucial angiogenic pathways simultaneously.

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