Inhibition of High Affinity Basic Fibroblast Growth Factor Binding by Oligonucleotides*

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Oligonucleotides can be used to inhibit the binding of basic fibroblast growth factor to cells. Though standard phosphodiester oligonucleotides show a slight inhibition of binding, the oligonucleotides with phosphorothioate internucleoside linkages have inhibition levels equivalent to that of the polyanion heparin. Variations in sequence of the oligonucleotides does lead to differences in the inhibitory action of the oligonucleotides. This inhibition of basic fibroblast growth factor by phosphorothioate oligonucleotides may account for much of the published data on inhibition of various genes by proposed antisense oligonucleotides and needs to be taken into account when considering the mechanism of action of oligonucleotides in biological systems.

Oligonucleotides have been used extensively in the past several years to inhibit gene expression. Compounds are most often designed as antisense agents and, as such, have shown efficacy in cell culture and in some animal models of disease. Efficacious oligonucleotides have been used against viruses, including human cytomegalovirus, herpes simplex virus, human immunodeficiency virus, and papillomavirus, and against various cellular targets, including the oncogenes c-myc, c-myc, and c- abl (1–12). In addition, investigators have used anti-c-myc/myb antisense oligonucleotides to prevent restenosis in animal models (13–15). Several of these compounds are progressing into human drug trials based on their proposed antisense inhibition of viral or oncogene targets in disease. Oligonucleotide therapeutics are targeted to human cytomegalovirus in retinitis, papillomavirus in genital warts, human immunodeficiency virus in AIDS, c- abl in myelogenous leukemia, and c-myc and c-myc in restenosis. At the same time, increased numbers of unexplained, nonspecific effects have been reported especially for the phosphorothioate (PS) oligonucleotides (16–20). Phosphorothioate oligonucleotides contain internucleoside linkages in which one of the nonbridging oxygen atoms has been replaced by a sulfur atom in order to enhance nuclease resistance.

Our own studies progressed from the initial use of oligonucleotides as anti-herpesvirus agents on Vero and MRC-5 cells (9). The action of oligonucleotides against herpes simplex virus and human cytomegalovirus have been reported previously (3, 8, 9), but the actual analysis of the data has been complicated by the ability of the oligonucleotides to operate on the exterior of the cell as inhibitors of viral adsorption. The binding of herpes simplex virus to the cell involves an initial attachment to cell surface heparan sulfate molecules and can be competed by the addition of heparin to the media (21). Phosphorothioate oligonucleotides are particularly potent inhibitors of viral adsorption with effective concentrations in the nanomolar range (9, 22). Because basic fibroblast growth factor (bFGF) binding, like herpes simplex virus adsorption, involves interaction with cell surface heparan sulfate, we investigated whether oligonucleotides would also inhibit the binding of bFGF to cells.

bFGF is a member of the heparin binding family of growth factors (23, 24). It is a widely distributed growth factor found in all organs, solid tissues, tumors, and cultured cells examined (25). bFGF binds to high affinity protein receptors in association with cellular matrix heparan sulfate. This association, which is competed by the presence of exogenously added heparin, is necessary for the high affinity and functional binding of bFGF. Heparin-induced dimerization of the growth factor/receptor is required for full activity of the growth factor (26). Heparin and heparin subfragments can be used to inhibit the biological activity of FGF (27, 28), although it may not be required for all of the FGF effects (29). The bFGF receptors are members of the group of tyrosine kinase receptors capable of autophosphorylation and dimerization; it is this dimerization that results in an increase in receptor/ligand interaction and signal transduction (30, 31). In this model of bFGF receptor activity, exogenously added heparin competes with cell surface heparan sulfate and inhibits bFGF activity through the disruption of the dimerization of the receptor. This is similar in concept to the disruption of ligand binding to receptors by the uncoupling of the receptor and the G protein complex for those receptors, which signal through G protein complex formation (32, 33). FGF binding/dimerization and signaling results in a variety of cellular responses including myc, fos, collagenase, and platelet-derived growth factor receptor up-regulation, increases in intracellular pH, hydrolysis of polyphosphoinositol, and phosphorylation of cellular proteins. It induces growth in various cell types including astrocytes, glioma cells, fibroblasts, and, in synergy with hematopoietic growth factors, stem cells. In animal models it is most directly connected with angiogenesis and wound healing.

Our studies show that oligonucleotides, especially PS oligonucleotides, are able to inhibit bFGF binding to cells. These observations on bFGF and oligonucleotides, taken together, indicate that although the oligonucleotides studied to date may have therapeutic potential, their level of biologic activity may not be due to the original rational “antisense” design.

**EXPERIMENTAL PROCEDURES**

**Materials**—125I-bFGF was obtained from DuPont, unlabeled bFGF was obtained from Life Technologies Inc., Vero cells were obtained from the ATCC, and heparin was obtained from Sigma.

**Oligonucleotide Synthesis**—Oligonucleotides were synthesized on an Applied Biosystems Inc. DNA synthesizer model 380B or 394, using standard phosphoramidite methods. 5'-protected nucleoside phos-
The binding of basic FGF to Vero cell low (A) and high (B) affinity receptors was assayed as described under "Experimental Procedures." For the competition with cold FGF, 100 ng/mL unlabeled bFGF was added. Heparin was used at 100 μg/mL; the phosphorothioate oligonucleotide ODN-1 was used at 0.6 μM (7 μg/mL). Each value is the average of duplicate samples of a typical experiment. All points were assayed in at least two separate experiments.

**RESULTS AND DISCUSSION**

bFGF binding to cells is assayed by the incubation of radiolabeled bFGF with cell monolayers with subsequent wash conditions able to distinguish the low affinity heparin sulfate binding from the high affinity protein receptor-specific binding (29). The bFGF bound to the heparan sulfate is removed with 2 M NaCl at neutral pH. This heparan sulfate binding is not saturated and is not competed away at 100-fold concentrations of unlabeled bFGF. It is however competed away by exogenous heparin or by the addition of other polyanions such as pentosan polysulfate and suramin. Phosphorothioate oligonucleotides can also act as appropriate polyanions and inhibit the low affinity heparan sulfate binding of bFGF (Fig. 1A) at submicromolar (0.6 μM) concentrations.

The bFGF bound to the high affinity receptors is then removed from the cells with a subsequent low pH rinse. It is this high affinity binding that is specific for the bFGF receptor proteins. This high affinity receptor binding is effectively competed away with unlabeled bFGF (Fig. 1B). Heparan sulfate binding is necessary for the dimerization of the bFGF receptor; therefore, an inhibition of the bFGF low affinity heparan sulfate binding results in a change in the binding to the high affinity protein receptor. The binding constant has been reported to shift from 50 μM with heparan sulfate involvement to 175 μM without such involvement (37). If the bFGF concentration is kept low, then only the high affinity receptor binding resulting from dimerization is detected; the concentration of bFGF is not sufficient for the receptor binding that occurs in the absence of dimerization. Both heparin and PS oligonucleotides inhibit high affinity binding under such conditions (Fig. 1B).

When a standard saturation curve is plotted, the difference in binding over an increasing range of bFGF concentrations is seen for both the high and low affinity receptors (Fig. 2). The binding to the high affinity receptors increases more slowly in the presence of the oligonucleotides or heparin, approaching a plateau only at higher concentrations. The concentrations are too low to saturate the binding in the presence of heparin or PS oligonucleotides. At higher bFGF concentrations the bFGF may bind in the presence of heparin, but this binding is not expected to result in signal transduction due to the inability of the receptors to dimerize. It has been shown that although cell surface heparan sulfate is not required for receptor specific binding, it does increase the affinity of basic bFGF for its receptor and facilitates dimerization and activity of FGF (37, 38).

Because a variation in the effect of different antisense oligonucleotides is seen when they are assayed for efficacy in the various systems employed, we tested a variety of different oligonucleotides to determine if this variability could be attributed to differences in bFGF binding inhibition. A series of competition experiments was performed in which the concentration of 125I-bFGF was kept constant while the dose of oligonucleotide used in competition was varied. In this way the relative binding affinities of the various oligonucleotides could be compared. Representative results are shown (Fig. 3). Though unlabeled bFGF competed for binding at only the high affinity receptors, as expected, the oligonucleotides and heparin showed similar competition at both the low and the high affinity binding sites. Although the oligonucleotides varied in their ability to block bFGF binding, we were unable to distinguish any sequence or structure that was responsible. The concentration at which 50% of the high affinity binding was inhibited (IC50) was calculated for several representative oligonucleotides and in several conditions (Table I). The PS oligonucleotide monomers and other reagents were obtained from Milligen with the exception of acetonitrile, which was obtained from Baxter. All oligonucleotides were synthesized with a 3’ Amino Modifier (Glen Research), which results in the covalent attachment of a propanolamine group to the 3’ hydroxyl group (34, 35). Phosphorothioate-containing oligonucleotides were prepared using the suruzifying agent TETD, which produces a random mixture of R and S isomers (36). The purity of the oligonucleotides was confirmed by analytical high pressure liquid chromatography, electrophoresis of 32P-labeled oligonucleotide on a polyacrylamide gel, or capillary gel electrophoresis. The oligodeoxynucleotides (ODNs) used were: ODN-1 (GTGGTGGTGGTGGTGGG), ODN-2 (GATCCATGTCAGTGACAC-TGCGTAGATCCGATGATC), and ODN-4 (GGGTGGTTTGGGGGGTGGGGG), ODN-1, ODN-3 and ODN-4 contain phosphorothioate linkages. ODN-2 has a sequence identical to ODN-1 but contains phosphodiester linkages. ODN-5 (GGSgcsCTGcCsCoAsgOsGsOsTsCsCsCs) and ODN-6 (GSGSgcsAcsAsCTGcCsCoAsgOsGsCsCsCs) contain both phosphodiester and phosphorothioate linkages (designated “o” and “s” in the sequence, respectively).

**FIG. 1. Inhibition of basic FGF binding to low and high affinity receptors.** The binding of 125I-bFGF to Vero cell low (A) and high (B) affinity receptors was assayed as described under "Experimental Procedures." For the competition with cold FGF, 100 ng/mL unlabeled bFGF was added. Heparin was used at 100 μg/mL; the phosphorothioate oligonucleotide ODN-1 was used at 0.6 μM (7 μg/mL). Each value is the average of duplicate samples of a typical experiment. All points were assayed in at least two separate experiments.
gonucleotides were consistently competitive at lower concentrations than the phosphodiester or partial phosphorothioate oligonucleotides, though the phosphodiester oligonucleotides did have some effect at concentrations greater than 10 µM. A comparison of PS oligonucleotides of various lengths (12–36 bases) showed no direct correlation of length to ID50, but the exact sequence did influence bFGF binding inhibition; two PS oligonucleotides (ODN-1 and ODN-3) of identical length but different sequence both diminish bFGF binding, but the ID50 values differ by 10-fold. The oligonucleotides we used that were composed of G's and T's were generally more effective than mixed sequences, but no firm rules could be established for predicting the relative binding inhibition of the phosphorothioates. The oligonucleotides and heparin are both polymeric anions, but the phosphorothioate modification of the oligonucleotide backbone presumably causes a change in charge or secondary structure that renders the oligonucleotides even more effective in their inhibition of bFGF binding.

In an effort to more closely mimic the various antisense experiments, we also varied the time of addition and incubation of the oligonucleotide. In the standard protocol, the oligonucleotides were mixed with the bFGF prior to the addition to the cells. If the oligonucleotide was preincubated with the cells, instead of with the growth factor, the ID50 was only slightly higher (Table I). The ID50 was significantly higher if the oligonucleotide was removed prior to the addition of the bFGF but was still less than 5 µM. Although there is some variation in the extent of bFGF inhibition depending on whether the growth

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**TABLE I**

Comparison of oligonucleotide inhibition of high affinity bFGF binding

| Backbone | ID50 (µM) |
|----------|-----------|
| ODN-1    | 0.08      |
| ODN-2    | 0.40      |
| ODN-3    | >20       |
| ODN-4    | 1.2       |
| ODN-5    | 0.15      |
| ODN-6    | >20       |

* PO, phosphodiester backbone.

* The dose of oligonucleotide that inhibited 50% of the bFGF binding (ID50) was calculated for several oligonucleotides based on competition curves of 125I-bFGF binding to Vero cells.

* Additional assay variations were performed on ODN-1. For cellular preincubation, oligonucleotide was added to cells 1 h prior to the addition of bFGF, and for cellular preincubation/wash, oligonucleotide was incubated with cells for 1 h but removed by washing prior to the addition of bFGF.
factor or the cells are preincubated with oligonucleotide, there is a significant level of activity against bFGF under the conditions of most antisense assays. It should be kept in mind that many cell lines synthesize and secrete bFGF and that endogenously produced bFGF may have significant effect on cell proliferation, and the full effect of the binding inhibition may be seen.

Inhibition of bFGF binding by phosphorothioate oligonucleotides may account for some of the experimental results attributed to antisense mechanisms. Antisense experiments often report results using concentrations of PS oligonucleotides in the range of 0.1–50 μM, which we would expect to influence bFGF binding. The 10-fold variation in the activity of the PS oligonucleotides is in the same range as the specific/non-specific activity of antisense and control oligonucleotides reported for gene disruption experiments. This would be especially true when the assays are for those activities known to be influenced by bFGF, including myc and fos regulation, platelet-derived growth factor and bFGF receptor phosphorylation, hematopoiesis, and cell growth, especially that of vascular smooth muscle cells. Other cellular factors known to influence bFGF binding. The 10-fold variation in the activity of these cell culture and animal trials of antisense data must be made in light of this effect of binding inhibition and the full effect of the binding inhibition may be seen.

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