Acidic Fibroblast Growth Factor (FGF-1) and FGF Receptor 1 Signaling in Human Y79 Retinoblastoma

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Objectives: Fibroblast growth factors (FGFs) represent potent effectors and play essential roles in both normal development and many pathological processes. Little is known about their possible implication in retinoblastoma growth. We sought to examine FGF high- and low-affinity receptor (FGFR) expression, activation of FGFR1 by acidic FGF (FGF-1), and proliferative effects on Y79 cells.

Methods: Expression of FGFR1 to FGFR4 was screened in Y79 cells by means of immunochemical and reverse transcriptase polymerase chain reaction techniques. Tyrosine phosphorylation of FGFR1 induced by FGF was examined by immunoprecipitation after stimulation with FGF-1 in the presence or absence of heparin. Retinoblastoma proliferation was monitored by radiolabeled thymidine incorporation or a vital dye–based assay, after addition of FGF-1 with or without inclusion of a specific FGFR1 neutralizing antibody or FGFR1 antisense oligonucleotides. Low-affinity heparan sulfate proteoglycan coreceptors were blocked through sodium chlorate or heparinase treatment of Y79 cells.

Results: Y79 retinoblastoma expressed all 4 FGFRs, at both the protein and messenger RNA levels. The FGFR1 was differentially phosphorylated in a time- and heparin-dependent manner by FGF-1. Proliferation of Y79 cells induced by FGF-1 was entirely mediated by FGFR1, since inclusion of specific neutralizing antibodies or antisense oligonucleotides completely prevented tumor cell multiplication. Finally, FGF-1–induced proliferation was dependent on the presence and sulfation of heparan sulfate proteoglycan.

Conclusions: Y79 retinoblastoma expresses all 4 FGFRs, but FGFR1 activation entirely accounts for FGF-1–driven cell proliferation.

Clinical Relevance: These studies demonstrate a role for the FGF-1/FGFR1 pathway in retinoblastoma proliferation, and may contribute to developing therapeutic strategies to limit retinoblastoma growth.

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The biological activities of FGFs are mediated by 5 distinct but closely homologous high-affinity cell-surface tyrosine kinase receptors (designated FGFR1-FGFR5). As for other tyrosine kinase receptors, ligands induce receptor clustering and autophosphorylation followed by recruitment of intracellular substrates to activate gene expression. Besides interacting with high-affinity FGFRs, FGFs bind to the glycosaminoglycan heparin and to cell-surface and extracellular matrix heparan sulfate proteoglycans (HSPGs). The HSPGs are synthesized within the retina and have recently been implicated in modulating binding of pigment epithelium–derived factor to Y79 cells, and are therefore placed to play potential roles in modulating FGF signaling in both normal physiology and pathology.

Y79 cells have been used as model systems to study roles of some growth factors, and apart from a single study demonstrating the synthesis of FGFR2, have not been used to investigate the expression or effects of the FGF/FGFR signaling pathways. The present study reports the robust expression of FGFR within Y79 retinoblastoma, and through the use of specific neutralizing antibodies and antisense oligonucleotides, we show that FGFR1 controls FGF-1–stimulated proliferation, showing a possible role in tumor progression.

METHODS

CELL CULTURE

Y79 cells were purchased from the European Collection of Cell Cultures (CAMR, Salisbury, England) and maintained in Roswell Park Memorial Institute (RPMI) medium with 1-glutamine (Gibco Life Technologies SARL, Cergy Pontoise, France) supplemented with 15% fetal bovine serum (Gibco Life Technologies SARL), penicillin, 100 U/mL; and streptomycin, 100 µg/mL. The incubation medium was changed twice weekly. Cells were passaged for experimentation on reaching confluence.

IMMUNOCYTOCHEMISTRY

Y79 cultures were fixed for 15 minutes in 4% paraformaldehyde, rinsed in phosphate-buffered saline (PBS) (0.1M, pH 7.3), and centrifuged at 900 rpm onto polylysine-treated coverslips. Cells were permeabilized with 0.1% Triton X-100 in PBS for 5 minutes, then blocked for 10 minutes in PBS containing 0.1% bovine serum albumin and 0.2% Tween 20 (buffer A). Cells were incubated for 2 hours with anti-FGFR1, anti-FGFR2, anti-FGFR3, or anti-FGFR4 rabbit polyclonal antibodies (No. sc 121, 122, 123, and 124; Santa Cruz Biotechnology Inc, Santa Cruz, Calif) diluted 1:100 in buffer A. In parallel, primary antibodies that had been preabsorbed with the corresponding immunizing peptides (Santa Cruz Biotechnology Inc) were used to show antibody specificity. After washing, antibody binding was visualized with fluorescent tagged secondary antibody (donkey anti-rabbit IgG/Alexa594; Molecular Probes Ltd, Eugene, Ore), 10 µg/mL in buffer A for 1 hour. Cells were incubated in 4,6-diamidino-2-phenylindole (Sigma Aldrich Life Sciences, St Louis, Mo) to demonstrate nuclear staining. Coverslips were washed, mounted in PBS-glycerol 1:1, and observed with a photomicroscope (Nikon Optiphot 2; Nikon, Tokyo, Japan) equipped with differential interference contrast objectives.

WESTERN BLOTTING

Western blotting studies were performed essentially as previously published. Y79 cells were pellet centrifugation (900 rpm for 5 minutes) and suspended in lysis buffer (100mM Tris, 150mM sodium chloride, 1mM EDTA, 1mM sodium vanadate, 1mM sodium fluoride, 0.2M phenylmethanesulfonyl fluoride, 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, and protease inhibitor cocktail, 100 µL/sample), to which was added an equal volume of Laemml buffer. A human breast cancer cell line (SUM44PE, generous gift of S. Ethier, PhD, University of Michigan Breast Cell/Tissue Bank and Database, Ann Arbor) that overexpresses FGFR1 was also collected as a positive control.

Table. Primer Sequences Used for FGFR Screening

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer Pairs (5’ , 3’)</th>
<th>Expected Size, Base Pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGFR1</td>
<td>AAGGTCCGTTATGGCACCT</td>
<td>297</td>
</tr>
<tr>
<td>FGFR2</td>
<td>CAGTTTGTCTGGGCACCACCT</td>
<td>244</td>
</tr>
<tr>
<td>FGFR3</td>
<td>GGAAGACGGACGCTAAAT</td>
<td>270</td>
</tr>
<tr>
<td>FGFR4</td>
<td>CAGTGGCTACAGGATGGA</td>
<td>149</td>
</tr>
</tbody>
</table>

St Louis, Mo) to demonstrate nuclear staining, Coverslips were washed, mounted in PBS-glycerol 1:1, and observed with a photomicroscope (Nikon Optiphot 2; Nikon, Tokyo, Japan) equipped with differential interference contrast objectives.

FGFR MESSENGER RNA EXPRESSION IN Y79 CELLS

Total RNA was extracted from cell cultures by the acid guanidium thiocyanate–phenol–chloroform extraction method. Fragments of DNA of B-actin and FGFR1 to FGFR4 were amplified from total RNA by reverse transcriptase polymerase chain reaction (RT-PCR) with the use of specific primers (Table). After 35 cycles of amplification according to the manufacturer’s specifications (Promega, Lyon, France), PCR products were separated by electrophoresis on 1% agarose gels and visualized by ethidium bromide staining.

TYROSYNE PHOSPHORYLATION OF FGFR1: FGF-1 STIMULATION, IMMUNOPRECIPITATION, AND WESTERN BLOTTING

Y79 cells (10⁶ cells/mL of RPMI medium) were left unstimulated or incubated with recombinant FGF-1 (R&D Systems, Abing-
don, England) (100 ng/mL) for 0, 1, 2, 5, 10, and 30 minutes at 37°C. In a second set of experiments, Y79 cells were incubated with a single concentration (100 ng/mL) of FGFR-1 in the absence or presence of 2 different concentrations of heparin, 10 ng/mL and 10 µg/mL. Subsequent to incubation, cells were immediately frozen in liquid nitrogen, thawed on ice, and centrifuged (900g for 5 minutes). After removing a small aliquot for protein measurement, pellets were resuspended in cold lysis buffer (100mM TRIS, 150mM sodium chloride, 1mM EDTA, 1mM sodium vanadate, 1mM sodium fluoride, 0.2M phenylmethylsulfonyl fluoride, 0.1% SDS, 1% Triton X-100, and protease inhibitor cocktail, 100 µL per sample) and stored on crushed ice.

Antibody-tagged beads were prepared by incubating anti-FGFR1 (No. sc 121, 20 µg in 100 µL of lysis buffer) with protein A–agarose beads (Sigma Aldrich SARL, Saint Quentin Fallavier, France) (200 µL of slurry, prerinsed twice in PBS and lysis buffer) for 90 minutes with gentle agitation at 4°C. After rinsing and centrifugation to remove unbound antibody (10,000 rpm, 2 × 5 seconds), antibody-coated beads were incubated with Y79 lysates (1 mg of total protein in 200 µL of lysis buffer) overnight with gentle agitation at 4°C. Beads were washed in excess lysis buffer and sedimented (10,000 rpm, 3 × 5 seconds), then resuspended in 10 µL of lysis buffer to which was added an equal volume of Laemmli buffer. Solubilized proteins were separated by 10% SDS-PAGE, and proteins were transferred to nitrocellulose membranes and blocked with TBS, 5% dry milk, 3% bovine serum albumin, and 0.2% Tween 20 for 1 hour at room temperature, then incubated with mouse monoclonal phosphotyrosine antibody (Upstate Biotechnology Inc, Lake Placid, NY) (0.1 µg/mL) in TBS and 0.2% Tween 20 overnight at 4°C. Bound primary antibody was detected by means of peroxidase-conjugated goat anti–mouse secondary antibody (Jackson Immunoresearch Laboratories), 1:15,000 dilution in TBS and 0.2% Tween 20.

EFFECT OF FGF-1 AND HEPARIN ON Y79 CELL PROLIFERATION

Cells were seeded at an initial density of 10⁵/mL in serum-free RPMI medium to which FGF-1 was added directly; this was tested at a wide range of dilutions (10 pg/mL to 10 µg/mL), and cell numbers were determined after 3 days in vitro. Cell numbers were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay using the nonradioactive cell proliferation assay outlined earlier. Cell numbers were assayed by the cell titer nonradioactive cell proliferation assay (Promega), by the addition of a premixed optimized 15-µL dye solution to 96-well tissue culture plates containing living cells in each well was determined by measuring the optimized 15-µL dye solution was then added to 9.5 mL of scintillation cocktail and counted by means of a scintillation spectrometer (Beckman Coulter, Inc, Fullerton, Calif). All determinations were performed from a minimum of triplicate independent experiments.

ANTIGENIC Neutralization of FGFR1 Activation

A specific FGFR1 neutralizing antibody (MAB125; Chemicon International Inc, Temecula, Calif) was used to block FGF-1 signaling through this receptor. For phosphorylation studies, cells were preincubated for 3 hours in MAB125 (used at 1 and 10 µg/mL), then treated with a single dose of FGF-1 (100 ng/mL) for 5 minutes, lysed, and immunoprecipitated with anti-FGFR1 antibody as described in the preceding section. Samples were loaded onto SDS-PAGE and transferred to nitrocellulose membranes, after which they were subjected to phosphotyrosine immunoblotting by the same procedures. For cell proliferation studies, Y79 cells were grown for 3 days in the presence of blocking antibody (1 and 10 µg/mL) or nonspecific IgG (10 µg/mL), then cell numbers were assayed by the cell titer nonradioactive cell proliferation assay outlined earlier.

ANTIGENIC SENSE Oligonucleotide Administration

The FGFR1 function was also probed through the use of antisense oligonucleotide technology. To control for nucleotide accessibility, Y79 cells were plated at 10⁶ cells per well in RPMI medium supplemented with 5% serum. Fluorescein-labeled phosphorothioate oligonucleotides (Biognostik, Göttingen, Germany) (1µM) were added at different time points (0, 1, 2, 4, 8, 24, and 48 hours), and cells were then fixed with 4% paraformaldehyde for 15 minutes. Fixed cells were washed in PBS, centrifuged onto coverslips, and examined under epifluorescence illumination on a photomicroscope (Nikon Optiphot 2). Y79 cells were plated at 10⁵/mL in serum-supplemented medium (RPMI medium, 5% fetal bovine serum). After 8 hours, cells were treated with phosphorothioate-modified antisense nucleotide (FGFR1-AS) or randomized-sequence phosphorothioate oligonucleotide (FGFR1-S) (Biognostik). Oligonucleotides were used at a final concentration of 2µM, being added to cells again after 3 and 5 days. Cultures were maintained for 7 days; one half of each sample was taken for Western blotting and the other half was stimulated by FGF-1 (10 ng/mL). Samples for Western blotting were solubilized in lysis buffer as described already, migrated on SDS-PAGE, transferred to nitrocellulose, and blotted with anti-FGFR1 antibody. For the remaining dishes, after 24 hours in culture, tritiated thymidine (Amersham Biosciences Ltd, Little Chalfont, England), 3 µCi/mL (0.11 MBq/mL), was added to each sample and incubated for 4 hours at 37°C. Cultures were washed 3 times with PBS to remove excess radioactive probe, and incorporated tritiated thymidine was solubilized with 500 µL of sodium hydroxide (0.1M) for 4 hours at room temperature. This solution was then added to 9.5 mL of scintillation cocktail and counted by means of a scintillation spectrometer (Beckman Coulter, Inc, Fullerton, Calif). All determinations were performed from a minimum of triplicate independent experiments.

PERTURBATION OF HEPARAN SULFATE PROTEOGLYCAN SYNTHESIS AND EFFECTS OF HEPARIN

Sodium chloride is a competitive inhibitor of adenylyltransferase and inhibits sulfation of glycoconjugates. Cells were cultured for 24 hours in RPMI medium supplemented with 0.1% FBS containing 30mM sodium chloride. Medium was changed to serum-free RPMI medium with 30mM sodium chloride for an additional 24 hours, and then cells were trypsinized (0.09% trypsin, 1:250, 5 minutes at 37°C) and replaced in RPMI medium with 30mM sodium chloride. In control experiments, cells were cultured with RPMI medium alone and were not trypsinized. Cells were then stimulated for 3 days by FGF-1 (10 ng/mL) with or without heparin (10 ng/mL or 10 µg/mL) (Sigma Aldrich SARL).

For enzymatic degradation experiments, pretreatment of cells with 30mM sodium chloride was performed as in the preceding paragraph, then cultures were digested with protease-free heparinase III (Sigma-Aldrich SARL) for 2 hours at 37°C in RPMI medium. Typically, 0.2 U/mL of heparinase III was used for 10⁶ cells. In control experiments, cells were trypsinized before stimulation. Cells were then cultured in RPMI medium with 30mM sodium chloride and trypsinized (0.09% trypsin, 1:250, 5 minutes at 37°C) and replaced in RPMI medium with 30mM sodium chloride.
sodium chlorate and stimulated by FGF-1 as in the preceding paragraph.

STATISTICS

Each figure shows the results of experiments repeated a minimum of 3 times, with each data point in duplicate or triplicate. All statistical analyses were performed with the 2-tailed t test for 2 independent populations. A value of \( P < .05 \) was considered statistically significant.

RESULTS

EXPRESSION OF FGFR IN Y79 CELLS

Y79 cells were immunolabeled with antibodies directed against the different forms of FGFR1 to FGFR4. Cell-surface staining was observed for FGFR1 and FGFR4 (Figure 1A, B, G, and H) and was completely abolished by preadsorption of FGFR1 antibody with the immunizing peptide (Figure 1I and J). There was also staining with antibodies against FGFR2 (Figure 1C and D) and FGFR3 (Figure 1E and F), although this appeared more diffuse. As for FGFR1, staining for each specific antibody was completely blocked by preadsorption with the corresponding competing peptide (data not shown).

Immunoblotting studies using the same antibodies gave largely similar results. Positive immunoreactive bands were observed for FGFR1, FGFR2, and FGFR4 in all 3 samples, showing different molecular masses. For FGFR1, Y79 cells exhibited strongly immunoreactive bands at approximately 120 and 65 kDa, with additional lower mass forms. There was also a more faintly stained band at 80 kDa. These same bands were also observed in breast cancer cells, with additional bands at approximately 130, 80, and 70 kDa. Human retina showed the 65-kDa band, as well as lower mass forms, but bands could not be visualized at higher masses (Figure 2A). The FGFR2 showed a band at approximately 90 kDa for all 3 samples, with Y79 cells showing a band at 130 kDa and breast cancer cells and a human retina band at 65 kDa (Figure 2B). No detectable immunoreactivity was observed for FGFR3 in any of the 3 samples (Figure 2C). The FGFR4 immunoreactivity was prominent in Y79 and breast cancer cells, and visible at greater than 240, 130, and 80 kDa, with additional fainter bands. The FGFR4 immunoreactivity in human retina was visible at greater than 240 and 70 kDa, with faint bands in between (Figure 2D).

To confirm FGFR expression in Y79 cells, RT-PCR amplification of transcripts for FGFR1 to FGFR4 was performed (Figure 3). Specific bands were obtained for each receptor, at the expected molecular weight for each primer pair (see Table).

TYROSYL PHOSPHORYLATION OF FGFR1 IN Y79 CELLS

To determine the kinetics of FGF-1–induced phosphorylation, Y79 cells were stimulated for increasing times with a single concentration of FGF-1, known to bind efficiently to FGFR1. Antiphosphotyrosine blotting of FGFR1-immunoprecipitated protein showed a single band with a molecular mass of 80 kDa, with a minor additional band of 70 kDa observed only 5 minutes after stimulation (Figure 4A). Signal could be detected already 1 minute after stimulation, becoming maximal around 5 minutes, decreasing by 10 minutes, and being undetectable by 30 minutes.

Heparin has been strongly implicated in modulating FGFR activation, and the effects of heparin alone or in addition to FGF-1 on tyrosyl phosphorylation of FGFR1 were examined. On the basis of the kinetic profile obtained for FGF-1, Y79 cells were stimulated for 5 minutes with heparin alone (10 ng/mL), FGF-1 alone (100 ng/mL), and FGF-1 (100 ng/mL) plus heparin at 10 ng/mL or 10 µg/mL. No phosphotyrosine immunolabeling was
The FGF-1–induced stimulation was already significantly decreased at higher doses (Figure 5A). When used at 1 µg/mL, MAB125 had no observable effect on FGF-1–stimulated cells (not significantly different from controls). In contrast, at higher heparin concentrations, the mitogenic response was progressively inhibited to below untreated baseline levels. Heparin alone completely abolished phosphorylation at 10 µg/mL. Figure 5B shows that at concentrations from 2 to 50 ng/mL, heparin showed a weak additive effect on FGF-1–stimulated cells (not significantly different from controls). In contrast, at higher heparin concentrations, the mitogenic response was progressively inhibited to below untreated baseline levels. Heparin alone did not stimulate proliferation.

PROLIFERATIVE RESPONSE TO FGF-1 ENTIRELY MEDIATED BY FGFR1

To determine whether FGF-1–induced proliferation was mediated by FGFR1, signaling through this receptor was inhibited in Y79 cultures by means of a specific neutralizing antibody, MAB125. The specificity and activity of MAB125 were verified by immunoprecipitation and immunoblotting with antiphosphotyrosine antibodies. The MAB125 at 1 µg/mL reduced FGF-1–induced tyrosyl phosphorylation of FGFR1 by approximately 50% and completely abolished phosphorylation at 10 µg/mL (Figure 6A). Cultures were grown for 3 days with either no growth factor, FGF-1 alone, FGF-1 and MAB125, or FGF-1 and nonimmune IgG. The FGF-1 induced a significant increase in proliferation as seen earlier (Figure 6B). When used at 1 µg/mL, MAB125 had no effect on this proliferation, consistent with the incomplete inhibition of FGFR1 activation at this concentra-
Stimulation of Y79 proliferation by fibroblast growth factor 1 (FGF-1). A, FGF-1 showed dose-dependent proliferation of Y79 after 3 days in vitro. Values obtained for cell proliferation at doses greater than 300 pg/mL were all significantly greater \((P<.05)\) than those obtained for untreated controls and doses less than 300 pg/mL. B, Dose-dependent effects of heparin on FGF-1–induced proliferation of Y79. A fixed dose of FGF-1 (4 ng/mL, corresponding to the half-maximal effective dose calculated from panel A as the median between baseline and maximal stimulation) was supplemented with varying heparin concentrations. Values obtained for heparin doses at and greater than 3 µg/mL were all significantly lower \((P<.05)\) than those obtained for untreated controls and doses equal to or less than 1 µg/mL. Each data point represents average \((\text{mean} \pm \text{SEM})\) of triplicate wells from 3 independent experiments.

A different approach to verify the implication of FGFR1 in Y79 mitogenic pathways concerned the use of antisense oligonucleotides. We previously ensured that fluorescence oligonucleotide constructs were able to enter cells in a time- and dose-dependent manner, and that cells remained viable after oligonucleotide incorporation (data not shown). Samples treated with either sense (S) or antisense (AS) oligonucleotides were probed with antifibroblast growth factor receptor 1 antibody to ensure that there was a reduction in protein expression. As shown in Figure 7A, the intensity of the 120-kDa immunoreactive band was markedly reduced after antisense treatment. Subsequent to incubation in FGFR1-S or FGFR1-AS, FGF-1 was added to Y79 cells and the proliferative response measured after 24 hours. As shown in Figure 7B, FGF-1–induced tritiated thymidine uptake was approximately the same in cultures treated with FGF-1 alone and in the presence of FGFR1-S. Precubulation of cells with FGFR1-AS probe completely blocked FGF-1 mitogenic activity. To ensure that inhibition in the presence of FGFR1-AS was not due to cellular toxicity, viability of control and FGFR1-AS–treated cultures was measured at the end of the experimental period. Although there was a small decrease in viable cell number (95% viable cells in control cultures, 88% viable cells in treated cultures), this difference was not statistically significant.

INFLUENCE OF HEPARAN SULFATE PROTEOGLYCAN ON FGF-1–INDUCED PROLIFERATION

Sodium chloride pretreatment of Y79 cells was used to assess whether sulfation was required for FGF-1 to exert mitogenic effects. Cultures were grown for 3 days under control conditions or in the presence of chloride and were...
exposed to no growth factors (control), FGF-1 alone (10 ng/mL), or both FGF-1 and heparin at low (10 ng/mL) and high (10 µg/mL) concentrations. In the absence of exogenous factors, untreated cells exhibited a low level of proliferation during the in vitro period, this process being significantly increased in the presence of FGF-1. Supplementation of FGF-1 with heparin (10 ng/mL) did not further enhance proliferation, while high doses of heparin led to small decreases compared with FGF-1 alone (Figure 8A). In sodium chlorate–treated cells, baseline proliferation after 3 days was markedly reduced, and FGF-1 addition had only a small stimulatory effect over these baseline values. Addition of heparin led to a significant restoration of mitogenic activity (Figure 8A).

In a second series of experiments, in which de novo DNA synthesis rather than cell number increase was measured, slightly different results were obtained. To assess the specific role of HSPGs in the generation of mitogenic responses to FGF-1, cells were pretreated with sodium chlorate, then digested with heparinase III and maintained in the presence of chlorate, and DNA synthesis was measured by tritiated thymidine incorporation. Whereas undigested Y79 cells this time showed significant FGF-1– and FGF-1/heparin–induced DNA replication, heparinase III treatment completely inhibited the mitogenic response to FGF-1. Heparin alone had no effect, but if added simultaneously with FGF-1, the mitogenic effect was almost fully restored with heparin doses of 10 ng/mL, whereas high doses of heparin had no effect (Figure 8B). In all cases, microscopic observation and vital dye exclusion at the end of experiments showed that none of the treatments used were toxic for Y79 cells (data not shown).

Figure 7. Prevention of fibroblast growth factor 1 (FGF-1)–induced proliferation of Y79 cells by antisense oligonucleotides specific for fibroblast growth factor receptor 1 (FGFR1) messenger RNA. A, Western blotting of Y79 cells treated with either sense control probe (R1-S) or antisense (R1-AS) with anti-FGFR1 antibody showed a large decrease in intensity of labeling (arrow, 120 kDa). B, As before, FGF-1 (10 ng/mL) treatment significantly stimulated cell multiplication, and this effect was not diminished by a 1-week pretreatment with missense oligonucleotides for FGFR1 (R1-S). However, a 1-week preincubation of cells in antisense oligonucleotides for FGFR1 (R1-AS) completely prevented FGF-1–induced stimulation. Asterisk indicates P<.01 relative to controls. Values are the average (mean±SEM) calculated from 3 independent cultures, triplicate wells for each; cpm indicates counts per minute.

Figure 8. Suppression of fibroblast growth factor 1 (FGF-1)–induced Y79 proliferation by perturbation of heparan sulfate proteoglycan coreceptors. A, As before, addition of FGF-1 (10 ng/mL, depicted in first row below the x-axis) led to increased Y79 proliferation after 3 days in vitro compared with growth in unstimulated controls. Addition of low or high doses of heparin (shown in the second row under the x-axis) did not greatly alter the inductive effect when FGF-1 was used at this dose. After sodium chlorate (NaClO3) block of sulfation, Y79 cells were no longer able to respond mitotically to FGF-1. However, this effect was restored in the presence of low or high doses of heparin, the latter being significantly less elevated than the former. Black bars indicate cell density at start of experiment; gray bars, cell density after 3 days in culture. Asterisk indicates P<.05, and dagger, P<.01, with respect to untreated control; double dagger, P<.01 with respect to chlorate-treated controls; section mark, P<.05 with respect to low-dose heparin treatment. B, When DNA synthesis was measured instead of cell number increase, FGF-1 still stimulated DNA replication after chlorate treatment, presumably acting through preexisting heparan sulfate proteoglycans. Combined heparinase and chlorate treatment abolished FGF-1–induced DNA synthesis, although this effect could be efficiently rescued by coaddition of low but not high doses of heparin. Black bars indicate chlorate treatment alone; stippled bars, heparinase and chlorate treatments. Asterisk indicates P<.05, and dagger, P<.01, with respect to untreated control; double dagger, P<.01 compared with FGF-1 alone (10 ng/mL). Values are the average (mean±SEM) from 4 independent cultures, triplicate wells for each; cpm indicates counts per minute.
In the present study we investigated the expression profile of FGFR within Y79 retinoblastoma, and examined in detail the effects of FGF-1 on FGFR1 phosphorylation and cell proliferation. The FGF-1 signaling through FGFR1 accounted entirely for cell proliferation.

Previous analyses by us and investigators at other laboratories have determined the composition in FGFR of the neural retina, and in particular the photoreceptors. These cells express principally FGFR1 and FGFR4 in roughly equal amounts, and no detectable FGFR3. The FGFR2 was observed by immunochemical and RT-PCR screening; it should be noted that we previously localized FGFR2 to human cone photoreceptors, and some studies have indicated a cone phenotype in retinoblastoma. We also detected FGFR3 by immunochemistry and RT-PCR (although the antibody used did not give detectable staining by Western blotting). The FGFR4 is also highly expressed in many cancer cell lines, and we reasoned that tumors originating from retina should likely express high levels of FGFR4. We did not screen for the newly described FGFR5, which lacks a carboxyl terminal to which no function is currently ascribed.

We chose to examine in greater detail the effects of FGF-1 on FGFR1 activation and Y79 growth, since it has been shown to bind with high affinity to FGFR1, is endogenous to the eye, and is known to exert effects on photoreceptors in vitro. Stimulation of Y79 cells with FGF-1 and subsequent immunoprecipitation with anti-FGFR1 antibody showed an apparent molecular mass of approximately 80 kDa. Reported values for FGFR1 vary between 55 and 150 kDa depending on isoform, antibody used, and tissue of origin. Since full-length FGFR1 is approximately 150 kDa, the band observed in these studies may represent a proteolytic fragment, a truncated isoform (a faint band of approximately 80 kDa was seen in FGFR1 immunoblots of Y79 and breast cancer cells [Figure 2], indicating the existence of a shorter isoform or proteolytic fragment of FGFR1), or a communoprecipitated protein.

The FGF-1 induced robust tyrosine phosphorylation of FGFR1 in these cells, and FGF-1 induced tumor cell proliferation in a dose- and heparin-dependent manner, in general agreement with FGF stimulatory effects on many cancer cell lines. As has also been observed in previous studies, high doses of FGF-1 actually led to reduced cell growth, probably because of FGFR downregulation in the presence of excess ligand. The binding of FGF-1 and activation of FGFR have been widely shown to be strongly enhanced by both heparin and HSPGs. Endogenous HSPGs are both necessary and sufficient to permit FGF-1 activation of FGFR1 and generation of a biological response in Y79 cells. Chlorate treatment resulting in blockade of sulfation greatly reduced proliferative responses under both baseline and FGF-1–supplemented conditions. It should be noted that, whereas this treatment prevented cell division, DNA replication did continue but, presumably, cells were not able to complete the entire cell cycle. Heparinase treatment and maintenance in chlorate-supplemented media to prevent sulfation of surface HSPGs completely removed the ability of these cells to respond to exogenously applied FGF-1, and provision of low doses of heparin partly replaced endogenous HSPGs and permitted signaling. High doses of heparin were less effective, because of competition for binding sites as has been previously observed. Heparin addition only slightly affected FGF-1–induced tyrosine phosphorylation of FGFR4. Future studies will address the identity of intracellular second messenger proteins recruited after FGF-1–stimulated FGFR activation in Y79 cells.

To determine the FGFR responsible for mediating FGF-1–induced proliferation in Y79 cells, we blocked FGFR1 by means of either specific neutralizing antibodies or antisense oligonucleotides. The proliferative response to exogenous FGF-1 was entirely abolished by both treatments, strongly suggesting that the proliferative response to FGF-1 was mediated in a crucial manner by FGFR1. However, since FGFRs have been shown to form heterodimers with the other FGFRs on ligand binding, receptors other than FGFR1 may also be involved.

In conclusion, Y79 retinoblastomas reliably express detectable levels of high-affinity FGF, and by a variety of tests, FGFR1 activation leads to cellular multiplication. Tumor progression in vivo may hence be partly regulated by FGF ligands either released by retinoblastoma cells themselves, by normal or damaged neural retina, by neighboring tissues such as pigmented epithelium, or all 3. Since neutralizing antibodies and antisense oligonucleotides against FGFR1 can effectively prevent cell division in vitro, similar studies in animal models of retinoblastoma would be predicated. An additional potential line of research may involve perturbing the low-affinity HSPG coreceptors expressed by retinoblastoma, since the quantity and degree of sulfation of these molecules present at the cell surface can strongly regulate sensitivity to FGF.

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