

## Interaction of Fibroblast Growth Factor-2 (FGF-2) with Free Gangliosides: Biochemical Characterization and Biological Consequences in Endothelial Cell Cultures

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Exogenous gangliosides affect the angiogenic activity of fibroblast growth factor-2 (FGF-2), but their mechanism of action has not been elucidated. Here, a possible direct interaction of sialo-glycolipids with FGF-2 has been investigated. Size exclusion chromatography demonstrates that native, but not heat-denatured, <sup>125</sup>I-FGF-2 binds to micelles formed by gangliosides GT<sub>1b</sub>, GD<sub>1b</sub>, or GM<sub>1</sub>. Also, gangliosides protect native FGF-2 from trypsin digestion at micromolar concentrations, the order of relative potency being GT<sub>1b</sub> > GD<sub>1b</sub> > GM<sub>1</sub> = GM<sub>2</sub> = sulfatide > GM<sub>3</sub> = galactosyl-ceramide, whereas asialo-GM<sub>1</sub>, neuraminic acid, and *N*-acetylneuramin-lactose were ineffective. Scatchard plot analysis of the binding data of fluoro-chrome-labeled GM<sub>1</sub> to immobilized FGF-2 indicates that FGF-2/GM<sub>1</sub> interaction occurs with a *K<sub>d</sub>* equal to 6 μM. This interaction is inhibited by the sialic acid-binding peptide mastoparan and by the synthetic fragments FGF-2(112–129) and, to a lesser extent, FGF-2(130–155), whereas peptides FGF-2(10–33), FGF-2(39–59), FGF-2(86–96), and the basic peptide HIV-1 Tat(41–60) were ineffective. These data identify the COOH terminus of FGF-2 as a putative ganglioside-binding region. Exogenous gangliosides inhibit the binding of <sup>125</sup>I-FGF-2 to high-affinity tyrosine-kinase FGF-receptors (FGFRs) of endothelial GM 7373 cells at micromolar concentrations. The order of relative potency was GT<sub>1b</sub> > GD<sub>1b</sub> > GM<sub>1</sub> > sulfatide a = sialo-GM<sub>1</sub>. Accordingly, GT<sub>1b</sub>, GD<sub>1b</sub>, GM<sub>1</sub>, and GM<sub>2</sub>, but not GM<sub>3</sub> and asialo-GM<sub>1</sub>, prevent the binding of <sup>125</sup>I-FGF-2 to a soluble, recombinant form of extracellular FGFR-1. Conversely, the soluble receptor and free heparin inhibit the interaction of fluoro-chrome-labeled GM<sub>1</sub> to immobilized FGF-2. In agreement with their FGFR antagonist activity, free gangliosides inhibit the mitogenic activity exerted by FGF-2 on endothelial cells in the same range of concentrations. Also in this case, GT<sub>1b</sub> was the most effective among the gangliosides tested while asialo-GM<sub>1</sub>, neuraminic acid, *N*-acetylneuramin-lactose, galactosyl-ceramide, and sulfatide were ineffective. In conclusion, the data demonstrate the capacity of exogenous gangliosides to interact with FGF-2. This interaction involves the COOH terminus of the FGF-2 molecule and depends on the structure of the oligosaccharide chain and on the presence of sialic acid residue(s) in the ganglioside molecule. Exogenous gangliosides act as FGF-2 antagonists when added to endothelial cell cultures. Since gangliosides are extensively shed by tumor cells and reach elevated levels in the serum of tumor-bearing patients, our data suggest that exogenous gangliosides may affect endothelial cell function by a direct interaction with FGF-2, thus modulating tumor neovascularization.

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## INTRODUCTION

Gangliosides are neuraminic acid (NeuAc)<sup>1</sup>-containing glycosphingolipids. Under physiological conditions, gangliosides are mainly associated to the cell membranes where they play different roles in controlling cell growth, cell adhesion, and cell-cell interaction (Hakomori, 1990; Zeller and Marchase, 1992). Gangliosides shed in the microenvironment during tumor growth and metastasis (Merritt *et al.*, 1994; Chang *et al.*, 1997) possibly as a consequence of their aberrant overproduction by tumor cells induced by various cytokines. Indeed, IL-1 (Kjaer *et al.*, 1992), interferon- $\gamma$  (IFN- $\gamma$ ), IL-2, IL-4 (Hoons *et al.*, 1991; Ando *et al.*, 1996), tumor necrosis factor- $\alpha$  (Furukawa *et al.*, 1990), PDGF (Pilkington *et al.*, 1993), fibroblast growth factor-2 (FGF-2), and EGF (Drago *et al.*, 1989) affect the synthesis and surface expression of different gangliosides. Conversely, both free and cell-associated gangliosides can modulate the expression of cytokines. For instance, gangliosides inhibit the production of IL-1  $\beta$ , tumor necrosis factor- $\alpha$ , and IL-6 (Ziegler-Heitbrock *et al.*, 1992; Dumontet *et al.*, 1994) while GD<sub>3</sub> stimulates the production of vascular endothelial growth factor in human glioma cells (Koochekpour *et al.*, 1996).

Gangliosides modulate the biological activity of growth factors and cytokines. Exogenous GM<sub>1</sub>, GM<sub>3</sub>, and GT<sub>1b</sub> inhibit neurite outgrowth induced by PDGF, insulin, nerve growth factor, and insulin-like growth factor-1 (Hynds *et al.*, 1997). They also inhibit neuroblastoma cell proliferation induced by PDGF (Hynds *et al.*, 1995; Zhang *et al.*, 1995). GM<sub>1</sub> and GM<sub>3</sub> affect EGF- and PDGF-dependent fibroblast proliferation (Bremer *et al.*, 1986). Moreover, gangliosides modulate IL-2- and IL-3-dependent proliferation of different cell types of the immune system (Sharom *et al.*, 1991; Nakamura *et al.*, 1996). Also, GM<sub>2</sub> and GT<sub>1</sub> are able to modulate the antiviral activity of human IFN (Besancon and Ankel, 1974; Vengris *et al.*, 1976). Finally, glucosylceramide synthesis has been demonstrated to be required for FGF-2 to stimulate axonal growth (Boldin and Futerman, 1997). Accordingly, gangliosides influence FGF-2-dependent mitogenesis and migration of glial cells (Meuillet *et al.*, 1996a,b), and GM<sub>3</sub> inhibits the proliferation of fibroblasts exposed to FGF (Bremer and Hakomori, 1982).

The mechanisms by which gangliosides modulate the biological activity of growth factors and cytokines are not fully elucidated. Experimental evidence indicates that exogenous gangliosides are incorporated into the plasma membrane and may affect the activity

of tyrosine kinase receptors and intracellular signaling. For instance, membrane-incorporated GM<sub>3</sub> inhibits ligand-induced autophosphorylation of EGF receptor. This occurs in the absence of a direct interaction of the ganglioside with the growth factor or modifications of the binding of EGF to its receptor (Bremer *et al.*, 1986; Hanai *et al.*, 1988a,b; Weis and Davis, 1990; Song *et al.*, 1991). Gangliosides inhibit ligand-induced dimerization and autophosphorylation of PDGF receptor (Nojiri *et al.*, 1991; Van Brocklyn *et al.*, 1993; Hynds *et al.*, 1995) and prevent the activation of downstream second messengers (Saqr *et al.*, 1995; Sachinidis *et al.*, 1996). On the other hand, the incorporation of GM<sub>1</sub> and GM<sub>3</sub> into the cell membrane of 3T3 fibroblasts increases the affinity of PDGF binding in the absence of a direct interaction with PDGF (Bremer *et al.*, 1984), whereas exogenous GM<sub>1</sub> and GM<sub>2</sub> inhibit PDGF binding to its receptors, suggesting an interaction of free gangliosides with the growth factor and/or the receptor (Sachinidis *et al.*, 1996). Indeed, exogenous gangliosides have been shown to bind directly to IFN (Besancon and Ankel, 1974), IL-2 (Chu and Sharom, 1990), IL-4 (Chu and Sharom, 1995), and to the nerve growth factor receptor Trk (Mutoh *et al.*, 1995). In conclusion, gangliosides play an important role in regulating the biological activity of growth factors and cytokines by different mechanisms of action. In turn, growth factors regulate the ganglioside composition of the plasma membranes and of the extracellular environment.

Angiogenesis is the process of generating new capillary blood vessels. Uncontrolled endothelial cell proliferation is observed in tumor neovascularization. Several growth factors and cytokines have been shown to stimulate endothelial cell proliferation *in vitro* and *in vivo*, and FGF-2 was one of the first among them to be characterized (Moscatelli *et al.*, 1986). FGF-2 is a Mr 18,000 heparin-binding cationic polypeptide that induces proliferation, migration, and protease production in endothelial cells in culture and neovascularization *in vivo* (Basilico and Moscatelli, 1992). FGF-2 interacts with endothelial cells through two distinct classes of receptors, the high-affinity tyrosine-kinase receptors (FGFRs) and low-affinity heparan sulfate proteoglycans (HSPGs) present on the cell surface and in the extracellular matrix (Jonhson and Williams, 1993). Both classes of receptors are necessary for the transduction of the signal generated by the growth factor (Yayon *et al.*, 1991) and for its internalization inside the cell (Roghani and Moscatelli, 1992; Rusnati *et al.*, 1993).

Gangliosides are highly expressed in the hypervascularized areas of gliomas (Koochekpour and Pilkington, 1996), and they regulate the neovascularization process *in vivo* (Ziche *et al.*, 1989, 1992; Gullino *et al.*, 1990; Cockerill *et al.*, 1995; Gullino, 1995). Interestingly, GM<sub>2</sub> and GM<sub>3</sub> inhibit FGF-2-mediated endothelial cell proliferation, and the addition of GD<sub>3</sub> restores

<sup>1</sup> Abbreviations: FGF, fibroblast growth factor; FGFR, tyrosine-kinase FGF receptor; HSPGs, heparan sulfate proteoglycans; IFN, interferons; MAE cells, mouse aortic endothelial cells; NeuAc, neuraminic acid; xcFGFR-1, soluble extracellular form of FGFR-1/flg. Gangliosides are named according to the nomenclature of Svennerholm (1964).

**Table 1.** Structural characteristics of gangliosides and related compounds utilized in this study

Molecule <sup>a</sup>	Schematic structure
N-Acetylneuramin-lactose	NeuAc 2→3Gal 1→4Glc 1
Galactosyl-ceramide	3Gal 1→1'Cer
Sulfatide	SO <sub>3</sub> <sup>-</sup> →3Gal 1→1'Cer
asialo-GM <sub>1</sub>	Gal 1→3GalNAc 1→4Gal 1→4Glc 1→1'Cer
GM <sub>1</sub>	Gal 1→3GalNAc 1→4Gal(3←2NeuAc) 1→4Glc 1→1'Cer
GM <sub>2</sub>	GalNAc 1→4Gal(3←2NeuAc) 1→4Glc 1→1'Cer
GM <sub>3</sub>	NeuAc 2→3Gal 1→4Glc 1→1'Cer
GD1 <sub>b</sub>	Gal 1→3GalNAc 1→4Gal (3←2NeuAc8←2NeuAc) 1→4Glc 1→1'Cer
GT1 <sub>b</sub>	NeuAc 2→3Gal 1→3GalNAc 1→4Gal(3←2NeuAc8←2NeuAc) 1→4Glc 1→1'Cer

Glc, glucose; Gal, galactose; GalNAc, N-acetyl-galactosamine; NeuAc, neuraminic-acid; Cer, ceramide.

<sup>a</sup> Ganglioside nomenclature according to Svennerholm (1964).

optimal levels of cell growth (Alessandri *et al.*, 1992; Ziche *et al.*, 1992). In contrast, GD<sub>3</sub> enhances the chemotactic activity exerted by FGF-2 on endothelial cells, which is counteracted by GM<sub>3</sub> (Ziche *et al.*, 1992). Moreover, GM<sub>1</sub>, GD<sub>1b</sub>, and GT<sub>1b</sub> act synergistically with FGF-2 in favoring survival, growth, and motility of capillary endothelial cells (De Cristian *et al.*, 1990). Finally, angiogenesis induced by FGF-2 in the rabbit cornea assay can be stimulated or repressed by modulating the GM<sub>3</sub>:GD<sub>3</sub> molar ratio (Ziche *et al.*, 1992). An increase of the angiogenic activity of FGF-2 can also be obtained by increasing the local concentration of GM<sub>1</sub> and GT<sub>1b</sub> (Ziche *et al.*, 1989).

Little is known about the mechanism(s) by which gangliosides affect the angiogenic activity of FGF-2 during tumor growth. The shedding of gangliosides by tumor cells can be so extensive as to alter the ganglioside composition of the extracellular environment of the tumor and to cause an increase of their serum levels (Kloppel *et al.*, 1977). Different observations have shown the capacity of gangliosides to bind directly to certain growth factors (see above). Moreover, the heparin-binding properties of FGF-2 and its capacity to interact with various polysulfated/polysulfonated compounds point to the possibility that anionic NeuAc groups of sialo-gangliosides may mimic sulfated/sulfonated groups of glycosaminoglycans in FGF-2 interaction. In the present article we investigated the capacity of exogenous free gangliosides to interact directly with FGF-2 and to affect the biological activity of the growth factor in endothelial cells.

## MATERIALS AND METHODS

### Chemicals

Human recombinant FGF-2 was expressed and purified from transformed *Escherichia coli* cells by heparin-Sepharose chromatography (Isacchi *et al.*, 1991). Recombinant FGF-1 and FGF-4 were gifts from C. Basilio (New York University Medical Center, New York, NY). The recombinant, soluble form of the extracellular domain of FGFR-1/βg (xcFGFR-1) (Bergonzoni *et al.*, 1992) was provided by A. Isacchi (Pharmacia-Upjohn, Nerviano, Italy). Synthetic peptides repre-

senting different fragments of human FGF-2 (Schubert *et al.*, 1987) were kindly donated by A. Baird (Prizm Pharmaceuticals, San Diego, CA). The synthetic peptide representing the basic domain of HIV-1 Tat protein was from the Medical Research Council AIDS Reagent Project (Potters Bar, Herts, United Kingdom). 4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoil acid (BODIPY-dodecanoil acid) was obtained from Molecular Probes (Eugene, OR). Gangliosides, N-acetylneuramin-lactose, and mastoparan were from Sigma (St. Louis, MO). Sulfatide was prepared from pig brain by the method of Hara and Radin (1979), and its chromatographic purity and conversion to the sodium salt were determined as described (Cestaro *et al.*, 1982). Details about the structure of the gangliosides and ganglioside-related molecules utilized in this study are detailed in Table 1.

### Cell Cultures

Transformed fetal bovine aortic endothelial GM 7373 cells were obtained from the N.I.G.M.S. Human Genetic Mutant Cell Repository (Institute for Medical Research, Camden, NJ). They correspond to the BFA-1c multilayered transformed clone described by Grinspan *et al.* (1983). GM 7373 cells were grown in Eagle's minimal essential medium containing 10% FCS, vitamins, and essential and nonessential amino acids. Spontaneously immortalized BALB/c mouse aortic endothelial 22106 cells (MAE cells) were grown in DMEM containing 10% FCS (Bastaki *et al.*, 1996).

### <sup>125</sup>I-FGF-2 Cell Binding and Internalization

FGF-2 was iodinated as described (Neufeld and Gospodarowicz, 1985) at a specific radioactivity equal to 800 cpm/fmol. GM 7373 were incubated at 4°C in serum-free medium containing 10 ng/ml <sup>125</sup>I-FGF-2, 0.15% gelatin, 20 mM HEPES buffer (pH 7.5), and the indicated concentrations of the ganglioside under test. After 2 h, the amount of <sup>125</sup>I-FGF-2 bound to low- and high-affinity binding sites was evaluated as described (Moscatelli, 1987). Briefly, after a PBS wash, cells were rinsed twice with 2 M NaCl in 20 mM HEPES buffer (pH 7.5) to remove <sup>125</sup>I-FGF-2 bound to low-affinity binding sites and twice with 2 M NaCl in 20 mM sodium acetate (pH 4.0) to remove <sup>125</sup>I-FGF-2 bound to high-affinity binding sites. Nonspecific binding was measured in the presence of a 100-fold molar excess of unlabeled FGF-2 and subtracted from all the values.

In some experiments, GM 7373 cells were preloaded with GM<sub>1</sub> before the <sup>125</sup>I-FGF-2 cell-binding assay. To this purpose, cells were seeded at 70,000 cells/cm<sup>2</sup> in 24-well dishes. After 16 h, cells were incubated for an additional 72 h in fresh medium containing 0.4% FCS in the absence or in the presence of 100 μM GM<sub>1</sub>. At the end of incubation, cells were extensively washed with PBS and incubated



at 4°C in serum-free medium containing 10 ng of  $^{125}\text{I}$ -FGF-2 per ml in the absence of free ganglioside. After 2 h, the amount of  $^{125}\text{I}$ -FGF-2 bound to low- and high-affinity binding sites was evaluated as described above. To assess the amount of ganglioside incorporated during the preloading incubation period, parallel cultures were trypsinized and cells were sonicated at 50 W for 2 min at 4°C. Then, samples were centrifuged for 20 min at  $40,000 \times g$ , and the amount of NeuAc was evaluated in the cell membrane and cytosolic fractions as described previously (Svennerholm, 1956).

For cell internalization assays, GM 7373 cells were incubated with  $^{125}\text{I}$ -FGF-2 exactly as described above. After 2 h, cell cultures were shifted at 37°C and incubated for an additional 6 or 24 h. At the end of incubation, surface-bound  $^{125}\text{I}$ -FGF-2 was removed as described above, and cell-internalized  $^{125}\text{I}$ -FGF-2 was recovered by lysing the cells with 0.1 mM Tris-HCl (pH 8.1) containing 0.5% Triton X-100.

### Cell Proliferation and DNA Synthesis Assays

Cell proliferation assay on GM 7373 cells was performed as described (Presta *et al.*, 1989). Briefly, GM 7373 cells were seeded at 70,000 cells/cm<sup>2</sup> in 24-well dishes. Plating efficiency was higher than 90%. After overnight incubation, cells were incubated for 24 h in fresh medium containing 0.4% FCS in the absence or in the presence of 10 ng/ml FGF-2 and the indicated concentrations of gangliosides. At the end of incubation, cells were trypsinized and counted in a Burkner chamber. For DNA synthesis assay, MAE cells were seeded at 25,000 cells/cm<sup>2</sup> in 24-well dishes and incubated for 2 d with 0.5% FCS. Quiescent cell cultures were then supplemented with the different mitogens in the absence or in the presence of GT<sub>1b</sub> and incubated for 16 h at 37°C. At the end of incubation, cells were pulse labeled with [<sup>3</sup>H]thymidine (1 μCi/ml) for 6 h. The amount of radioactivity incorporated into the trichloroacetic acid-precipitable material was then measured.

### Size Exclusion Chromatography

To assess the association between FGF-2 and micellar gangliosides, 100-μl samples containing 3 pmol of  $^{125}\text{I}$ -FGF-2 were incubated for 10 min at 4°C with 125 nmol of the different gangliosides. Then, samples were chromatographed on a size-exclusion fast protein liquid chromatography Superose-12 column (Pharmacia, Piscataway, NJ) in PBS with a flow rate equal to 1.0 ml/min. Elution profiles of FGF-2 and of the ganglioside were obtained by quantification of the radioactivity and of the NeuAc content of the different fractions, respectively. Ferritin ( $M_r$  440,000), immunoglobulin G ( $M_r$  150,000), ovalbumin ( $M_r$  45,000), soybean trypsin inhibitor ( $M_r$  20,100), and cytochrome C ( $M_r$  12,000) were chromatographed under the same experimental conditions as molecular size standards.

### Proteolytic Digestion and SDS-PAGE

The protective effect of gangliosides on tryptic digestion of FGF-2 was evaluated as described (Coltrini *et al.*, 1993). Briefly, FGF-2 aliquots (55 pmol) were equilibrated at 37°C for 5 min in 50 mM Tris-HCl (pH 7.5) in the presence of increasing amounts of the ganglioside under test. Then, 60 ng of trypsin (Sigma, St. Louis, MO) were added in a final volume of 100 μl, and digestion was allowed to proceed at 37°C for 3 h. At the end of trypsin digestion, samples were added with an equal volume of SDS-reducing sample buffer, boiled at 100°C for 2 min, and subjected to 15% SDS-PAGE. Gels were stained with the silver staining procedure. The amount of undigested protein in a given line was estimated by soft-laser scanning of the gel.

### Preparation of BODIPY-12-labeled GM<sub>1</sub>

BODIPY-12-GM<sub>1</sub> was synthesized and purified according to previously described procedures (Marchesini *et al.*, 1994) by acylation of lyso-GM<sub>1</sub> with the *N*-hydroxy succinimide ester of BODIPY-dodecanoic acid.

### Coating of FGF-2 to Plastic and Binding Assay

Aliquots (100 μl) of 100 mM NaHCO<sub>3</sub> (pH 9.6) (carbonate buffer), containing 20 μg/ml native or heat-denatured FGF-2, were added to polystyrene nontissue culture microtiter plates. After 16 h of incubation at 4°C, the solution was removed and wells were washed three times with PBS. Experiments using  $^{125}\text{I}$ -FGF-2 as a tracer revealed that up to 10% of the protein binds to plastic under these experimental conditions (Rusnati *et al.*, 1997a).

For competition binding assays, the indicated amounts of BODIPY-12-GM<sub>1</sub> were incubated for 10 min at room temperature into wells coated with 20 μg/ml native or heat-denatured FGF-2 in the absence or in the presence of the indicated concentrations of unlabeled GM<sub>1</sub>, heparin, xcFGFR-1, or synthetic FGF-2 fragments. At the end of incubation, wells were washed three times with PBS, and FGF-2-associated GM<sub>1</sub> was eluted from the wells with 100 μl of methanol-chloroform solution (40:60, vol/vol) and measured with a FCT-150 spectrofluorimeter (Jasco Spectroscopic, Tokyo, Japan) at its optimal excitation and emission wavelengths. Nonspecific binding was measured in wells incubated with carbonate buffer and was subtracted from all the data.

For the determination of the  $K_d$  of the interaction of BODIPY-12-GM<sub>1</sub> with FGF-2, 100-μl aliquots of PBS containing different concentrations of labeled GM<sub>1</sub> were added into wells coated with 20 μg of FGF-2 per ml. Then samples were processed exactly as described above. Nonspecific binding was subtracted from all the data, which were then analyzed by the Scatchard plot procedure (Scatchard, 1949).

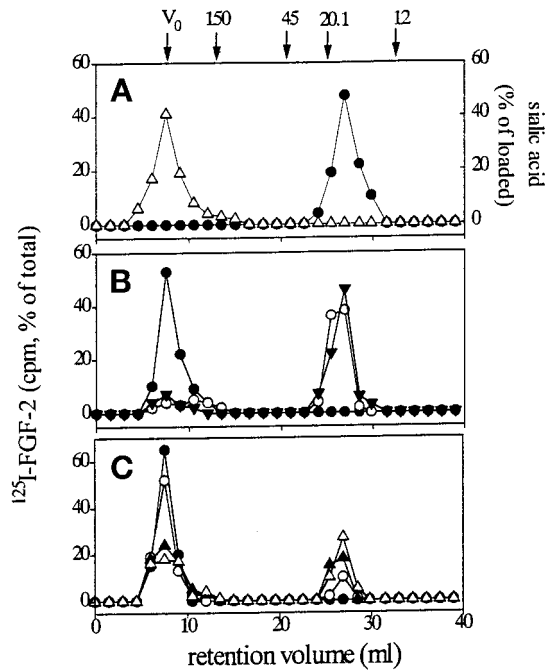
### Cross-Linking of $^{125}\text{I}$ -FGF-2 to xcFGFR-1

$^{125}\text{I}$ -FGF-2 (0.3 pmol) was incubated in PBS for 2 h at 37°C with 3 pmol of the soluble extracellular form of FGFR-1/flg (xcFGFR-1) in the absence or in the presence of 1.5 nmol of the ganglioside under test. At the end of the incubation, the complexes between xcFGFR-1 and  $^{125}\text{I}$ -FGF-2 were cross-linked by adding 1 mM bis[2-(succinimidoxycarbonyloxy)ethyl] sulfone (BSOCOES, Pierce Chemical, Rockford, IL). After 30 min of incubation at room temperature, the reaction was stopped by the addition of reducing SDS-PAGE sample buffer. Samples were boiled and analyzed by 10% SDS-PAGE. Gels were dried and exposed to Kodak X-OMAT AR film (Eastman Kodak, Rochester, NY) at -70°C for 1 wk.

## RESULTS

### Size Exclusion Chromatography of $^{125}\text{I}$ -FGF-2-Ganglioside Complexes

Gangliosides form high-molecular-weight micelles when dissolved in aqueous solutions at concentrations higher than the critical micellar concentration (which usually ranges from  $10^{-8}$  to  $10^{-5}$  M) (Formisano *et al.*, 1979; Ulrich-Bott and Wiegandt, 1984; Saqr *et al.*, 1993). Accordingly, gel filtration chromatography performed on a Superose-12 size-exclusion fast protein liquid chromatography column (Pharmacia) demonstrates that gangliosides dissolved in PBS at  $1.25 \times 10^{-3}$  M form high-molecular-weight micelles that elute with the void volume of the column (7 ml) (Figure 1A). Conversely, low-molecular-weight  $^{125}\text{I}$ -FGF-2 ( $M_r$  18,000) elutes with a retention volume equal to 27 ml. On this basis, to assess a possible interaction of FGF-2 with gangliosides, 3 pmol of  $^{125}\text{I}$ -FGF-2 were preincubated for 10 min at room temperature with 125 nmol of GM<sub>1</sub> (final concentration of the ganglioside equal to  $1.25 \times 10^{-3}$  M) and then loaded onto the Superose-12



**Figure 1.** Size-exclusion chromatography of FGF-2-ganglioside complexes. (A) 100  $\mu$ l samples containing 3 pmol of  $^{125}$ I-FGF-2 ( $\bullet$ ) or 125 nmol of GM<sub>1</sub> ( $\Delta$ ) in PBS were incubated for 10 min at room temperature, loaded separately onto size-exclusion fast protein liquid chromatography Superose-12 column, and eluted in PBS at 1 ml/min flow rate. Radioactivity or NeuAc concentration was measured in each fraction for the evaluation of  $^{125}$ I-FGF-2 or ganglioside content, respectively. (B) 3 pmol of native ( $\bullet$ ,  $\circ$ ) or of heat-denatured  $^{125}$ I-FGF-2 ( $\blacktriangledown$ ) were incubated for 10 min at room temperature with 125 nmol of GM<sub>1</sub> (closed symbols) or of asialo-GM<sub>1</sub> ( $\circ$ ). Then, samples were subjected to size-exclusion chromatography as in panel A, and radioactivity was measured in each fraction. (C) 3 pmol of  $^{125}$ I-FGF-2 were incubated for 10 min at room temperature with decreasing concentrations of GM<sub>1</sub> [12.5 nmol ( $\bullet$ ), 5 nmol ( $\circ$ ), 1.25 nmol ( $\blacktriangle$ ), or 0.125 nmol ( $\Delta$ )]. Then, samples were subjected to size-exclusion chromatography as in panel A, and radioactivity was measured in each fraction. Molecular size standards (in thousands) were ferritin ( $M_r$  440,000), that eluted with the void volume of the column ( $V_0$ ), IgG ( $M_r$  150,000), ovalbumin ( $M_r$  45,000), soybean trypsin inhibitor ( $M_r$  20,100), and cytochrome C ( $M_r$  12,000).

column. Under these experimental conditions,  $^{125}$ I-FGF-2 preincubated with GM<sub>1</sub> dramatically changes its chromatographic behavior and coelutes with the ganglioside in the void volume of the column, thus indicating the formation of  $^{125}$ I-FGF-2-GM<sub>1</sub> complexes (Figure 1B). Similar results were obtained when  $^{125}$ I-FGF-2 was preincubated with the same doses of GM<sub>2</sub>, GM<sub>3</sub>, GD<sub>1b</sub>, or GT<sub>1b</sub> (our unpublished results), whereas asialo-GM<sub>1</sub> was unable to complex the growth factor (Figure 1B). Also, no  $^{125}$ I-bFGF-GM<sub>1</sub> complexes were observed when the growth factor was heat denatured before incubation with the ganglioside (Figure 1B), thus indicating that NeuAc and a correct three-dimensional structure of FGF-2 are required for ganglioside interaction.

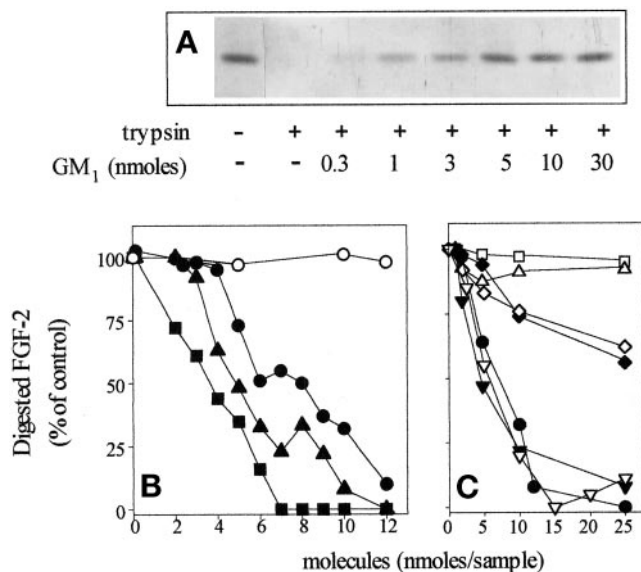
The capacity of gangliosides to complex with FGF-2 is dose dependent, as shown by the progressive reduction of the high-molecular-weight peak corresponding to the  $^{125}$ I-bFGF-GM<sub>1</sub> complex paralleled by the appearance of a retained peak of free  $^{125}$ I-bFGF when the growth factor was preincubated with decreasing doses of GM<sub>1</sub> (Figure 1C).

#### Gangliosides Protect FGF-2 from Trypsin Digestion

Sulfated glycosaminoglycans bind to FGF-2 and protect it from tryptic digestion (Coltrini *et al.*, 1993). This capacity has been utilized to study the structural features of FGF-2-binding polysulfated/polysulfonated compounds (Coltrini *et al.*, 1993). On this basis, the possibility that ganglioside interaction can prevent FGF-2 proteolysis was investigated. To this purpose, aliquots of FGF-2 (55 pmol) were equilibrated at 37°C for 5 min in the presence of increasing amounts of the different gangliosides. Then, 60 ng of trypsin were added, and proteolytic digestion was allowed to proceed at 37°C for 3 h. At the end of incubation, samples were analyzed by SDS-PAGE followed by silver staining of the gel (Figure 2A), and the amount of undigested FGF-2 was quantified by soft laser scanning. As shown in Figure 2, A and B, gangliosides protect FGF-2 from tryptic digestion in a dose-dependent manner as a function of the number of NeuAc residues of the molecule, the order of relative potency of the gangliosides tested being GT<sub>1b</sub> > GD<sub>1b</sub> > GM<sub>1</sub>. It should be pointed out that GT<sub>1b</sub> was unable to protect heat-denatured FGF-2 from trypsin digestion (Figure 3), thus confirming that the protective effect of gangliosides depends on the interaction with FGF-2, and not with the proteolytic enzyme, and that this interaction occurs only when the growth factor is present in the proper native conformation.

The above data suggest that NeuAc residue(s) are of importance in gangliosides-FGF-2 interaction. Accordingly, asialo-GM<sub>1</sub> does not prevent tryptic digestion of FGF-2 (Figure 2B). In addition, however, free NeuAc and *N*-acetylneuramin-lactose, a disaccharide bearing one NeuAc group, do not protect FGF-2 from tryptic digestion at doses up to 250  $\mu$ M (Figure 2C), thus suggesting that NeuAc residue(s) associated with defined glycosphingolipidic structures are required for optimal FGF-2 interaction. Relevant to this point is the observation that GM<sub>3</sub> shows a reduced capacity to bind and protect FGF-2 from trypsin digestion when compared with GM<sub>2</sub> and GM<sub>1</sub> (Figure 2C). Since these monosialo-gangliosides differ in the length of their oligosaccharide chain (see Table 1), the data point to the importance of the saccharide structure in presenting NeuAc to FGF-2.

Anionic groups as sulfates can equal the protein-recognition properties of sialic acids (Rosen and Bertozzi, 1994), and specific sulfated glycolipids have



**Figure 2.** Protection of FGF-2 from tryptic digestion by gangliosides and related molecules. (A) Representative experiment in which 1  $\mu$ g aliquots of FGF-2 were incubated at 37 $^{\circ}$  C for 3 h with 60 ng of trypsin in the absence or in the presence of the indicated amounts of GM<sub>1</sub>. Then samples were analyzed by 15% SDS-PAGE followed by silver staining of the gel. (B and C) Aliquots (1  $\mu$ g) of FGF-2 were incubated with trypsin in the presence of the indicated amounts of asialo-GM<sub>1</sub> (○), GM<sub>1</sub> (●), GD<sub>1b</sub> (▲), GT<sub>1b</sub> (■) (panel B) or GM<sub>1</sub> (●), NeuAc (□), N-acetylneuramin-lactose (Δ), GM<sub>2</sub> (▼), GM<sub>3</sub> (◆), sulfatide (▽), galactosyl-ceramide (◇) (panel C). Then samples were analyzed by 15% SDS-PAGE followed by silver staining of the gel. The amount of nondigested FGF-2 was evaluated by soft-laser scanning of the gel, and data are expressed as percentage of digested FGF-2 in respect to samples in which trypsin was omitted. Each point is the mean of two to five determinations in duplicate. SEM never exceeded 13% of the mean value.

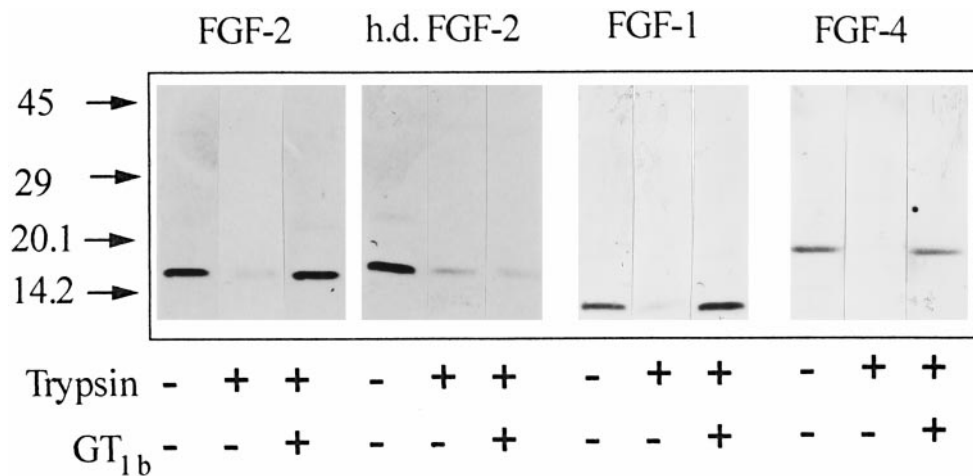
been demonstrated to bind to hepatocyte growth factor (Kobayashi *et al.*, 1994). Accordingly, sulfatide was able to protect FGF-2 from proteolytic cleavage with a

potency similar to GM<sub>1</sub> and GM<sub>2</sub>, whereas galactosyl-ceramide exerted a limited effect (Figure 2C). Taken together, the data indicate that NeuAc residue(s), the oligosaccharide chain, and, to a limited extent, the ceramide moiety of the ganglioside play a role in FGF-2 interaction.

FGF-2 belongs to a family of heparin-binding growth factors (Basilico and Moscatelli, 1992). To assess whether the ganglioside-binding capacity is limited to FGF-2, trypsin digestion experiments were also performed with FGF-1 and FGF-4. As shown in Figure 3, all FGFs tested are protected from trypsin digestion by GT<sub>1b</sub>, suggesting that various members of the FGF family share structural features responsible for ganglioside interaction.

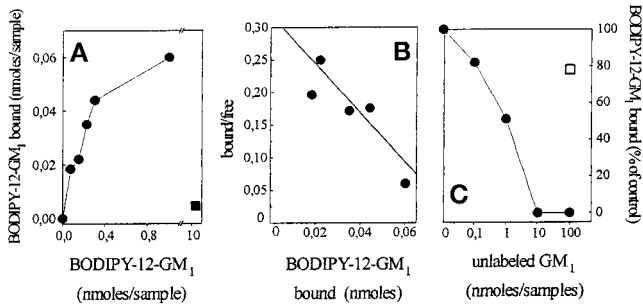
**Gangliosides Bind to Immobilized FGF-2**

FGF-2 immobilized onto nontissue culture plastic retains its cell-binding capacity and biological activity (Presta *et al.*, 1992; Rusnati *et al.*, 1997a). On this basis, FGF-2 was adsorbed to plastic and evaluated for its capacity to bind to BODIPY-12-labeled GM<sub>1</sub>. As shown in Figure 4A, fluorochrome-labeled GM<sub>1</sub> binds to immobilized FGF-2. The binding was dose dependent and saturable, specificity being demonstrated by the incapacity of BODIPY-12-GM<sub>1</sub> to interact with immobilized heat-denatured FGF-2. Scatchard plot analysis of the binding data indicates that BODIPY-12-GM<sub>1</sub> binds to immobilized FGF-2 with a  $K_d$  equal to  $6.3 \pm 2 \mu$ M (Figure 4B). Unlabeled GM<sub>1</sub> competed for the binding of BODIPY-12-GM<sub>1</sub> to FGF-2 in a dose-dependent manner, half-maximal inhibition being observed at equimolar concentrations of the two compounds (Figure 4C). Asialo-GM<sub>1</sub> did not exert any inhibitory effect on the binding of the labeled ganglioside to the growth factor. These data demonstrate that the BODIPY fluorochrome group does not inter-



**Figure 3.** Ganglioside-mediated protection of FGF-1 and FGF-4 from tryptic digestion. Aliquots (1  $\mu$ g) of native or heat-denatured (h.d.) FGF-2, FGF-1, or FGF-4 were incubated at 37 $^{\circ}$ C for 3 h with 60 ng of trypsin in the absence or in the presence of 8 nmol of GT<sub>1b</sub>. Then samples were analyzed by 15% SDS-PAGE followed by silver staining of the gel.



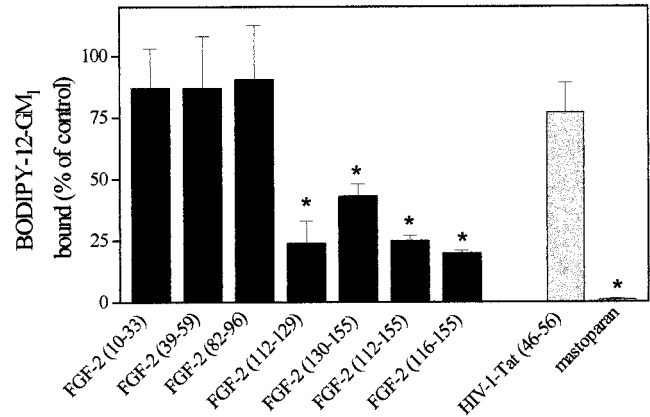


**Figure 4.** Binding of fluorochrome-labeled GM<sub>1</sub> to immobilized FGF-2. (A) Increasing concentrations of BODIPY-12-GM<sub>1</sub> were incubated for 5 min at room temperature into wells coated with 20 μg/ml native (●) or heat-denatured (■) FGF-2. At the end of incubation, the BODIPY-12-GM<sub>1</sub> bound to the immobilized growth factor was extracted and measured with a spectrofluorimeter. In panel B, binding data were analyzed by the Scatchard plot procedure. They are representative of three independent experiments that gave similar results. (C) Aliquots (50 μl) containing 1 nmol of BODIPY-12-GM<sub>1</sub> were incubated for 5 min at room temperature into FGF-2-coated wells in the presence of 100 nmol of asialo-GM<sub>1</sub> (□) or of increasing concentrations of unlabeled GM<sub>1</sub> (●). At the end of incubation, the amount of BODIPY-12-GM<sub>1</sub> bound to the immobilized growth factor was extracted, measured, and compared with the amount of BODIPY-12-GM<sub>1</sub> bound to immobilized FGF-2 in the absence of any competitor. Each point is the mean of two determinations in duplicate. SEM never exceeded 5% of the mean value.

with FGF-2-GM<sub>1</sub> interaction. On this basis, BODIPY-12-GM<sub>1</sub> was utilized for further studies.

#### *Gangliosides Interact with the COOH Terminus of FGF-2*

To identify the region(s) of the FGF-2 molecule responsible for ganglioside interaction, 2.4 nmol of BODIPY-12-GM<sub>1</sub> were incubated for 10 min at room temperature with equimolar concentrations of synthetic peptides representing different regions of the FGF-2 molecule (in the present article, amino acid numbering 1–155 was utilized for FGF-2). Then, the mixtures were added to FGF-2-coated wells, and the capacity of the different FGF-2 fragments to prevent the binding of BODIPY-12-GM<sub>1</sub> to the immobilized growth factor was evaluated. An irrelevant basic peptide, represented by the basic domain (amino acid residues 41–60) of HIV-1 Tat, a protein able to bind heparin and other polyanionic compounds (Rusnati *et al.*, 1997b), and mastoparan, a peptide from wasp venom that binds to sialic acid residue(s) of gangliosides (Bueb *et al.*, 1990), were used as negative and positive controls, respectively. Among the FGF-2 peptides tested, only FGF-2(112–129) and, to a lesser extent, FGF-2(130–155) inhibit the binding of BODIPY-12-GM<sub>1</sub> to immobilized FGF-2 (Figure 5). Accordingly, two synthetic peptides containing both FGF-2 fragments and corresponding to amino acid sequences FGF-2(112–155) and FGF-2(116–155) inhibited the

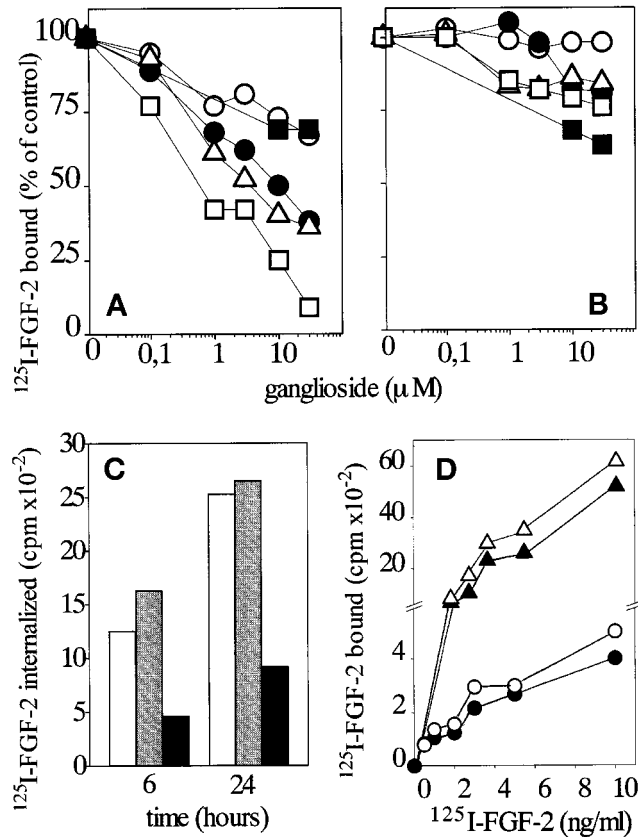


**Figure 5.** Mapping of the ganglioside-binding region of FGF-2. Aliquots (50 μl) containing 2.4 nmol of BODIPY-12-GM<sub>1</sub> were incubated for 5 min at room temperature into FGF-2-coated wells in the presence of 2.5 nmol of various synthetic peptides representing different fragments of FGF-2, of the basic domain of HIV-1-Tat, or of mastoparan. At the end of incubation, the amount of BODIPY-12-GM<sub>1</sub> bound to the immobilized growth factor was extracted, measured, and compared with the amount of fluorescent GM<sub>1</sub> bound to immobilized FGF-2 in the absence of any peptide. Each point is the mean ± SEM of two to three determinations in duplicate. \*, Statistically different from control (p < 0.05).

binding of BODIPY-12-GM<sub>1</sub> (Figure 5). Under the same experimental conditions, mastoparan abolished FGF-2-GM<sub>1</sub> interaction while the basic peptide HIV-1 Tat(41–60) was ineffective. Thus, the data implicate the COOH terminus of the FGF-2 molecule in ganglioside interaction.

#### *Gangliosides Inhibit FGF-2 Interaction with Tyrosine-Kinase FGF Receptor*

The above data prompted us to investigate whether the interaction of free gangliosides with FGF-2 is able to modulate the ability of the growth factor to bind to high-affinity tyrosine-kinase FGFRs and/or to low-affinity HSPGs in endothelial cells. To this purpose, experimental conditions (i.e., low temperature and short time of incubation) were adopted to minimize possible alterations of ligand-receptor interaction due to ganglioside uptake and incorporation into the cell membrane (Saqr *et al.*, 1993). On this basis, subconfluent cultures of endothelial GM 7373 cells were incubated at 4°C with 10 ng/ml <sup>125</sup>I-FGF-2 in the absence or in the presence of increasing concentrations of the different gangliosides. After 2 h, the amount of <sup>125</sup>I-FGF-2 associated with FGFRs and HSPGs was evaluated. As shown in Figure 6A, gangliosides inhibit the binding of <sup>125</sup>I-FGF-2 to FGFR in a dose-dependent manner. Among the gangliosides tested, GT<sub>1b</sub> showed the strongest antagonist activity and fully inhibited <sup>125</sup>I-FGF-2 binding to FGFRs at the dose of 30 μM. GM<sub>1</sub> and GD<sub>1b</sub> showed intermediate inhibitory capac-



**Figure 6.** Effect of exogenous gangliosides on the binding of  $^{125}\text{I}$ -FGF-2 to FGFRs and HSPGs and its internalization in endothelial cells. (A and B) Subconfluent cultures of GM 7373 cells were incubated for 2 h at  $4^\circ\text{C}$  with 10 ng/ml  $^{125}\text{I}$ -FGF-2 in the presence of the indicated concentrations of asialo-GM<sub>1</sub> (○), GM<sub>1</sub> (●), GD<sub>1b</sub> (△), GT<sub>1b</sub> (□), and sulfatide (■). At the end of incubation,  $^{125}\text{I}$ -FGF-2 bound to FGFRs (A) and to HSPGs (B) was evaluated as described in MATERIALS AND METHODS and expressed as percentage of the radioactivity measured in cell cultures incubated in the absence of ganglioside. Each point is the mean of three determinations in duplicate. SEM never exceeded 9% of the mean value. (C) Subconfluent cultures of GM 7373 cells were incubated for 2 h at  $4^\circ\text{C}$  with 10 ng/ml  $^{125}\text{I}$ -FGF-2 with no addition (open bars) or in the presence of asialo-GM<sub>1</sub> (gray bars) or of GT<sub>1b</sub> (black bars), both at  $30\ \mu\text{M}$ . At the end of incubation, cell cultures were shifted at  $37^\circ\text{C}$  and incubated at this temperature for the indicated periods of time. At the end of incubation, cell surface-associated  $^{125}\text{I}$ -FGF-2 was removed, and the amount of internalized  $^{125}\text{I}$ -FGF-2 was measured. The data are representative of two independent experiments in duplicate. (D) GM 7373 cells were incubated for 72 h at  $37^\circ\text{C}$  in the absence (●, ▲) or in the presence (○, △) of GM<sub>1</sub> ( $100\ \mu\text{M}$ ). At the end of the incubation, cell cultures were washed extensively and incubated for 2 h at  $4^\circ\text{C}$  with increasing concentrations of  $^{125}\text{I}$ -FGF-2 in the absence of free ganglioside. At the end of incubation,  $^{125}\text{I}$ -FGF-2 bound to FGFRs (circles) and to HSPGs (triangles) was evaluated as described in MATERIALS AND METHODS. The data are representative of four independent experiments in duplicate.

ity, whereas asialo-GM<sub>1</sub> and sulfatide were the least effective. At variance with the FGFR binding data, the gangliosides tested did not inhibit significantly the

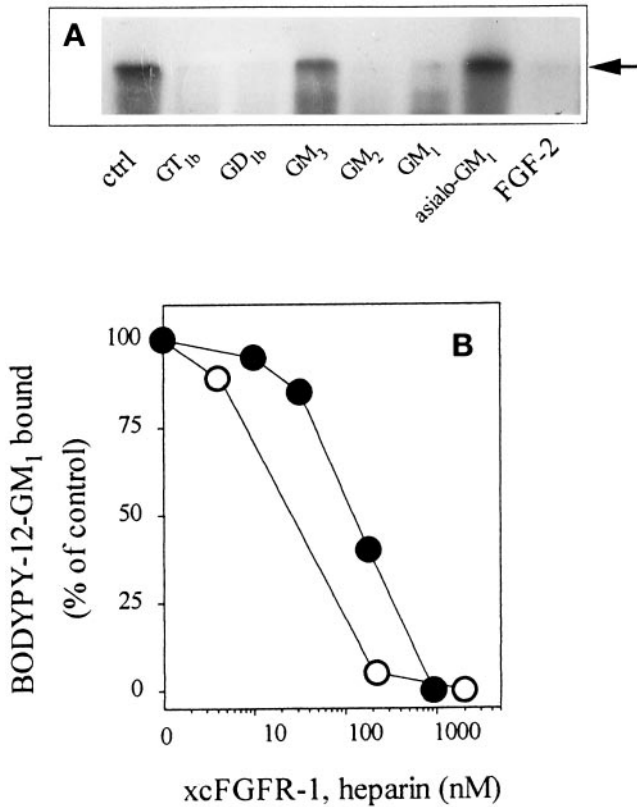
binding of  $^{125}\text{I}$ -FGF-2 to endothelial HSPGs, a limited effect being exerted by sulfatide only (Figure 6B). Finally, free NeuAc did not affect the binding of  $^{125}\text{I}$ -FGF-2 to FGFRs nor to HSPGs, even when tested at doses as high as  $300\ \mu\text{M}$  (our unpublished results).

FGF-2 interaction with the endothelial cell surface leads to its internalization (Roghani and Moscatelli, 1992; Rusnati *et al.*, 1993). On this basis, we investigated the effect of free gangliosides on FGF-2 internalization in GM 7373 cells. As shown in Figure 6C, GT<sub>1b</sub> inhibits both early and late internalization of  $^{125}\text{I}$ -FGF-2 into GM 7373 cells, while the control ganglioside asialo-GM<sub>1</sub> was ineffective. It must be pointed out that, because of the contribution of HSPGs to FGF-2 cell entry (Roghani and Moscatelli, 1992; Rusnati *et al.*, 1993), a limited internalization of FGF-2 occurs also in the presence of concentrations of GT<sub>1b</sub> ( $30\ \mu\text{M}$ ) sufficient to cause a complete inhibition of FGF-2 binding to FGFRs (see Figure 6A).

To rule out the possibility that the observed effects of gangliosides on FGF-2-FGFR interaction were due to plasma membrane alterations consequent to a limited incorporation of the ganglioside during the short-term binding assay, GM 7373 cells were exposed for 72 h to fresh medium containing 0.4% FCS in the absence or in the presence of  $100\ \mu\text{M}$  GM<sub>1</sub>. Under these conditions, gangliosides are efficiently incorporated into the cell (Saqr *et al.*, 1993). Accordingly, a significant increase of the content of plasma membrane-associated NeuAc (2.7 vs. 1.2 nmol of NeuAc/ $10^6$  cells) and of cytosolic NeuAc (6.0 vs. 4.0 nmol of NeuAc/ $10^6$  cells) was observed in GM<sub>1</sub>-treated cells in respect to control cells. This corresponds to an incorporation into the cells of  $\sim 2\%$  of the originally added exogenous GM<sub>1</sub>. After loading with the ganglioside, control and GM<sub>1</sub>-loaded cells were washed with ganglioside-free medium and incubated for 2 h at  $4^\circ\text{C}$  with increasing concentrations of  $^{125}\text{I}$ -FGF-2. As shown in Figure 6D, no significant differences were observed between control and GM<sub>1</sub>-loaded cells in the capacity of  $^{125}\text{I}$ -FGF-2 to bind to low-affinity HSPGs and high-affinity FGFRs. Taken together, the data demonstrate that exogenous free ganglioside, but not membrane-incorporated GM<sub>1</sub>, affects FGF-2-FGFR interaction in intact cells.

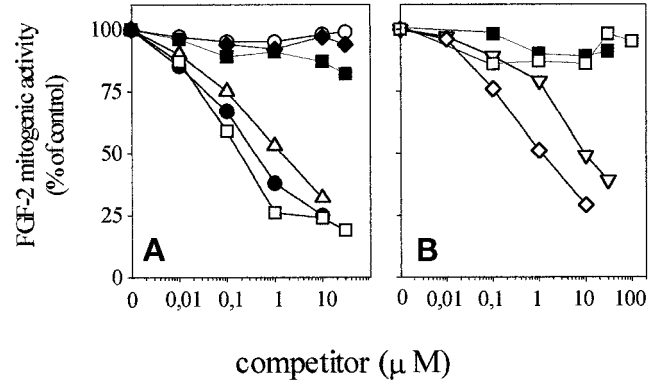
The capacity of gangliosides to prevent the binding of  $^{125}\text{I}$ -FGF-2 to cell-associated FGFRs prompted us to assess their ability to affect FGF-2 interaction with the extracellular domain of FGFR in a cell-free system. To this purpose,  $0.3\ \text{pmol}$  of  $^{125}\text{I}$ -FGF-2 was incubated for 2 h at  $37^\circ\text{C}$  with  $3\ \text{pmol}$  of a soluble extracellular form of FGFR-1/flg (xcFGFR-1) (Bergonzoni *et al.*, 1992) in the absence or in the presence of  $1.5\ \text{nmol}$  of the different gangliosides. At the end of incubation  $^{125}\text{I}$ -FGF-2-xcFGFR-1 complexes were chemically cross-linked and analyzed by SDS-PAGE followed by autoradiography of the gel. GM<sub>1</sub>, GM<sub>2</sub>, GD<sub>1b</sub>, and GT<sub>1b</sub>,





**Figure 7.** Effect of gangliosides on the binding of FGF-2 to soluble xcFGFR-1. (A)  $^{125}\text{I}$ -FGF-2 (0.3 pmol) was incubated for 2 h at 37°C with 3 pmol of recombinant, soluble xcFGFR-1 in the absence (ctrl) or in the presence of 1.5 nmol of the indicated gangliosides. Then,  $^{125}\text{I}$ -FGF-2-xcFGFR-1 complexes were chemically cross-linked with BSOCOES and analyzed by 10% SDS-PAGE followed by autoradiography of the gel. Arrow points to the cross-linked  $M_r$  69,000 kDa  $^{125}\text{I}$ -FGF-2-xcFGFR-1 complex (Rusnati *et al.*, 1994), which is abrogated by incubation with a 100-fold excess of unlabeled growth factor (FGF-2). (B) Aliquots (50  $\mu\text{l}$ ) containing 1 nmol of BODIPY-12-GM<sub>1</sub> were incubated into plastic dishes coated with 20  $\mu\text{g}/\text{ml}$  FGF-2 in the presence of increasing concentrations of xcFGFR-1 (●) or of heparin (○). At the end of incubation, fluorescent GM<sub>1</sub> bound to the immobilized growth factor was extracted, measured with a spectrofluorimeter, and compared with the amount of fluorescent GM<sub>1</sub> bound to immobilized FGF-2 in the absence of the competitor.

but not asialo-GM<sub>1</sub> and GM<sub>3</sub>, were able to prevent the binding of  $^{125}\text{I}$ -FGF-2 to xcFGFR-1, as demonstrated by the lack of appearance on the gel of the  $M_r$  68,000 radiolabeled band corresponding to the  $^{125}\text{I}$ -FGF-2-xcFGFR-1 complex (Figure 7A). Conversely, xcFGFR-1 is able to prevent the binding of 1 nmol of BODIPY-12-GM<sub>1</sub> to immobilized FGF-2 in a dose-dependent manner, a complete inhibition being observed at  $\sim 1$   $\mu\text{M}$  (corresponding to 50 pmol of soluble receptor per sample, Figure 7B). Under the same experimental conditions, free heparin prevents the interaction of BODIPY-12-GM<sub>1</sub> with FGF-2 at  $\sim 200$  nM (correspond-



**Figure 8.** Effect of exogenous gangliosides and related compounds on the mitogenic activity of FGF-2 in endothelial cells. Subconfluent cultures of GM 7373 cells were incubated for 24 h at 37°C with 10 ng/ml FGF-2 in the presence of increasing concentrations of asialo-GM<sub>1</sub> (○), GM<sub>1</sub> (●), GD<sub>1b</sub> ( $\Delta$ ), GT<sub>1b</sub> ( $\square$ ), galactosyl-ceramide (■), or sulfatide ( $\blacklozenge$ ) (panel A) or of GM<sub>2</sub> ( $\diamond$ ), GM<sub>3</sub> ( $\nabla$ ), NeuAc ( $\square$ ), N-acetylneuramin-lactose ( $\blacksquare$ ) (panel B). At the end of incubation, cells were trypsinized and counted in a Burker chamber. For the different experimental conditions, data have been calculated as cell population doublings during the 24-h incubation period and expressed as percentage of the mitogenic activity exerted by FGF-2 in the absence of any competitor (equal to 0.8 cell population doublings, see also Figure 9A). Each point is the mean of two to six determinations in duplicate. SEM never exceeded 16% of the mean value.

ing to 10 pmol per sample, Figure 7B). The fivefold weaker potency of xcFGFR-1, when compared with free heparin, in preventing the binding of BODIPY-12-GM<sub>1</sub> to immobilized FGF-2 is in keeping with the relative affinity of the two molecules for the growth factor ( $K_d$  equal to 5–10 and 1 nM for FGF-2-xcFGFR-1 and FGF-2-heparin interaction, respectively) (Bergonzoni *et al.*, 1992; Li and Seddon, 1994).

#### Gangliosides Affect the Biological Activity of FGF-2 in Endothelial Cells

To assess the biological consequences of FGF-2-ganglioside interaction, we evaluated the effects of free gangliosides onto the mitogenic activity exerted by FGF-2 on cultured endothelial cells in a short-term cell proliferation assay (Presta *et al.*, 1989). Confluent cultures of GM 7373 cells were incubated for 24 h with 10 ng/ml FGF-2 in the absence or in the presence of increasing concentrations of different gangliosides. At the end of incubation, cells were trypsinized and counted. As shown in Figure 8, sialo-gangliosides inhibit FGF-2 mitogenic activity in a dose-dependent manner. In contrast, asialo-GM<sub>1</sub>, sulfatide, and galactosyl-ceramide do not inhibit FGF-2-mediated cell proliferation, in keeping with their inability to inhibit FGF-2-FGFR interaction (see Figure 6A). As observed for FGF-2-ganglioside interaction (see above), the inhibitory potency of the various gangliosides depends,

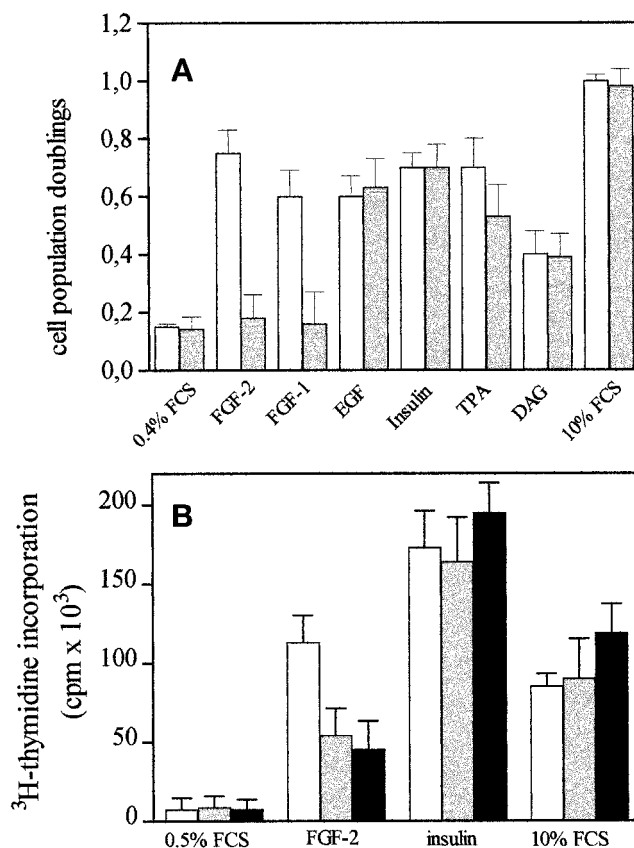
at least in part, on the number of NeuAc residues (GT<sub>1b</sub> being the most potent inhibitor, Figure 8A), to the length of the oligosaccharide chain (GM<sub>1</sub> being more potent than GM<sub>2</sub> and GM<sub>3</sub>, Figure 8B), and to the presence of a ceramide portion (free NeuAc and *N*-acetylneuramin-lactose being inactive, Figure 8B). The inhibitory effect exerted by gangliosides on the mitogenic activity of FGF-2 appears to be specific and restricted to the members of the FGF family. Indeed, 10  $\mu$ M GM<sub>1</sub> inhibits the mitogenic activity of FGF-2 and FGF-1 without affecting cell proliferation induced by the phorbol ester 12-*O*-tetradecanoyl phorbol 13-acetate, 1,2-dioctanoyl-*sn*-glycerol, 10% FCS, EGF, or insulin (Figure 9). It must be pointed out that the lack of inhibitory activity of GM<sub>1</sub> on FGF-independent stimuli does not reflect the relative potency of the mitogen under test, the ganglioside being equally ineffective when cells were stimulated by a potent inducer (e.g., 10% FCS) or by a much weaker mitogen (e.g., 1,2-dioctanoyl-*sn*-glycerol).

The FGF-2-antagonist activity of gangliosides is not restricted to GM 7373 cells. Indeed, GT<sub>1b</sub> inhibits DNA synthesis induced by FGF-2 in MAE cells in culture (Figure 9B). Also, in this case the inhibitory effect appears to be specific since GT<sub>1b</sub> does not affect [<sup>3</sup>H]thymidine incorporation stimulated by insulin or 10% FCS.

## DISCUSSION

Previous observations had shown that gangliosides can modulate the biological activity of FGF-2 in vitro (Bremer and Hakomori, 1982; De Cristian *et al.*, 1990) and in vivo (Ziche *et al.*, 1989, 1992). Here we demonstrate that FGF-2 binds to gangliosides in solution. This interaction is able to prevent the binding of FGF-2 to tyrosine-kinase FGFRs with a consequent inhibition of the mitogenic activity exerted by the growth factor on endothelial cells.

FGF-2-ganglioside interaction depends upon defined structural features of both molecules. Binding to FGF-2 and consequent inhibition of receptor binding and mitogenic activity of the growth factor occur in the micromolar range of concentrations of ganglioside, above its critical micellar concentration (Formisano *et al.*, 1979; Ulrich-Bott and Wiegandt, 1984). Under these experimental conditions the oligosaccharide chain of the glycosphingolipid is exposed to the aqueous environment and available for FGF-2 interaction. Several observations point to the importance of NeuAc residues of the oligosaccharide chain in this interaction. Indeed, the relative potency of the ganglioside in protecting FGF-2 from trypsin digestion, in inhibiting its interaction with cell surface FGFRs, and in preventing its mitogenic action appears to be related, at least in part, to the number of sialic acid residues present on the glycosphingolipid, GT<sub>1b</sub> being usually the most effective. Moreover, the lack of NeuAc groups in the



**Figure 9.** Effect of GM<sub>1</sub> on the mitogenic activity of different endothelial cell mitogens. (A) Subconfluent cultures of GM 7373 cells were incubated for 24 h at 37°C in 0.4% FCS with no addition (control) or with FGF-2 (10 ng/ml), FGF-1 (30 ng/ml), EGF (30 ng/ml), insulin (10  $\mu$ g/ml), 10% FCS, 12-*O*-tetradecanoyl phorbol 13-acetate (TPA, 30 ng/ml), or 1,2-dioctanoyl-*sn*-glycerol (DAG, 5  $\mu$ g/ml) in the absence (white bars) or in the presence (gray bars) of 10  $\mu$ M GM<sub>1</sub>. At the end of incubation, cells were trypsinized and counted in a Burker chamber. Data are expressed as cell population doublings during the 24-h incubation period. Each point is the mean  $\pm$  SEM of two to six determinations in duplicate. All mitogens induce a statistically significant increase of cell proliferation rate (Student's *t* test, *p* < 0.05). (B) MAE cells were incubated for 2 d with 0.5% FCS. Quiescent cell cultures were then treated with vehicle (0.5% FCS), FGF-2 (30 ng/ml), insulin (10  $\mu$ g/ml), or 10% FCS in the absence (white bars) or in the presence of 30  $\mu$ M (gray bars) or 100  $\mu$ M (black bars) of GT<sub>1b</sub>. After 16 h, cells were pulse labeled with [<sup>3</sup>H]thymidine (1  $\mu$ Ci/ml) for 6 h. The amount of radioactivity incorporated into the trichloroacetic acid-precipitable material was measured. Each point is the mean  $\pm$  SEM of three determinations in triplicate. At both concentrations, GT<sub>1b</sub> causes a statistically significant decrease of DNA synthesis induced by FGF-2 (Student's *t* test, *p* < 0.05).

oligosaccharide chain impairs the capacity of the ganglioside to interact with FGF-2, as observed for asialo-GM<sub>1</sub>. However, sialic acid alone fails to bind to the growth factor. Also, the presence of one sialic acid residue in *N*-acetylneuramin-lactose is not sufficient to confer to this molecule the capacity to interact with FGF-2. Taken together, these data indicate that NeuAc

must be presented to FGF-2 in the context of a defined glycolipidic structure to exert its FGF-2 binding capacity.

In *N*-acetylneuramin-lactose, which is unable to bind FGF-2, NeuAc is linked to a short glucose-galactose disaccharide. This structure is comparable to that of the oligosaccharide chain of GM<sub>3</sub> (see Table 1) that, among the monosialo-gangliosides tested, has the shortest oligosaccharide chain and the poorest FGF-2 antagonist activity. This suggests that the length and structure of the oligosaccharide chain are also of importance in determining the FGF-2-binding activity of the ganglioside. The lack of FGF-2-binding activity of *N*-acetylneuramin-lactose, when compared with GM<sub>3</sub>, may also suggest that the ceramide portion of the ganglioside is involved in FGF-2 interaction, as supported by the observation that galactosyl-ceramide protects FGF-2 from trypsin digestion with a potency similar to that of GM<sub>3</sub>. Taken together, the results indicate that FGF-2-ganglioside interaction occurs via NeuAc residue(s) and is strictly regulated by other components of the glycolipidic structure. Similar conclusions have been drawn from sialic acid recognition studies of selectins to sialylated Lewis blood group epitopes (McEver *et al.*, 1995). Also in this case, selectin interaction depends not only on the presence and structure of the sialic acid residue but also on that of other saccharide residues (i.e., fucose and galactose) in the oligosaccharide chain.

Sulfation of sialyl Lewis X is of importance for selectin interaction (Rosen and Bertozzi, 1994), and anionic groups as sulfates can equal the protein-recognition properties of sialic acids, as shown by the capacity of L- and P-selectins to bind to sulfatides and subsets of heparin fragments (Rosen and Bertozzi, 1994). Accordingly, our data demonstrate that sulfatide can bind FGF-2 and protect it from proteolytic cleavage with a potency similar to mono-sialo gangliosides. These observations are of particular relevance when the heparin-binding properties of FGF-2 and its capacity to interact with various polysulfated/polysulfonated compounds are considered (Coltrini *et al.*, 1993). On this basis, the possibility that negatively charged NeuAc groups might interact with the strongly cationic FGF-2 molecule could be anticipated. However, our observations indicate that the binding of sialo-gangliosides to FGF-2 is not the consequence of a mere electrostatic interaction but depends upon specific structural features of the growth factor. Indeed, FGF-2 must be present in a proper three-dimensional conformation to interact with gangliosides. Also, a ganglioside-binding region has been identified in the COOH terminus of the FGF-2 molecule by synthetic peptide-binding experiments. Peptide FGF-2(112–129) and to a lesser extent peptide FGF-2(130–155), as well as peptides FGF-2(112–155) and FGF-2(116–155), were able to prevent the binding of BODIPY-12-GM<sub>1</sub> to the immobilized growth factor, whereas peptides FGF-2(10–

34), FGF-2(39–59), and FGF-2(82–96) were ineffective (note that in the present work amino acid numbering 1–155 has been used for FGF-2). The specificity of these observations is confirmed by the inability of the highly charged basic peptide HIV-1 Tat(41–60) to bind GM<sub>1</sub> under the same experimental conditions.

Peptide FGF-2(112–129) and larger FGF-2 fragments containing this amino acid sequence had been shown to bind heparin and to prevent the binding of FGF-2 to its high-affinity FGFRs, suggesting that this region (formerly known as the putative receptor-binding loop) is involved in receptor recognition and binding (Baird *et al.*, 1988). More recent observations, based on site-directed mutagenesis of the FGF-2 molecule, x-ray crystallography data, isothermal titrating calorimetry, and computer modeling, have indicated that two separate receptor-binding sites adjacent to a discontinuous heparin-binding domain exist in FGF-2 (Pantoliano *et al.*, 1994; Springer *et al.*, 1994; Thompson *et al.*, 1994). This allows the formation of heparin-FGF-2-FGFR ternary complexes (Pantoliano *et al.*, 1994). The primary, high-affinity receptor-binding site is comprised of six discontinuous residues that are located on the same face of the FGF-2 molecule. The second, low-affinity receptor-binding site is a surface-exposed type I  $\beta$ -turn within the putative receptor-binding loop and is composed of residues FGF-2(120–124). This region is required for receptor dimerization *in vitro*, and mitogenic signal transduction in cultured cells (Springer *et al.*, 1994). We have observed that gangliosides hamper the capacity of <sup>125</sup>I-FGF-2 to complex in solution with the recombinant form of xcFGFR-1 and prevent its interaction with FGFRs present on the endothelial cell surface. However, none of the gangliosides tested prevent the binding of <sup>125</sup>I-FGF-2 to GM 7373 cell surface HSPGs, even though this interaction occurs with a much lower affinity than FGF-2-FGFR interaction ( $K_d$  equal to 300 and 20 pM for the two interactions, respectively). The higher abundance of cell-surface HSPGs in respect to FGFRs ( $4.4 \times 10^5$  vs.  $1.6 \times 10^4$  binding sites/cell, respectively) may explain this apparent discrepancy (Rusnati *et al.*, 1993). Indeed, under appropriate experimental conditions, both heparin and soluble xcFGFR-1 can prevent the binding of fluorochrome-labeled GM<sub>1</sub> to immobilized FGF-2 (see Figure 7B). Thus, gangliosides bind to COOH-terminal region(s) of FGF-2 overlapping or adjacent to those involved in heparin-heparan sulfate and FGFR interactions.

These observations raise the possibility that exogenous gangliosides may exert a FGF-2 antagonist activity by a direct interaction with the growth factor, thus preventing its binding to tyrosine-kinase FGFRs. This hypothesis is supported by experimental evidence indicating that the structural features of the ganglioside required to bind FGF-2 and protect it from trypsin digestion are similar to those required to prevent



FGFR binding and mitogenic activity. Moreover, both GM 7373 cells and MAE cells proliferate when exposed to different tyrosine-kinase- and protein kinase C-dependent mitogens, including phorbol ester, diacyl-glycerol, serum, EGF, or insulin, in the presence of concentrations of ganglioside sufficient to inhibit the mitogenic activity exerted by FGF-2. This demonstrates that the inhibitory activity exerted by gangliosides is specific for FGF-2 and is not the consequence of a general impairment of the capacity of endothelial cells to respond to mitogenic stimuli. Interestingly, gangliosides are also able to inhibit the mitogenic activity of FGF-1, another member of the FGF family that shares with FGF-2 various structural and biological features, including FGFR- and HSPG-binding capacity (Jonhson and Williams, 1993). These findings, together with the observation that FGF-1, FGF-2, and FGF-4 are protected from trypsin digestion by GT<sub>1b</sub>, suggest that different members of the FGF family share structural features responsible for ganglioside interaction.

As stated above, sulfatide binds FGF-2 and protects it from proteolytic cleavage. Nevertheless, free sulfatide is unable to prevent FGF-2–FGFR interaction and to inhibit FGF-2–mediated cell proliferation. These observations indicate that the capacity of a molecule to interact with FGF-2 *in vitro* does not necessarily reflect its FGF-2 antagonist potential. Similar conclusions had been drawn for FGF-2–binding heparin derivatives (Ishihara *et al.*, 1993; Coltrini *et al.*, 1994). For instance, *N*-desulfated/*N*-acetylated beef lung heparin is as potent as unmodified heparin in preventing the proteolytic digestion of FGF-2, but it is highly inefficient in inhibiting the receptor-binding and mitogenic activity of the growth factor (Coltrini *et al.*, 1994). Thus, the capacity of a molecule to bind FGF-2 in a cell-free system and to modulate its biological activity can be dissociated at the structural level. This appears to be of importance for the development of synthetic FGF-2 inhibitors.

Here we have shown that a short-term incubation of GM 7373 cells with FGF-2 in the presence of free gangliosides causes an inhibition of the receptor-binding and mitogenic activity of the growth factor. In apparent contrast with these observations, De Cristian *et al.* (1990) demonstrated that a 6-h preincubation of endothelial cells with GT<sub>1b</sub> followed by a further 72-h incubation in the presence of both GT<sub>1b</sub> and FGF-2 increases the mitogenic activity of the growth factor. The addition of exogenous gangliosides to cell cultures is a widely used approach to investigate their effects on cell behavior. However, the diversified conditions under which they are added to cultured cells cause different degrees of ganglioside incorporation into cell membrane, making comparison among experiments difficult (Saqr *et al.*, 1993). Conflicting results may therefore depend on the free or cell-associated

status of the ganglioside and reflect different mechanisms of action of these glycolipids. Our data demonstrate that free gangliosides present in their micellar form in the cell culture medium bind and sequester FGF-2, preventing its interaction with cell-surface FGFRs. In contrast, cell membrane-incorporated glycolipids may regulate the biological activity of FGF-2 in the absence of free gangliosides by different mechanisms of action, possibly by affecting the activity of tyrosine kinase receptors and intracellular signaling, as already demonstrated for various growth factors, including PDGF and EGF (see INTRODUCTION). Accordingly, we have observed that a 72-h incubation of GM 7373 cells with GM<sub>1</sub> leads to a significant incorporation of the glycolipid into the cell membrane. Even though this does not result in a significant modification of the capacity of the cells to bind FGF-2 (see above), GM<sub>1</sub> preloaded cells proliferate more efficiently than control cells in response to FGF-2 with a consequent 10-fold increase of the potency of the growth factor (ED<sub>50</sub> equal to 1.0 and 10 ng/ml FGF-2 for GM<sub>1</sub> preloaded and control cells, respectively) (Rusnati and Urbinati, unpublished data). In agreement with this hypothesis is the observation that membrane-associated gangliosides modulate the biological activity of FGF-2 in fibroblasts and glial cells in the absence of a direct interaction with the growth factor (Bremer and Hakomori, 1982; Meuillet *et al.*, 1996a,b), probably by regulating tyrosine autophosphorylation of FGFR (Meuillet *et al.*, 1996a,b).

Taken together, the data suggest that exogenous free gangliosides and membrane-incorporated glycolipids can modulate the activity of FGF-2 by different mechanisms of action. It is interesting to note that soluble and cell-associated sulfated glycosaminoglycans have also been demonstrated to play contrasting roles in modulating the biological activity of FGF-2 (Rusnati and Presta, 1996). As observed for exogenous gangliosides, soluble glycosaminoglycans protect FGF-2 from proteolytic cleavage and inhibit FGF-2–FGFR interaction and FGF-2–dependent cell proliferation (Coltrini *et al.*, 1994; Rusnati *et al.*, 1994). In contrast, cell-associated HSPGs increase the local concentration of FGF-2 and modulate FGFR binding, dimerization, and signaling, thus promoting the biological activity of the growth factor (Rusnati and Presta, 1996).

In conclusion, we have demonstrated that exogenous free gangliosides 1) protect FGF-2 from proteolytic degradation; 2) modulate the binding of the growth factor to tyrosine kinase FGFRs; 3) modulate cell internalization of FGF-2; and 4) inhibit FGF-2–dependent endothelial cell proliferation. All these effects occur at concentrations of free ganglioside between 0.3 and 30  $\mu$ M. During tumor growth and metastasis, gangliosides shed in the microenvironment (Kloppel *et al.*, 1977; Merritt *et al.*, 1994; Chang *et al.*, 1997). This process can be so extensive as to alter

the ganglioside composition of the extracellular environment of the tumor (Kloppel *et al.*, 1977). It has been demonstrated that tumor cells can shed up to 0.5% of their membrane ganglioside content per hour (Li and Ladisch, 1991) and that gangliosides are present at concentrations as high as 10  $\mu$ M in the serum of tumor-bearing patients (Valentino and Ladisch, 1992). On this basis, because of the possible role of FGF-2 in tumor angiogenesis (Rak and Kerbel, 1997), gangliosides shed by tumor cells may affect endothelial cell function by interacting with FGF-2, thus modulating tumor neovascularization.

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