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, $^{125}$I-FGF-2 binds to micelles formed by gangliosides GT1b, GD1b, or GM1. Also, gangliosides protect native FGF-2 from trypsin digestion at micromolar concentrations, the order of relative potency being GT1b $>$ GD1b $>$ GM1 = GM2 $>$ sulfatide $>$ GM3 = galactosyl-ceramide, whereas asialo-GM1, neuraminic acid, and N-acetylneuramin-lactose were ineffective. Scatchard plot analysis of the binding data of fluorochrome-labeled GM1 to immobilized FGF-2 indicates that FGF–2/GM1 interaction occurs with a $K_d$ equal to 6 $\mu$M. 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 $^{125}$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 GT1b $>$ GD1b $>$ GM1 $>$ sulfatide a = sialo-GM1. Accordingly, GT1b, GD1b, GM1, and GM2, but not GM3 and asialo-GM1, prevent the binding of $^{125}$I-FGF-2 to a soluble, recombinant form of extracellular FGFR-1. Conversely, the soluble receptor and free heparin inhibit the interaction of fluorochrome-labeled GM1 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, GT1b was the most effective among the gangliosides tested while asialo-GM1, 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.
INTRODUCTION

Gangliosides are neuraminic acid (NeuAc) 1-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 interactions (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-γ (IFN-γ), IL-2, IL-4 (Hoons et al., 1991; Ando et al., 1996), tumor necrosis factor-α (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 β, tumor necrosis factor-α, and IL-6 (Ziegler-Heitbrock et al., 1992; Dumontet et al., 1994) while GD3 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 GM3, GM2, and GT1b 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). GM1 and GM3 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, GM2 and GT1 are able to modulate the antiviral activity of human IFN (Besancon and Ankel, 1974; Vengris et al., 1976). Finally, glycosylceramide synthesis has been demonstrated to be required for FGF-2 to stimulate axonal growth (Boldin and Futterman, 1997). Accordingly, gangliosides influence FGF-2-dependent mitogenesis and migration of glial cells (Meuillet et al., 1996a,b), and GM3 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 GM3 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 (Noji et al., 1991; Van Brocklyn et al., 1993; Hyndes 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 GM1 and GM3 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 GM1 and GM3 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; Cockrell et al., 1995; Gullino, 1995). Interestingly, GM3 and GM4 inhibit FGF-2–mediated endothelial cell proliferation, and the addition of GD3 restores

1 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).
optimal levels of cell growth (Alessandri et al., 1992; Ziche et al., 1992). In contrast, GD3 enhances the chemotactic activity exerted by FGF-2 on endothelial cells, which is counteracted by GM3 (Ziche et al., 1992). Moreover, GM3, GD1b, and GT1b 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 GM3:GD3 molar ratio (Ziche et al., 1989). An increase of the angiogenic activity of FGF-2 can also be obtained by increasing the local concentration of GM1 and GT1b (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/polyuronated compounds point to the possibility that anionic NeuAc groups of sialo-gangliosides may mimic sulfated/polysulfonated 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. Basilico (New York University Medical Center, New York, NY). The recombiant, soluble form of the extracellular domain of FGFR-1/βg (scFGFR-1) (Bergonzoni et al., 1992) was provided by A. Isacchi (Pharmacia-Upjohn, Nerviano, Italy). Synthetic peptides 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-dodecanoyl acid (BODIPY-dodecanoil acid) was obtained from Molecular Probes (Eugene, OR). Glycans, N-acetylneuraminic-lactose, and mastoparan were from Sigma (St. Louis, MO). Sulfatide was prepared from pig brain by the method of Harano 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.

Interaction of FGF-2 with Gangliosides

FGF-2 was iodinated as described (Neufeld and Gospodarowicz, 1985) at a specific radioactivity equal to 800 cpm/fmol. GM 7373 cells were incubated at 4°C in serum-free medium containing 10% FCS. The 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).

125I-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 cells were incubated at 4°C in serum-free medium containing 10 ng/ml 125I-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 125I-FGF-2 bound to low- and high-affinity binding sites was evaluated as described (Moscattelli, 1987). Briefly, after a PBS wash, cells were rinsed twice with 2 M NaCl in 20 mM HEPES buffer (pH 7.5) to remove 125I-FGF-2 bound to low-affinity binding sites and twice with 2 M NaCl in 20 mM sodium acetate (pH 4.0) to remove 125I-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 GM1 before the 125I-FGF-2 cell-binding assay. To this purpose, cells were seeded at 70,000 cells/cm² 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 GM1. At the end of incubation, cells were extensively washed with PBS and incubated...
at 4°C in serum-free medium containing 10 ng of $^{125}$I-FGF-2 per ml in the absence of free ganglioside. After 2 h, the amount of $^{125}$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 × 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 $^{125}$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 $^{125}$I-FGF-2 was removed as described above, and cell-internalized $^{125}$I-FGF-2 was recovered by lysing the cells with 0.1 mM Trits-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/cm² in 24-well dishes and incubated for 20,100 h with 0.5% FCS. Quiescent cell cultures were then supplemented with BODIPY-12-GM1 was synthesized and purified according to previously described procedures (Marchesini et al., 1994) by acylation of lyso-GM1 with the N-hydroxy succinimide ester of BODIPY-dodecanic acid.

Coating of FGF-2 to Plastic and Binding Assay

Aliquots (100 µl) of 100 mM NaHCO₃ (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 $^{125}$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-GM₁ 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 GM₁, heparin, xcFGFR-1, or synthetic FGF-2 fragments. At the end of incubation, wells were washed three times with PBS, and FGF-2-associated GM₁ was eluted from the wells with 100 µl of methanol-chloroform solution (40:60, vol/vol) and measured with a FCT-150 spectrophotofluorimeter (Jacso 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-GM₁ with FGF-2, 100-µl aliquots of PBS containing different concentrations of labeled GM₁ were added into wells coated with 20 µg/ml 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 $^{125}$I-FGF-2 to xcFGFR-1

$^{125}$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 FGF-1/Rg (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 $^{125}$I-FGF-2 were cross-linked by adding 1 mM bis[(succinimido-carbonyloxy)ethyl] sulfone (BSOCES, 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 $^{125}$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 Wiegangt, 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 × 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 $^{125}$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 $^{125}$I-FGF-2 were preincubated for 10 min at room temperature with 125 nmol of GM₁ (final concentration of the ganglioside equal to 1.25 × 10⁻³ M) and then loaded onto the Superose-12
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$_1$ 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$_1$ (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 in the molecule, the order of relative potency of the gangliosides tested being GT$_{1b}$ > GD$_{1b}$ > GM$_4$. It should be pointed out that GT$_{1b}$ 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$_1$ does not prevent tryptic digestion of FGF-2 (Figure 2B). In addition, however, free NeuAc and N-acetylmuramyl-lactose, a disaccharide bearing one NeuAc group, do not protect FGF-2 from tryptic digestion at doses up to 250 μM (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$_3$ shows a reduced capacity to bind and protect FGF-2 from trypsin digestion when compared with GM$_2$ and GM$_1$ (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

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**Figure 1.** Size-exclusion chromatography of FGF-2–ganglioside complexes. (A) 100 μl samples containing 3 pmol of $^{125}$I-FGF-2 (●) or 125 nmol of GM$_1$ (○) 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. Molecular size standards (in thousands) were ferritin (Mr 440,000), that eluted with the void volume of the column (V$_0$), IgG (Mr 150,000), ovalbumin (Mr 45,000), soybean trypsin inhibitor (Mr 20,100), and cytochrome C (Mr 12,000).

Column. Under these experimental conditions, $^{125}$I-FGF-2 preincubated with GM$_1$ 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$_1$ complexes (Figure 1B). Similar results were obtained when $^{125}$I-FGF-2 was preincubated with the same doses of GM$_2$, GM$_{3b}$, GD$_{1b}$, or GT$_{1b}$ (our unpublished results), whereas asialo-GM$_1$ was unable to complex the growth factor (Figure 1B). Also, no $^{125}$I-bFGF–GM$_1$ 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.
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 GM₁ and GM₂, whereas galactosylceramide 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 GT₁b, 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 GM₁. As shown in Figure 4A, fluorochrome-labeled GM₁ binds to immobilized FGF-2. The binding was dose-dependent and saturable, specificity being demonstrated by the incapacity of BODIPY-12-GM₁ to interact with immobilized heat-denatured FGF-2. Scatchard plot analysis of the binding data indicates that BODIPY-12-GM₁ binds to immobilized FGF-2 with a $K_d$ equal to 6.3 ± 2 μM (Figure 4B). Unlabeled GM₁ competed for the binding of BODIPY-12-GM₁ to FGF-2 in a dose-dependent manner, half-maximal inhibition being observed at equimolar concentrations of the two compounds (Figure 4C). Asialo-GM₁ 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 2](image1.png)  
**Figure 2.** Protection of FGF-2 from tryptic digestion by gangliosides and related molecules. (A) Representative experiment in which 1 μg aliquots of FGF-2 were incubated at 37°C for 3 h with 60 ng of trypsin in the absence or in the presence of the indicated amounts of GM₁. Then samples were analyzed by 15% SDS-PAGE followed by silver staining of the gel. (B and C) Aliquots (1 μg) of FGF-2 were incubated with trypsin in the presence of the indicated amounts of asialo-GM₁ (○), GM₁ (●), GD₁b (♦), GT₁b (□) (panel B) or GM₁ (●), NeuAc (□), N-acetylneuramin-lactose (△), GM₂ (◇), GM₃ (◆), 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.

![Figure 3](image2.png)  
**Figure 3.** Ganglioside-mediated protection of FGF-1 and FGF-4 from tryptic digestion. Aliquots (1 μg) of native or heat-denatured (h.d.) FGF-2, FGF-1, or FGF-4 were incubated at 37°C for 3 h with 60 ng of trypsin in the absence or in the presence of 8 nmol of GT₁b. Then samples were analyzed by 15% SDS-PAGE followed by silver staining of the gel.
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-GM1 were incubated for 5 min at room temperature into FGF-2-coated wells in the presence of 100 nmol of asialo-GM1 or of increasing concentrations of unlabeled GM1. At the end of incubation, the amount of BODIPY-12-GM1 bound to the immobilized growth factor was extracted, measured, and compared with the amount of BODIPY-12-GM1 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.

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 GM7373 cells were incubated at 4°C with 10 ng/ml125I-FGF-2 in the absence or in the presence of increasing concentrations of the different gangliosides. After 2 h, the amount of 125I-FGF-2 associated with FGFR and HSPGs was evaluated. As shown in Figure 6A, gangliosides inhibit the binding of 125I-FGF-2 to FGF in a dose-dependent manner. Among the gangliosides tested, GT1b showed the strongest antagonist activity and fully inhibited 125I-FGF-2 binding to FGFR 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 $^{125}$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 $^{125}$I-FGF-2 to FGFRs nor to HSPGs, even when tested at doses as high as 300 μM (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$_{1b}$ inhibits both early and late internalization of $^{125}$I-FGF-2 into GM 7373 cells, while the control ganglioside asialo-GM$_{1}$ 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$_{1b}$ (30 μM) 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 μM GM$_{1}$. 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$_{1}$-treated cells in respect to control cells. This corresponds to an incorporation into the cells of ~2% of the originally added exogenous GM$_{1}$. After loading with the ganglioside, control and GM$_{1}$-loaded cells were washed with ganglioside-free medium and incubated for 2 h at 4°C with increasing concentrations of $^{125}$I-FGF-2. As shown in Figure 6D, no significant differences were observed between control and GM$_{1}$-loaded cells in the capacity of $^{125}$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$_{1}$, affects FGF-2–FGFR interaction in intact cells.

The capacity of gangliosides to prevent the binding of $^{125}$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 pmol of $^{125}$I-FGF-2 was incubated for 2 h at 37°C with 5 pmol of a soluble extracellular form of FGF-1/flg (xcFGFR-1) (Bergonzoni et al., 1992) in the absence or in the presence of 1.5 nmol of the different gangliosides. At the end of incubation $^{125}$I-FGF-2–xcFGFR-1 complexes were chemically cross-linked and analyzed by SDS-PAGE followed by autoradiography of the gel. GM$_{1}$, GM$_{2}$, GD$_{1b}$, and GT$_{1b}$.
but not asialo-GM₁ and GM₃, were able to prevent the binding of ¹²⁵I-FGF-2 to soluble xcFGFR-1, as demonstrated by the lack of appearance on the gel of the Mr 68,000 radiolabeled band corresponding to the ¹²⁵I-FGF-2–xcFGFR-1 complex (Figure 7A). Conversely, xcFGFR-1 is able to prevent the binding of 1 nmol of BODIPY-12-GM₁ to immobilized FGF-2 in a dose-dependent manner, a complete inhibition being observed at ~1 µM (corresponding to 50 pmol of soluble receptor per sample, Figure 7B). Under the same experimental conditions, free heparin prevents the interaction of BODIPY-12-GM₁ with FGF-2 at ~200 nM (corresponding 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-GM₁ to immobilized FGF-2 is in keeping with the relative affinity of the two molecules for the growth factor (Kₐ 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). 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-GM₁ (○), GM₁ (●), GD₁b (■), GT₁b (□), galactosyl-ceramide (■), or sulfatide (△) (panel A) or of GM₂ (▲), GM₃ (◇), NeuAc (□), N-acetyllactosamin-lactose (■) (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.

Figure 7. Effect of gangliosides on the binding of FGF-2 to soluble xcFGFR-1. (A) ¹²⁵I-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, ¹²⁵I-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 Mr 69,000 kDa ¹²⁵I-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 µl) containing 1 nmol of BODIPY-12-GM₁ were incubated into plastic dishes coated with 20 µg/ml FGF-2 in the presence of increasing concentrations of xcFGFR-1 (●) or of heparin (○). At the end of incubation, fluorescent GM₁ bound to the immobilized growth factor was extracted, measured with a spectrofluorimeter, and compared with the amount of fluorescent GM₁ bound to immobilized FGF-2 in the absence of the competitor.
the glycosphingolipid, GT1b being usually the most part, to the number of sialic acid residues present on its mitogenic action appears to be related, at least in interaction with cell surface FGFRs, and in preventing detecting FGF-2 from trypsin digestion, in inhibiting its producing structural features of both molecules. Binding to the micromolar range of concentrations of ganglioside, and mitogenic activity of the growth factor occur in experimental conditions the oligosaccharide chain of above its critical micellar concentration (Formisano, 1979; Ulrich-Bott and Wiegandt, 1984). Under these conditions the ganglioside 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 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, GT1b being usually the most effective. Moreover, the lack of NeuAc groups in the oligosaccharide chain impairs the capacity of the ganglioside to interact with FGF-2, as observed for asialo-GM1. 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

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.

The FGF-2-antagonist activity of gangliosides is not restricted to GM 7373 cells. Indeed, GT1b inhibits DNA synthesis induced by FGF-2 in MAE cells in culture (Figure 9). Also, in this case the inhibitory effect appears to be specific since GT1b does not affect [3H]thymidine incorporation stimulated by insulin or 10% FCS.
must be presented to FGF-2 in the contest 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 GM₃ (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 GM₃, 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 GM₃. 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 that of GM₃. Taken together, these observations are of particular relevance when the heparin-binding properties of FGF-2 and its capacity to interact with various polysulfated/poly sulfonated 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-GM₁ 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 GM₁ 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, isotermal 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–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 β-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 ¹²⁵I-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 ¹²⁵I-FGF-2 to GM 7373 cell surface HSPGs, even though this interaction occurs with a much lower affinity than FGF-2–FGFR interaction (K₄ equal to 300 and 20 pM for the two interactions, respectively). The higher abundance of cell-surface HSPGs in respect to FGFRs (4.4 × 10⁵ vs. 1.6 × 10⁴ 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 GM₁ 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 FGFR-2. This demonstrates that the inhibitory activity exerted by gangliosides is specific for FGFR-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 FGFR-1, another member of the FGFR family that shares with FGFR-2 various structural and biological features, including FGFR- and HSPG-binding capacity (Jonhson and Williams, 1993). These findings, together with the observation that FGFR-1, FGFR-2, and FGFR-4 are protected from trypsin digestion by GT1b, suggest that different members of the FGFR family share structural features responsible for ganglioside interaction.

As stated above, sulfatide binds FGFR-2 and protects it from proteolytic cleavage. Nevertheless, free sulfatide is unable to prevent FGFR-2–FGFR interaction and to inhibit FGFR-2–mediated cell proliferation. These observations indicate that the capacity of a molecule to interact with FGFR-2 in vitro does not necessarily reflect its FGFR-2 antagonist potential. Similar conclusions had been drawn for FGFR-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 FGFR-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 FGFR-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 FGFR-2 inhibitors.

Here we have shown that a short-term incubation of GM 7373 cells with FGFR-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 GT1b followed by a further 72-h incubation in the presence of both GT1b and FGFR-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 FGFR-2, preventing its interaction with cell-surface FGFRs. In contrast, cell membrane-incorporated glycolipids may regulate the biological activity of FGFR-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 GM1 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 FGFR-2 (see above), GM1 preloaded cells proliferate more efficiently than control cells in response to FGFR-2 with a consequent 10-fold increase of the potency of the growth factor (ED50 equal to 1.0 and 10 ng/ml FGFR-2 for GM1 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 FGFR-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 FGFR-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 FGFR-2 (Rusnati and Presta, 1996). As observed for exogenous gangliosides, soluble glycosaminoglycans protect FGFR-2 from proteolytic cleavage and inhibit FGFR-2–FGFR interaction and FGFR–dependent cell proliferation (Coltrini et al., 1994; Rusnati et al., 1994). In contrast, cell-associated HS-PGs increase the local concentration of FGFR-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 FGFR-2 from proteolytic degradation; 2) modulate the binding of the growth factor to tyrosine kinase FGFRs; 3) modulate cell internalization of FGFR-2; and 4) inhibit FGFR-2–dependent endothelial cell proliferation. All these effects occur at concentrations of free ganglioside between 0.3 and 30 μM. 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 Ladish, 1991) and that gangliosides are present at concentrations as high as 10 μM in the serum of tumor-bearing patients (Valentino and Ladish, 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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