Neutralizing Antibodies Inhibit the Binding of Basic Fibroblast Growth Factor to Its Receptor but Not to Heparin*

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Polyclonal antibodies were prepared against recombinant basic fibroblast growth factor (bFGF) that reacted only with bFGF but not acidic FGF. These antibodies were able to inhibit various biological activities of bFGF such as the ability of bFGF to stimulate DNA synthesis in 3T3 cells, proliferation and migration of bovine capillary endothelial cells (BCEC), and neurite extension in pheochromocytoma (PC12) cells. The anti-bFGF antibodies also inhibited the mitogenic activity of subendothelial cell extracellular matrix for BCEC, demonstrating that the growth factor component in extracellular matrix required for supporting BCEC proliferation was bFGF. Anti-bFGF antibodies inhibited the cross-linking of bFGF to its high affinity receptor on BCEC cells. However, these antibodies did not inhibit the binding of bFGF to heparin-Sepharose or to the low affinity receptors of BCEC which have been demonstrated to be heparin-like molecules. These results suggest that bFGF has distinct domains for binding to high affinity cellular receptors and for binding to heparin.

bFGF has numerous biological activities that include the ability to stimulate cell migration, cell proliferation, and cell differentiation (1, 2). The biological effects of bFGF are presumably mediated by high affinity cell surface receptors that have been identified, for example, on 3T3 (3), endothelial (4), baby hamster kidney (5), and PC12 cells (6). The biological activity of bFGF might also be mediated by its interaction with heparin or heparin-like molecules. bFGF is a heparin-binding protein that adheres tightly to columns of heparin-Sepharose (7, 8) and is found to be associated with heparin-like molecules in subendothelial cell ECM (9, 10) and in basement membrane (11). Heparin has been shown to potentiate the activity of aFGF (12) and to protect bFGF from denaturation (12). Endothelial cell-derived heparan sulfate protects bFGF from proteolytic degradation (13). Moscatelli (4, 14) has recently demonstrated the existence of low affinity bFGF-binding sites on numerous cell types including capillary endothelial and baby hamster kidney cells which appear to be cell-associated heparin-like molecules. These observations suggest that bFGF binding to heparin-like molecules on the cell surface might be important in regulating cellular responses.

We have used another approach to analyze heparin-binding and receptor-binding sites. Highly specific polyclonal antibodies have been developed that neutralize the ability of bFGF to stimulate cellular responses such as cell migration, cell proliferation, and neurite outgrowth. These antibodies will also block the ability of subendothelial cell ECM to support endothelial cell (EC) proliferation. In this report, we demonstrate that these antibodies inhibit the cross-linking of bFGF to high affinity EC receptors. However, these antibodies do not inhibit the ability of bFGF to bind to immobilized heparin or to heparin-like low affinity EC receptors. Therefore, it is possible to discriminate between the receptor-binding and heparin-binding domains of bFGF.

MATERIALS AND METHODS

Production of Polyclonal Antibodies—To produce anti-bFGF antibodies, male New Zealand White rabbits were given injections at multidotal sites of human recombinant bFGF (140 μg/rabbit) which was emulsified in complete Freund's adjuvant (Difco). The rabbits were boosted after 2 months with subcutaneous injections of bFGF (140 μg/rabbit) emulsified in incomplete Freund's adjuvant. To produce anti-aFGF polyclonal antibodies, a peptide representing bovine aFGF (59-90) (17) was conjugated to keyhole limpet hemocyanin and the conjugates were injected into rabbits (18). The rabbits were bled from the ear. Polyclonal antibodies used in this study were collected 7 days after the second booster injection. Non-immunized serum collected from the same rabbit prior to the first injection was used as control serum. The titer of the serum was determined using an enzyme-linked immunosorbent assay in which a 96-well plate (Fal-
Neutralizing Anti-basic Fibroblast Growth Factor Antibodies

...con was coated with 50 ng of bFGF or synthetic aFGF peptide/50 μl phosphate buffered saline (PBS) in each well at 4 °C overnight as described previously (18). Anti-bFGF IgG and normal rabbit sera IgG were purified by protein A-Sepharose chromatography (19). 2 ml of anti-bFGF serum or normal serum was applied to a protein A-Sepharose column (Pharmacia LKB Biotechnology Inc.; 2 ml bed volume) which had been equilibrated with 0.1 M Tris-HCl, pH 7.4. After washing with 0.1 M sodium acetate buffer, pH 5.0, bound IgG was eluted with 0.1 M sodium acetate buffer, pH 3.5. The peak protein fractions were collected and diazylated against PBS. Protein concentration was measured by absorbance at 280 nm (A = 1.4 = 1 mg/ml IgG) and the IgG concentration was adjusted to obtain a stock dilution of 4 mg/ml.

Western Blot Analysis—Bovine brain bFGF and aFGF were electrophoresed on SDS-polyacrylamide gels (15%) and transferred electrophoretically (Trans-Blot cell, Bio-Rad) to BA-83 nitrocellulose paper (Schleicher & Schuell) as described previously (18). The nitrocellulose paper was incubated with anti-bFGF antisera (1:200 dilution) and anti-aFGF antisera (1:1000 dilution) as described above. The proteins were visualized by successive incubations with biotinylated goat anti-rabbit antibodies, peroxidase-conjugated streptavidin, and 4-chloro-1-naphthol substrate. Prestained molecular weight (Bio-Rad) markers were used to determine the molecular weights of transferred polypeptides.

Iodination of bFGF—Human recombinant bFGF iodinated by a modification of the method of Bolton and Hunter (20). A reaction mixture containing 6.7 μg (0.37 nmol) of bFGF, 0.61 nmol of monodified 1-Bolton-Hunter reagent (2200 Ci/mm) when first assayed on a protein A-Sepharose column (Pharmacia LKB Biotechnology Inc., PD-10, 9.1 ml bed volume) equilibrated with 50 mM Tris-HCl, 0.3 M NaCl, 0.1% gelatin, and 1 mM dithiothreitol, pH 7.5. The biological activity of 125I-bFGF was retained as determined by its ability to bind to heparin-Sepharose and to stimulate DNA synthesis in Balb/c 3T3 cells in culture. The specific radioactivity of 125I-bFGF was 55 Ci/ng, when first assayed. Aliquots of 125I-bFGF were stored at -20 °C in the buffer used for gel filtration.

Cross-linking of 125I-bFGF to BCEC Receptors—BCEC were cultured on gelatinized 100-mm dishes as described previously (21). Cross-linking was performed with subconfluent cell layers (about 1.5 × 10^6 cells/100-mm dish). Before addition of 125I-bFGF, cells were washed with PBS and preincubated at 4 °C for 10 min in 2 ml of binding buffer (PBS, 0.2% gelatin, pH 7.4) using gentle rotation on an orbital shaking platform. Subsequently, the cells were incubated for 5 h in 5 ml of freezing buffer containing 125I-bFGF (10 ng/ml) in serum-free medium at 4 °C. The cell layer was washed with cold PBS, fixed on ice, incubated on a microfuge shaker at room temperature for 30 min in 2 ml of containing 0.15 mM disuccinimidyl suberate. The disuccinimidyl suberate was prepared immediately prior to addition to a 20 mM stock solution in dimethyl sulfoxide. The excess disuccinimidyl suberate was quenched by addition of 250 μl of 0.2 M glycine, and incubating for an additional 30 min on ice. 20 ml of 1.5% gelatin was added to the vial, and the 125I-bFGF was purified by gel filtration on a column of Sephadex G-25 (LKB Biotechnology Inc.; 10.1 ml bed volume) equilibrated with 50 mM Tris-HCl, 0.3 M NaCl, 0.1% gelatin, and 1 mM dithiothreitol, pH 7.5. The biological activity of 125I-bFGF was determined in a 48-microwell chamber apparatus (Neuroprobe) (24). Test samples of bFGF (0.01–1000 ng/ml) in DMEM containing 1% calf serum, in a total volume of 27 μl, were loaded into each well bottom. Anti-bFGF IgG (400 μg/ml) was added in neutralization studies. BCEC were treated by mild trypsin treatment, washed with DMEM/10% CS, collected by centrifugation, and resuspended in fresh DMEM/10% CS. 7500 cells/50 μl were loaded into each top chamber of the microwell plate. The apparatus was incubated at 37 °C with 10% CO_2 for 4 h. The top (non-penetrating) layer of cells was removed from the membrane (8-μm pores, polycyvinylpyrolidone-free; Nucleopore) using rubber wraper blades and cotton swabs. The migrating cells were fixed by immersing the membrane for 45 min in 10% buffered formalin phosphate (Fisher Scientific) and stained with 0.5% Nuclear Fast Red solution overnight. The membrane was washed with distilled water and mounted on a slide. Cells in the entire area of each well (8-mm^2) were counted at magnification ×40.

Neurite Outgrowth—PC12 cells, kindly provided by Dr. S. Rogelj (Whitehead Institute, Cambridge, MA), were seeded (5 × 10^5 cells/20-mm dish) in DMEM, 50 units/ml penicillin, 25 μg/ml streptomycin. The following day the culture medium was replaced with fresh medium that contained 25 ng/ml bFGF in the presence of anti-bFGF antisera (10 μg/ml of antisera/1.5 ml medium). Photographs were taken 5 days later with a Nikon camera; magnification was ×100. Ectacellular Matrix—6-well cluster dishes (35 mm/35-mm well) containing monkey EC conditioned media prepared by subconfluent subendothelial ECM were generously provided by Dr. Israel Vlodavsky (Jerusalem, Israel), and had been described as prepared previously (9).

bFGF and aFGF—Human recombinant bFGF (25) was a generous gift of the Takeda Chemical Company (Osaka, Japan). It had a specific activity of about 1-5 units/ml in the Balb/c3T3 DNA synthesis assay and was stored above. SK hepato-cells derived bFGF was prepared as described previously (26). Bovine brain bFGF and aFGF (27) were kindly provided by Dr. Patricia D’Amore (Children’s Hospital, Boston, MA).
Neutralizing Anti-basic Fibroblast Growth Factor Antibodies

RESULTS

Specificity of Antibody of bFGF—Polyclonal antiserum directed against human recombinant bFGF was analyzed by SDS-PAGE for the ability to react with bFGF and aFGF (Fig. 1). Anti-bFGF antibodies reacted with bFGF (lane 4) but not aFGF (lane 3). On the other hand, anti-aFGF antibodies reacted with aFGF (lane 1) but not bFGF (lane 2). Thus, the anti-bFGF antibodies used in the studies to be described below are highly specific in that they cross-react with bFGF, but not aFGF, despite the 55% homology (17) that exists between these two proteins.

Inhibition of bFGF-induced Cell Proliferation by Anti-bFGF Antibody—The ability of the antibody preparation that cross-reacted with bFGF on a Western blot (Fig. 1) to neutralize bFGF-induced DNA synthesis in 3T3 cells and to neutralize BCEC proliferation in a dose-dependent manner was tested (Fig. 2). An IgG fraction of anti-bFGF at 400 μg/ml neutralized the mitogenic activity for 3T3 cells of human recombinant bFGF by 100% (Fig. 2A) and of human hepatoma cell-derived bFGF by 80% (Fig. 2B). The antibody preparation did not inhibit aFGF at all (Fig. 2C), nor platelet-derived growth factor (not shown), nor any other calf serum-derived mitogen for 3T3 cells (not shown). The slightly decreased ability of the antibodies prepared against recombinant bFGF to inhibit hepatoma-derived bFGF probably reflects the structural differences in the two bFGF preparations, the recombinant bFGF being the truncated bFGF-(1-146) (17) and the hepatoma-derived bFGF being the intact bFGF-(1-154) (26).

The antibodies also neutralized the ability of bFGF to stimulate the proliferation of BCEC (Fig. 2D).

Inhibition of ECM-induced Cell Proliferation by Anti-bFGF Antibody—Cultured bovine corneal and aortic EC deposit an ECM which replaces the requirement of exogenous FGF for supporting the proliferation of these cells at clonal densities (9). Since subendothelial cell ECM contains bFGF, it was suggested, but not demonstrated, that this growth factor may be one of the ECM components needed to support EC proliferation (9). In the present study, plates containing cell-free preparations of corneal subendothelial cell ECM were incubated with polyclonal antibodies directed against bFGF and BCEC proliferation on these plates was measured (Fig. 3). There was a 14-fold increase of BCEC when grown on subendothelial cell ECM in 5 days (Fig. 3A, lane 1). The presence of anti-bFGF IgG decreased ECM-stimulated BCEC proliferation by over 75% (Fig. 3A, lane 3). Normal rabbit IgG showed very little inhibition (Fig. 3A, lane 2). It was concluded that ECM-derived bFGF was required to support BCEC proliferation.

In a parallel study (Fig. 3B), neutralizing anti-bFGF antibody (lane 3), but not normal rabbit antibody (lane 2), inhibited the proliferation of BCEC stimulated by exogenous bFGF to the same extent that they inhibited proliferation of BCEC stimulated by ECM in the absence of exogenous bFGF (Fig. 3A). The antibodies inhibited bFGF-stimulated but not basal BCEC proliferation. The basal proliferation of BCEC in 10% CS and in the absence of exogenous bFGF is about 4-fold in 5 days, i.e. from 1000 cells plated to 4000 cells/dish. The inability of anti-bFGF antibodies to neutralize BCEC basal proliferation is evidence that the antibodies are inhibiting bFGF action rather than being toxic to BCEC.

Inhibition of bFGF-induced Chemotaxis by Anti-bFGF Antibodies—Increasing concentrations of bFGF were tested for their ability to stimulate BCEC migration in a multiple-well Boyden chamber (Fig. 4). bFGF-induced chemotaxis of BCEC followed a bell-shaped curve with maximal response at 0.1–1.0 ng/ml. Anti-bFGF antibodies inhibited the chemotaxis stimulated by 1 ng/ml bFGF (Fig. 4, bar graph) and by lower amounts of bFGF (not shown), but did not inhibit basal migration in 10% CS.

Inhibition of bFGF-induced Neurite Outgrowth by Anti-
Neutralizing Anti-basic Fibroblast Growth Factor Antibodies

FIG. 3. Inhibition of subendothelial extracellular matrix (ECM)-stimulated and bFGF-stimulated BCEC proliferation by anti-bFGF antibody. A, BCEC (7,500/well, 6-well cluster dish) were plated on dishes coated with subendothelial cell ECM (9). Just prior to seeding, either PBS control buffer (lane 1), 200 µg/ml normal rabbit IgG (lane 2), or 200 µg/ml anti-bFGF IgG (lane 3), were added and after 6 days in culture, the BCEC were trypsin-treated and counted. B, BCEC were plated on plastic dishes (10,000/well, 24-well cluster dish) and bFGF (2 ng/ml) was added along with PBS control buffer (lane 1), 200 µg/ml normal rabbit IgG (lane 2), or 200 µg/ml anti-bFGF IgG (lane 3). After 5 days the BCEC were trypsin-treated and counted.

FIG. 4. Inhibition of bFGF-induced chemotaxis by anti-bFGF antibody. BCEC (7500/well, 8 mm² well) in DMEM/1% CS were placed in the top wells of a microwell chamber chemotactic apparatus. Increasing concentrations of bFGF were placed in the bottom wells. After a 4-h incubation chemotaxis was measured by counting the cells on the lower surface of the filter separating top and bottom wells in duplicate (open circles). Some wells containing 1 ng/ml bFGF also received 400 µg/ml anti-bFGF IgG (bar graph). The amount of anti-bFGF antibody used was not effective against larger concentrations of bFGF, such as 10 ng/ml, which represented a 1000-fold excess of bFGF needed to stimulate chemotaxis.

bFGF Antibody—bFGF stimulates neurite outgrowth in PC12 cells (Fig. 5, middle) mimicking the effect of nerve growth factor. Anti-bFGF IgG (Fig. 5, bottom) inhibits the bFGF-induced stimulation of neurite outgrowth by PC12 cells.

Effects of Anti-bFGF Antibody on bFGF Cross-linking to Receptors—Cell migration and proliferation are thought to be mediated by growth factor interaction with cell surface receptors. Cross-linking experiments have previously identified a receptor for bFGF that appears as a doublet on SDS-PAGE with molecular sizes that range from 115,000 to 140,000 daltons (28). Biologically active ¹²⁵I-bFGF was cross-linked to BCEC cell surface receptors with disuccinimidyl suberate, and the radioactive species were analyzed by SDS-PAGE and autoradiography (Fig. 6). The resulting radioactive cross-linked species was a doublet with apparent molecular sizes of 130,000 and 150,000 daltons (Fig. 6, lane 1). This is in good agreement with the molecular mass characteristics of bFGF receptors identified in other cells (5, 28). Cross-linking of ¹²⁵I-bFGF to the high affinity receptor was abolished by the anti-bFGF antibodies (Fig. 6, lane 2). The ¹²⁵I-bFGF receptor cross-linking was also abolished by a 100-fold excess of unlabeled bFGF (not shown) but was not affected by the control preimmune IgG (not shown). In lane 2 the cross-linked bands with molecular mass of about 85–90 kDa are probably ¹²⁵I-bFGF linked to IgG heavy chains. The large amount of radioactivity running at the front line in both lanes is non-cross-linked ¹²⁵I-bFGF.
Effects of Anti-bFGF Antibody on bFGF Binding to Heparin—bFGF binds tightly to heparin as well as to its cell surface receptors. Samples of biologically active 125I-bFGF incubated with anti-bFGF IgG, normal rabbit IgG, or not incubated with either were applied to columns of heparin-Sepharose and eluted batchwise with 0.6 M NaCl followed by 2.0 M NaCl, the concentration of NaCl required to elute bFGF from the column (Fig. 7). Anti-bFGF antibodies did not inhibit the binding of FGF to heparin. These results suggest that the heparin-binding domain which is not blocked by anti-bFGF antibodies and the cell receptor-binding domain which is blocked by these same antibodies must represent different parts of the bFGF molecule.

It has been suggested that the low affinity binding sites for bFGF are heparin-like molecules (4, 14). The observation that anti-bFGF antibodies do not block the ability of bFGF to bind to immobilized heparin suggests that these antibodies will not block the binding of bFGF to the low affinity binding sites either. Binding of 125I-bFGF to low affinity binding sites was inhibited by heparin in a dose-dependent manner, confirming previous reports (4, 14), but not by anti-bFGF antibodies (Table I) at concentrations sufficient to inhibit bFGF-induced proliferation, migration, neurite extension, and receptor cross-linking.

**DISCUSSION**

bFGF binds strongly to two different types of macromolecules: cell surface receptors which are proteins, and heparin and heparin sulfate which are glycosaminoglycans. Polyclonal antibodies, capable of neutralizing a number of the biological activities of bFGF such as the ability to stimulate BCEC migration, BCEC proliferation and neurite outgrowth in PC12 cells, inhibit the cross-linking of bFGF to its cell surface receptors but do not prevent binding of bFGF to immobilized heparin or to heparin-like molecules on the surface of BCEC. These results suggest that the cell surface receptor-binding domains and heparin-binding domains of bFGF are not the same. In previous studies using synthetic peptides representing regions of bFGF, it was observed that some heparin-binding bFGF peptides could also bind cell surface receptors while others could not (15, 16). These authors concluded that heparin-binding and receptor-binding domains overlapped in certain regions of bFGF but not in others. One possible advantage in using antibodies as probes, as compared to using synthetic peptides, is that the antibodies might be more sensitive and accurate in recognizing multiple activities dependent on the three-dimensional conformation of bFGF. Because the synthetic peptides probably do not have the conformation that these regions have in native bFGF, they may be less specific in binding, inhibition, and mitogenic assays. For example, an amino acid that is available in a synthetic peptide to bind to heparin might be inaccessible in the folded native molecule.

Neutralizing antibodies block bFGF stimulation of BCEC cell migration, BCEC proliferation, and PC12 neurite extension, as well as cross-linking of bFGF to 130,000–150,000-dalton BCEC receptors. However, they do not inhibit bFGF binding to heparin. These results suggest strongly that it is the high affinity 150,000–150,000-dalton receptors and not heparin which mediate these biological activities of bFGF. There may be other biological activities of bFGF that are mediated by binding to heparin, although it is not clear at present what these biological activities may be. One biological role of heparin might be to bind bFGF to the cell surface with low affinity. Moscatelli (4) has provided evidence that cells such as baby hamster kidney and BCEC have low affinity receptors from which binding of 125I-bFGF is displaced by heparin (4). These low affinity binding sites appear to be heparin-like, number about 600,000–1,000,000/cell, and have a Kd of about 2,000 pm as compared to the high affinity binding sites which number 8,000–82,000/cell and have a Kd of about 20 pm. We also find that heparin inhibits binding of bFGF to low affinity sites on BCEC. It has been suggested that heparin-like molecules on the cell surface and in the extracellular matrix act as a reservoir of bFGF. "Stored" bFGF might be transferred from heparin-binding sites of relatively low affinity to cell surface receptor binding sites of relatively high affinity (2, 9, 14, 22). Thus, the heparin-binding domain of bFGF might act as a functional entity which allows it to bind to heparin-like molecules, but relatively weakly. Binding of bFGF to heparin-like molecules may be a preliminary step in the transfer of bFGF to high affinity receptors at another locus on the cell surface resulting in cell migration, proliferation, or differentiation.

The use of neutralizing antibodies directed against bFGF has given us several insights into the regulation of EC growth. For example, EC plated on subendothelial cell ECM no longer have an exogenous requirement for bFGF (9). The presence
of bFGF in subendothelial ECM suggested, but did not prove, that this growth factor might play a role in ECM-mediated EC proliferation (9, 10). As demonstrated in this report, the ability of anti-bFGF antibodies to inhibit ECM-stimulated BCEC cell growth suggests strongly that bFGF is the active mitogen in ECM required for supporting the proliferation of EC.

Another observation is that anti-bFGF antibodies inhibit exogenous bFGF-stimulated EC migration and proliferation but not basal EC migration and proliferation. Lack of inhibition of basal growth by anti-bFGF antibodies is consistent with previous reports (29). The inability of anti-bFGF antibodies to inhibit EC basal growth suggests two possibilities. Since EC synthesize relatively large amounts of bFGF which remain cell-associated (9, 30, 31), it may be that basal growth of EC is due to cell-associated bFGF which is not accessible to antibodies. Alternatively, basal EC growth may be due to growth factors that are not bFGF, for example, serum-derived high density lipoprotein (32).

bFGF is an ECM-associated protein that has at least two functional domains, a heparin-binding domain and a cell surface receptor-binding domain. Interestingly, bFGF has structural and biological features similar to the ECM proteins fibronectin and laminin. Both fibronectin and laminin have heparin-binding and cell-binding domains (33, 34) and both are adhesion molecules (33, 34), as is bFGF (15, 16). Fibronectin and laminin also have some of the biological properties normally associated with bFGF. For example, fibronectin promotes cell migration and proliferation (33, 34) while laminin stimulates neurite outgrowth (35). Our antibody studies suggest that bFGF might be structured like laminin and fibronectin to have different domains which are characteristic of ECM structural proteins in general. These are domains that interact with heparan sulfate proteoglycan and cell surface receptors as well as structural elements found on other ECM proteins.

While neutralizing antibody studies have helped to establish the presence of distinct functional domains in bFGF, they have not identified where these domains are. Studies which, for example, include the use of antibodies directed against specific sites in bFGF and techniques such as in vitro mutagenesis may be helpful in mapping these domains.

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