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EFFECT OF HEPARIN ON THE BINDING AFFINITY OF ACIDIC FGF FOR THE CLONED HUMAN FGF RECEPTORS, flg and bek

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SUMMARY: Heparin potentiates the mitogenic activity of acidic fibroblast growth factor (aFGF) by 20-100 fold but mechanisms detailing this potentiation have not yet been fully elucidated. We report that heparin increases the binding affinity of aFGF for the two cloned and overexpressed human FGF receptors, flg and bek, by 2-3 fold. This increase in binding affinity, together with previous data demonstrating a 3-5 fold increase in the stability of aFGF, are likely to account for a significant portion of heparin's potentiation of aFGF activity observed in biological assay systems. © 1990 Academic Press, Inc.

The fibroblast growth factor (FGF) family consists of seven polypeptide mitogens which share a 30-50% amino acid sequence identity, and are characterized and purified on the basis of their affinity for heparin (1-7). These growth factors are mitogenic for a variety of cells of mesenchymal and neuroectodermal origin (reviewed in ref. 8) including endothelial cells which partly accounts for their classification as angiogenic factors (9).

Many studies have shown that heparin potentiates the mitogenic effect of acidic FGF in vitro by 20-100 fold (10-12). Heparin is a naturally occurring highly sulfated glycosaminoglycan that has a broad distribution in mammalian connective tissues and fluids (13). Heparin has been shown to produce a 3-5 fold increase in the biological halflife of aFGF (14-15) and to increase the binding affinity of bovine aFGF for receptor(s) on murine lung endothelial cells approximately 2.5 fold (10).

We recently cloned two human FGF receptors, flg and bek, separately overexpressed them in NIH 3T3 cells, and demonstrated that aFGF binds to both bek and flg with high affinity (17). In

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this study, we have quantitated the effect of heparin on the binding affinity of recombinant human aFGF (raFGF) using cell lines that express these two distinct recombinant human FGF receptors.

MATERIALS AND METHODS

Cells and reagents. Cells were maintained in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% calf serum (Gibco, Grand Island, N.Y.) NIH 3T3 cells were purchased from ATCC (CRL 1658). NFlg26 and NBek13 cell lines, constructed to overexpress the FGF receptors flg and bek, respectively, are described in ref. 17. Human recombinant aFGF (raFGF) was purified as described (18). Purified raFGF was quantitated by amino acid analysis. Heparin sodium solution was purchased from Upjohn and used at 5 U/ml.

Iodination of raFGF. Human raFGF was labeled with [^{125}I] using a modified chloramine T procedure (20). Active [^{125}I]raFGF was isolated on heparin-Sepharose (18). The specific activity of [^{125}I]raFGF was determined by radioisotope dilution with known concentrations of cold raFGF and yields varied between 8 X 10^4 cpm/ng to 3 X 10^5 cpm/ng for independent iodinations.

Equilibrium binding assay. Experiments which included heparin had heparin (5 U/ml) added to all solutions and serial dilutions. Fibronectin coated 24 well Falcon dishes containing 1 X 10^5 cells/well were rinsed twice with 1 ml of cold binding buffer, (DMEM, 50 mM Hepes, pH 7.4, 1 mg/ml bovine serum albumin, Fraction V, [Sigma]) then placed on ice and incubated for 20 min. with 1 ml of cold binding buffer. The medium was replaced with 250 μl of binding buffer containing serial dilutions of [^{125}I]raFGF and incubated for 2 h at 4°C. Nonspecific binding was obtained using the same serial dilutions, but in the presence of 100 fold molar excess of nonradioactive raFGF. The dishes were rinsed twice in cold binding buffer and the cells were solubilized in 0.3 N NaOH for 15 min. at 37°C. Scatchard analysis of the saturation binding data was performed as described (17,21).

Mitogenic Assay. NIH 3T3 cells were seeded onto fibronectin coated 24 well Falcon dishes and grown to subconfluency in DMEM supplemented with 10% calf serum. The cells were then serum starved for 48 h in 1 ml of DMEM supplemented with 0.5% calf serum. raFGF or raFGF plus heparin (5 U/ml) were added to the wells and incubated for 16 h. 0.5 μCi/ml of [^{3}H]-thymidine (New England Nuclear, 6.7 Ci/mmol) was added for 4 h. The cells were then rinsed with Dulbecco's phosphate buffered saline (DPBS), fixed in 1 ml of 10% trichloroacetic acid, rinsed with DPBS, and solubilized in 0.5 ml of 0.5 N NaOH. Uptake of [^{3}H]thymidine was measured by scintillation counting of the acid insoluble material.

RESULTS and DISCUSSION

Equilibrium binding assays were performed with the flg and bek overexpressing cell lines, NFlg26 and NBek13, respectively (17). Binding of [^{125}I]raFGF to both cell lines is specific and

saturable in both the presence and absence of heparin (Fig. 1, insert). Scatchard analysis (21) of the binding data indicates that [^{125}I]raFGF binds to a single class of high affinity sites on NFlg 26 and NBek 13 cells both in the presence and absence of heparin (Fig.1, Panels A and B). In the experiment shown in Fig. 1, [^{125}I]raFGF exhibited apparent K_ds of 280 pM and 230 pM for flg and bek, respectively, in the absence of heparin. The presence of heparin throughout the binding assay resulted in apparent K_ds of 110 pM for flg and 127 pM for bek. Averaged results of four experiments indicates that heparin increased the

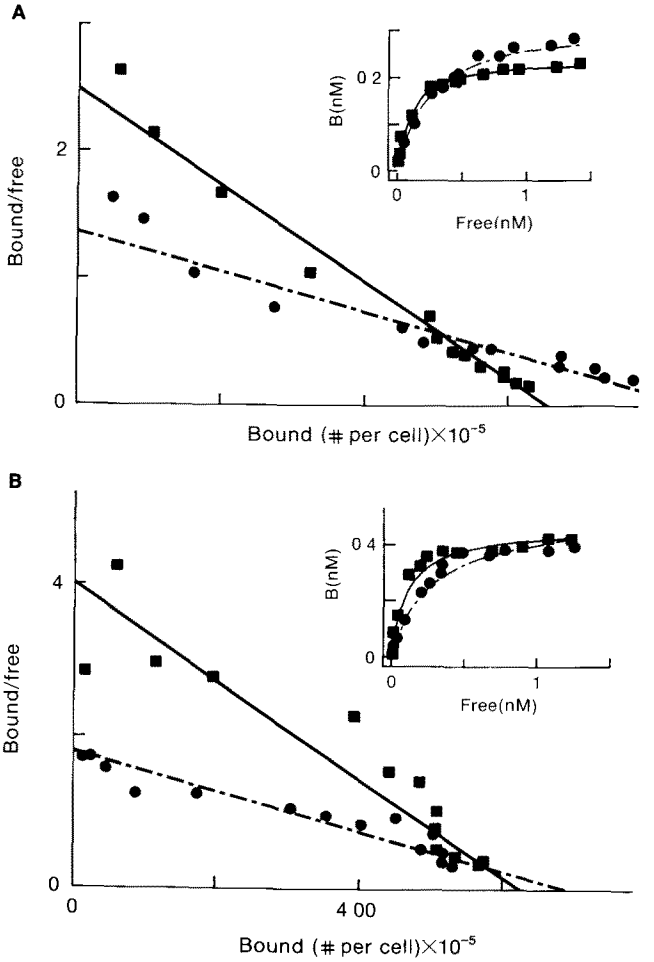

Fig. 1. Effect of Heparin on the Apparent K_ds of raFGF for flg and bek. Saturation binding analysis of [^{125}I]raFGF to NBek13 cells (Panel A) and NFlg26 cells (Panel B) in the presence (■--■) or absence (●--●) of heparin was performed as described in Materials and Methods. The background count in the presence of 100-fold molar excess of either ligand was less than 10% of the total cpm at each point. A single preparation of raFGF was used for the data described in Figs. 1 and 2 and Table 1.

TABLE 1

EFFECT OF HEPARIN ON INDEPENDENT PREPARATIONS OF [^{125}I] raFGF

FLG		BEK	
Hep-	Hep+	Hep-	Hep+
0.280 nM	0.111 nM	0.230 nM	0.127 nM
0.270	0.093	0.228	0.067
0.258	0.046	0.116	0.067
0.095	0.026	0.133	0.056

Note. The effect of heparin (5 U/ml) on the binding of independent preparations of [^{125}I]raFGF to NFlg 26 and NBek 13 cells was performed as described in Materials and Methods. The results from the Scatchard analysis of the binding data are compiled.

affinity of aFGF for bek 1.9 fold and for flg 3.2 fold. The increased affinities of aFGF for flg and bek are reproducible and statistically significant, $p < 0.05$ (Table 1), and are observed with independent preparations of [^{125}I]raFGF and [^{125}I]aFGF from bovine brain.

Schreiber et al. (10) noted a 2.5 fold increase in the affinity of bovine aFGF for FGF receptor(s) on murine lung capillary endothelial cells. Our results support their earlier work but have the advantage of being performed on cells expressing defined gene products rather than unidentified FGF receptors, which may be a mixed population of different gene products.

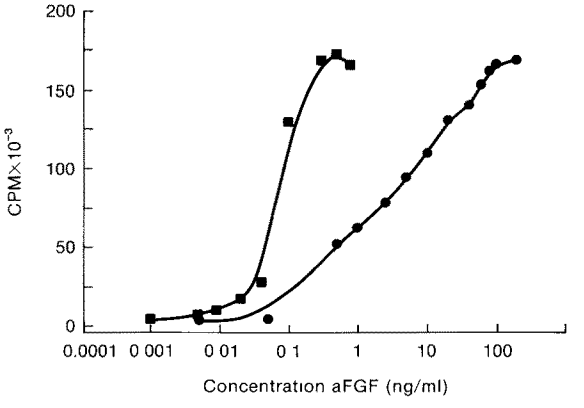

Fig. 2. Effect of Heparin on the Mitogenic Activity of Recombinant Human Acidic FGF. Increasing concentrations of raFGF were added to quiescent NIH 3T3 cells in the presence (■--■) or absence (●--●) of heparin (5 U/ml). Quantitation of the resulting [^{3}H]-thymidine incorporation was performed as described in Materials and Methods.

The interaction of aFGF with heparin is not completely understood. Heparin induces a conformational change in aFGF which alters the exposure of immunological epitopes (10). Although the details of this conformational change are unclear, heparin induces a 40% quenching of the primary fluorescence emitting tryptophan residue, Trp 102, suggesting the rotation of this residue into a more internal environment (18). The Trp 102 amino acid is located in acidic FGF just 12 residues amino terminal to the thrombin sensitive arginine residue; cleavage by thrombin is inhibited by heparin (22). This tryptophan residue is also included within a synthetic peptide derived from the sequence of basic FGF that has been shown to possess some affinity for heparin (23). Altogether these data indicate that this region in the FGFs is involved in interacting with heparin.

As a control, we demonstrated that heparin could stimulate our preparation of human raFGF in a NIH 3T3 [^{3}H]-thymidine incorporation assay. As has been reported earlier (18), heparin potentiated human raFGF stimulation of [^{3}H]-thymidine incorporation by 50-fold; i.e. EC_{50} was shifted from 0.08 ng/ml of growth factor to 4 ng/ml (Fig. 2). Therefore, the 2-3 fold increased affinity of aFGF for FGF receptors only partially accounts for potentiation of aFGF by heparin in a mitogenic assay.

The interaction of aFGF with heparin has several important consequences. It has been shown that heparin protects acidic FGF and basic FGF from acid and heat inactivation and confers protection against proteolytic digestion (14-16,19). Potentially, all of these heparin effects contribute to the enhancement of aFGF in mitogenic assays, which require prolonged exposure of the cells to mitogen for maximal effect. Although these effects of heparin have only been described *in vitro*, it is likely that they occur *in vivo* as well. *In vivo*, heparin-like molecules are found in the extracellular matrix and basement membranes of cells and probably serve as a storage reservoir for FGFs (24-26). The multiplicative effects of heparin on FGF availability, stability and enhanced binding to FGF receptors probably factor significantly in the *in vivo* regulation of FGF activity.

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