J. Biochem. Mol. Biol. Vol. 29, No. 3 pp. 205~209 May 31, 1996

Effect of Heparin on the High Affinity KGF and aFGF Binding to the Chimeric KGFR-HFc

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(Received December 22, 1995)

Abstract: To investigate the role of heparin in keratinocyte growth factor (KGF) and acidic fibroblast growth factor (aFGF) high affinity binding to the KGF receptor (KGFR), a cell free system was established which utilized a secreted chimeric molecule between the KGFR extracellular domain and the immunoglobulin heavy chain Fc domain (KGFR-HFc). KGFR-HFc was purified from NIH 3T3 cells and demonstrated the binding of [^{3}H]-heparin as well as heparin Sepharose. Scatchard analysis showed that the dissociation constant for heparin binding to KGFR-HFc was 140 nM. High affinity KGF and aFGF binding to KGFR-HFc remained unchanged after treatment with 0.6 M NaCl, which is the concentration sufficient to release any bound heparin to the KGFR-HFc. These results strongly suggest that although the KGFR interacts with heparin, the presence of heparin is not absolutely required for high affinity binding of either KGF or aFGF to the KGFR.

Key words: acidic fibroblast growth factor, heparin, immunoglobulin, keratinocyte growth factor, scatchard analysis.

Fibroblast growth factors (FGFs) have been shown to bind extracellular matrix or cell surface-associated heparin sulfate proteoglycans (HSPG) (Burgess and Maciag, 1989). These heparin binding properties have been exploited to identify and purify this family of growth factors using heparin Sepharose affinity chromatography (Shing *et al.*, 1984; Klagsbrun and Shing, 1985). The heparin binding domains of acidic FGF (aFGF) and basic FGF (bFGF) have been determined by biochemical techniques (Baird *et al.*, 1988; Harper and Lobb, 1988), and more recently, by x-ray crystallography (Eriksson *et al*, 1991; Zhang *et al.*, 1991; Zhu *et al.*, 1991).

Despite several investigations, the physiological relevance of FGF binding to heparin-like molecules has not been clearly elucidated (Yayon *et al.*, 1991). Ornitz *et al.* (1992) reported that high affinity bFGF binding to FGFR 1 and its mitogenic activity was dependent on the presence of heparin. In addition to bFGF, heparin-like molecules have been directly implicated in aFGF-mediated biological activity (Klagsbrun, 1989; Kaplow *et al.*, 1990; Ornitz and Leder, 1992). Alternatively, the possibility that heparin-like molecules act indirectly as a reservoir to protect FGF family members from degradation and regulate growth factor availability has also been suggested (Saksela *et al.*, 1988; Flaumenhaft *et al.*, 1989).

The fibroblast growth factor receptor family (FGFR) of tyrosine kinases also contains the KGFR, with high affinity binding for KGF and aFGF, but significantly lower affinity binding for bFGF (Bottaro *et al.*, 1990; Miki *et al.*, 1992). Although KGF binding has been shown to be associated with a low affinity heparin-like component in BALB/MK cells (Bottaro *et al.*, 1990), the importance of heparin binding to KGFR-mediated signaling has yet to be determined. Recently, a soluble chimeric molecule between the KGFR extracellular domain and the immunoglobulin heavy chain (KGFR-HFc) was engineered and shown to be functionally equivalent in binding to the native KGFR (Cheon *et al.*, 1994). Because KGFR-HFc is easily manipulated in a cell-free environment, the effect of heparin is more accurately evaluated than by using intact cells which express the KGFR. Therefore, the role of heparin in the high affinity binding of KGF and aFGF was examined by using KGFR-HFc in the present study. The results suggest that heparin-like molecules are not required for high affinity binding of KGF or aFGF to the KGFR.

Materials and Methods

Materials

Recombinant human KGF (M.W. 19 kDa), [^{125}I]-KGF,

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and bovine brain [^{125}I]-aFGF were obtained from LCMB laboratory, NCI, NIH. Bovine brain aFGF (M.W. 16 kDa) was obtained from Upstate Biotechnology, Inc. (Lake Placid, USA). Protein A Sepharose CL-4B, Gamma bind G Sepharose, Sepharose CL-6B, and heparin Sepharose were purchased from Pharmacia LKB Biotechnology, Inc. (Piscataway, USA). [^{3}H]-heparin (0.44 mCi/mg, M.W. 13 kDa) was purchased from NEN (Boston, USA). Heparin was purchased from Sigma (St. Louis, USA).

Preparation of KGFR-HFc chimeric proteins

The KGFR-HFc chimeric molecule was constructed, expressed, and enriched by protein A Sepharose chromatography as described previously (Cheon *et al.*, 1994). Bound heparin-like molecules were removed from the KGFR-HFc after binding to a protein A Sepharose column (bed volume, 2 ml) and washing with twenty-five column volumes of 0.6 M NaCl in phosphate buffered saline (PBS). The KGFR-HFc was then eluted with 100 mM sodium citrate buffer (pH 5.5), dialyzed against PBS, and concentrated using an Amicon concentrator.

KGFR-HFc binding to [^{3}H]-heparin and heparin Sepharose

KGFR-HFc was incubated at room temperature in 200 μl of PBS with varying concentrations of [^{3}H]-heparin (0.44 mCi/mg) in the presence or absence of a hundred-fold excess of unlabeled heparin. After 1 h, 50 μl of Gamma bind G Sepharose was added, shaken vigorously for 1 h, pelleted, and washed three times with PBS (0.5 ml each). Bound heparin recovered from the pellets was counted in 10 ml of Biofluor liquid scintillation cocktail. Specific binding was defined as the difference between the total binding and counts bound in the presence of excess unlabeled ligand.

To determine the KGFR-HFc binding to heparin Sepharose, [^{35}S]-labeled conditioned medium (500 μl) from the KGFR-HFc transfectant was precipitated with 300 μl of preswollen heparin Sepharose. The pellet complex was washed three times with PBS (0.5 ml each). The pellet was analyzed by SDS-PAGE and fluorography.

[^{125}I]-KGF and [^{125}I]-aFGF binding to KGFR-HFc

Binding assay was done in a similar manner to the heparin binding as described above. The incubation mixture (200 μl) contained varying concentrations of either [^{125}I]-KGF (270,000 cpm/ng) or [^{125}I]-aFGF (29,000 cpm/ng) in PBS/0.3% milk in the presence or absence of a hundred-fold excess unlabeled ligands, incubated for 1 h at room temperature, and the mixture was immunoprecipitated with 200 μl of preswollen *Staphylococcus aureus* protein A Sepharose CL-4B. After washing the pellets three times with 0.5 ml of PBS, bound KGF or aFGF recovered from the pellets was counted in a Beckman gamma counter.

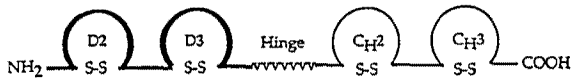

Fig. 1. Schematic structure of chimeric KGFR-HFc. Designations used are as follows: D2, KGFR immunoglobulin-like domain 2; D3, KGFR immunoglobulin-like domain 3; C_H, constant region of heavy chain of IgG1. The hinge region is depicted using a zigzag line.

Results and Discussion

KGFR-HFc binding to [^{3}H]-heparin and heparin Sepharose

Since the demonstration of the tight interaction between heparin and the FGF family members (Klagsbrun, 1989), it has been proposed that extracellular matrix associated heparin-like molecules are required for the binding as well as for the biological activity of the growth factors of this family. Previous studies have shown that the chimeric molecules consisting of the KGFR Ig-like domain two and three and the IgG heavy chain Fc domain (HFc) is functionally active and possesses the KGFR binding domain of intact cells (Cheon *et al.*, 1994). The results have indicated that the major binding sites for KGF and aFGF are localized to distinct KGFR Ig-like domains. Here, the effect of heparin on the binding properties of the KGF and aFGF to the KGFR was assessed in a cell free system by using secreted KGFR-HFc. The schematic structure of the KGFR-HFc is shown in Fig. 1.

KGFR-HFc gene products were expressed in NIH 3T3 cells, and purified with protein A Sepharose. Using soluble KGFR-HFc which is readily immunoprecipitated with Gamma bind G Sepharose, the binding experiment was done to examine whether KGFR-HFc bound to heparin. As shown in Fig. 2, binding of [^{3}H]-heparin to KGFR-HFc was saturable in the range of 0.5 to 13 μg/ml. The specificity of this reaction for heparin was in the KGFR portion of the chimera since heparin failed to bind the Fc portion of IgG, control monoclonal antibody MOPC21, nor to Gamma bind G Sepharose alone (result not shown). Scatchard analysis demonstrated that the dissociation constant for [^{3}H]-heparin binding to KGFR-HFc was 140 nM (Fig. 2).

As an independent approach for heparin binding to KGFR-HFc, KGFR-HFc was tested for the interaction with heparin Sepharose. As shown in Fig. 3, KGFR-HFc

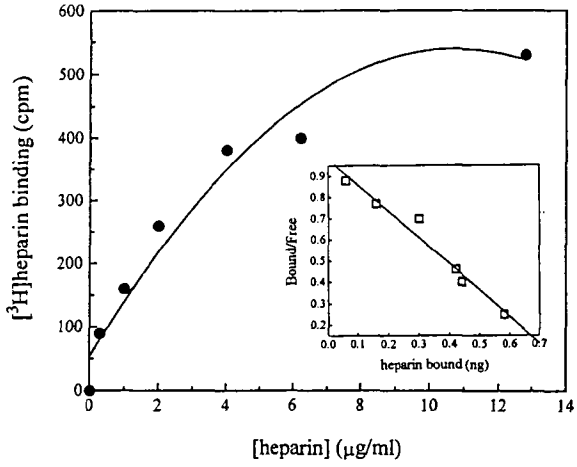

Fig. 2. [^{3}H]-heparin binding to KGFR-HFc. [^{3}H]-heparin at the indicated concentrations was incubated with KGFR-HFc for 1 h. After addition of Gamma bind G Sepharose for 1 h, complexes were immunoprecipitated, and specific [^{3}H]-heparin binding was determined as described in Materials and Methods. *Inset*: Scatchard analysis of [^{3}H]-heparin binding to KGFR-HFc.

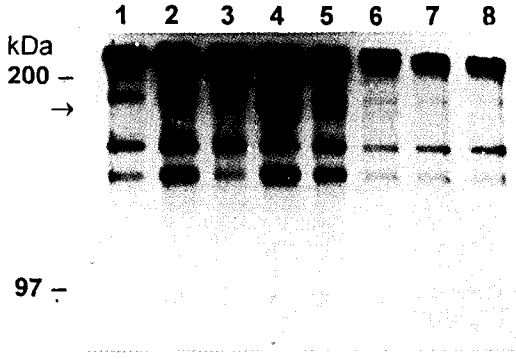

Fig. 3. Heparin Sepharose binding to KGFR-HFc. Heparin Sepharose (lanes 1~2 and 4~8) or Sepharose alone (lane 3) was incubated with metabolically labeled conditioned medium from NIH 3T3 cells (lane 1), NIH 3T3 transfectants expressing the KGFR-HFc (lanes 3~8), or NIH 3T3 cell transfectants expressing the HFc (lane 2). In some cases, the precipitation reaction was competed with 70 μM (lane 5), 250 μM (lane 6), 500 μM (lane 7), and 1 mM (lane 8) heparin. Pellets were analyzed by SDS-PAGE as described in Materials and Methods. The KGFR-HFc protein is indicated by arrow.

was readily precipitated with heparin Sepharose, but not Sepharose alone. As a further evidence for specificity, heparin Sepharose was used to immunoprecipitate the HFc portion of IgG and no corresponding immunoprecipitant was observed. In addition, the interaction between KGFR-HFc and heparin Sepharose was abrogated by free heparin (Fig. 3). Taken together, it was concluded that the KGFR-HFc binds heparin in a specific manner.

Next, attempts to remove the bound heparin, if any, to the KGFR-HFc while expressed and purified were carried out. Recent evidence has demonstrated that the

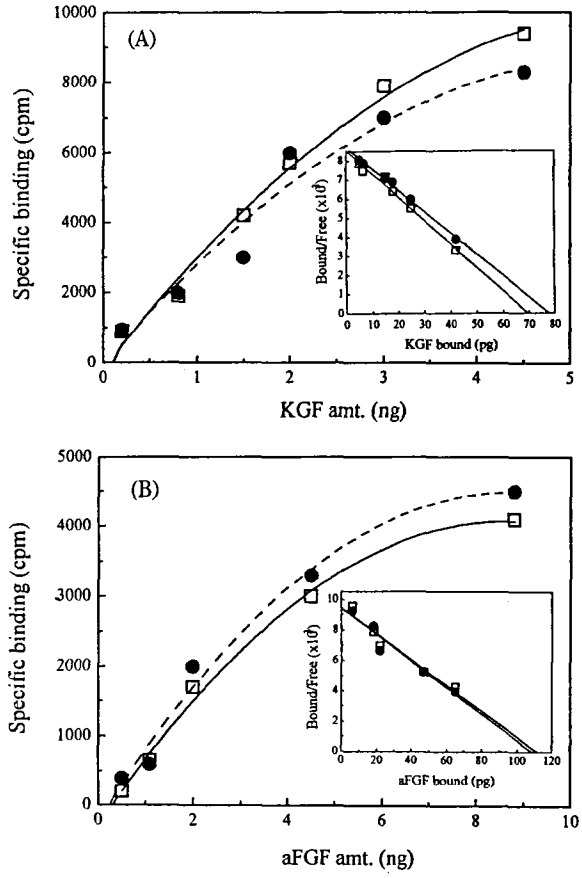

Fig. 4. Effect of 0.6 M NaCl washing on KGF and aFGF binding to the KGFR-HFc. 0.6 M NaCl washed (●) or unwashed (□) KGFR-HFc was incubated with the indicated concentration of [^{125}I]-KGF (panel A) or [^{125}I]-aFGF (panel B) in the presence or absence of a one hundred-fold excess of unlabeled KGF or aFGF respectively. Growth factor/KGFR-HFc complexes were pelleted with Gamma bind G Sepharose and counted as described in Materials and Methods. *Inset*: Scatchard analysis of [^{125}I]-KGF (panel A) or [^{125}I]-aFGF (panel B) binding to 0.6 M NaCl washed (●) or unwashed (□) KGFR-HFc.

interaction between heparin and FGFR 1 can be interrupted by the presence of high salt concentrations (Kan *et al.*, 1993). Therefore, the effect of high salt concentrations on the removal of [^{3}H]-heparin from KGFR-HFc was examined. The concentration of 0.6 M NaCl was sufficient to remove bound [^{3}H]-heparin from the KGFR-HFc/Gamma bind G Sepharose complex without interfering with the interaction between KGFR-HFc and Gamma bind G Sepharose (data not shown). Based on this result, the KGFR-HFc was treated with 0.6 M NaCl to remove any bound heparin-like molecules. [^{3}H]-heparin binding to the KGFR-HFc washed with 0.6 M NaCl also displayed a similar dissociation constant (230 nM) (data not shown). From the observation that salt washed KGFR-HFc exhibits a similar heparin binding pattern as compared with a salt unwashed preparation, KGFR-HFc expressed in NIH 3T3

cells and enriched by protein A Sepharose chromatography appeared not to contain heparin-like molecules.

KGF and aFGF binding to high salt treated and untreated KGFR-HFc

To determine whether heparin was required for KGF or aFGF binding to the KGFR, the KGF and aFGF binding properties to either salt washed or unwashed KGFR-HFc were compared. As shown in Fig. 4A, the binding saturation curves of KGF to either 0.6 M NaCl washed or unwashed KGFR-HFc were nearly identical. The dissociation constants of KGF binding to 0.6 M NaCl treated or untreated KGFR-HFc were 125 pM and 120 pM, respectively. In addition, aFGF bound 0.6 M NaCl treated or untreated KGFR-HFc with dissociation constants of 680 pM and 520 pM, respectively (Fig. 4B). Consistent with this, previous studies have reported that KGF and aFGF bind to native KGFR with dissociation constants of 400 pM and 350 pM, respectively (Bottaro *et al.*, 1990). Thus, removal of heparin does not appear to change the binding affinity of either KGF or aFGF to the KGFR. Furthermore, the addition of 300 ng/ml heparin to the salt washed KGFR-HFc had no effect on KGF binding (data not shown). Based on the present observation, although the KGFR-HFc interacts with heparin, it appears that the presence of heparin is not absolutely required for high affinity binding of either KGF or aFGF to the KGFR.

The possibility whether the low affinity (greater than 2.5 μM) bFGF binding to KGFR-HFc was affected by the presence of heparin was addressed. While 300 ng/ml heparin showed no effect on KGF binding, bFGF binding was enhanced. At heparan concentrations which showed a maximum enhancement of bFGF binding, bFGF was determined to possess an affinity constant of 12 nM to KGFR-HFc.

Since the interaction between the FGF family of growth factors and heparin has been demonstrated, it was surprising that the high affinity KGF and aFGF binding to KGFR-HFc was unchanged upon 0.6 M NaCl treatment which removes bound heparin from the KGFR-HFc. Interestingly, heparin seems to facilitate FGF dimerization which may lead to receptor dimerization and subsequent activation (Ornitz *et al.*, 1992). Previous studies demonstrated that the KGFR-HFc is expressed as a disulfide-linked dimer form (Cheon *et al.*, 1994). If heparin induces the growth factor dimerization, thus leading to its biological action, the heparin dispensability for KGF and aFGF high affinity binding can be explained by the presence of the dimeric form of the KGFR-HFc, formed independently from the growth factor dimerization. Alternatively, other related glycosaminoglycan may still be present in the KGFR-HFc despite high salt treatment, and thus participate in high affinity ligand binding.

Kan *et al.* (1993) recently reported that heparin interacts with the amino terminus of the second immunoglobulin-like domain of the FGFR 1. Consistent with the observed interaction between heparin Sepharose and KGFR-HFc, KGFR-HFc contains the indicated heparin binding site of the receptor. In addition, the chimeric molecule between the second Ig-like domain of the KGFR and HFc was able to precipitate with heparin Sepharose (unpublished result). Although it has been reported that aFGF and bFGF bind to cell-associated heparin sulfate with dissociation constants of 2~3 nM and 60~90 nM, respectively (Moscatelli, 1987; Walicke *et al.*, 1989; Lee and Lander, 1991), the affinity of heparin for the receptor has not been reported. In the studies reported here, the dissociation constants of heparin for the KGFR-HFc were estimated to be about 100~200 nM. Similar to the reported results that the interaction between heparin and the FGF family was disrupted by 1.0~1.5 M NaCl (Klagsbrun, 1989), the association between heparin and KGFR-HFc was blocked efficiently by 0.6 M NaCl. These results are also in agreement with those of Kan *et al.* (1993) who dissociated heparin from FGFR 1 at 0.6 M NaCl.

FGFR is known to exist in multiple forms. In contrast to the previously reported results supportive of the heparin involvement in the FGF biological action, the present study demonstrates that the cognate ligands of the KGFR, KGF and aFGF bind to the KGFR (alternative splicing form of the FGFR 2) with high affinity in the absence of heparin. However, it may be still possible that HSPG is implicated in KGFR-mediated events at stages following the direct binding of the growth factors. Further investigation awaits to clarify whether the observed differential effect of heparin on the high affinity binding of the FGF family is attributed to the different FGFR subtypes.

References

Baird, A., Schubert, D., Ling, N. and Guillemin, R. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 2324.

Bottaro, D. P., Rubin, J. S., Ron, D., Finch, P. W., Florio, C. and Aaronson, S. A. (1990) *J. Biol. Chem.* **265**, 127 67.

Burgess, W. H. and Maciag, T. (1989) *Annu. Rev. Biochem.* **58**, 575.

Cheon, H. G., LaRochelle, W. J., Bottaro, D. P., Burgess, W. H. and Aaronson, S. A. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 989.

Eriksson, A. E., Cousens, L. S., Weaver, L. H. and Matthews, B. W. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 3441.

Flaumenhaft, R., Moscatelli, D., Saksela, O. and Rifkin, D. B. (1989) *J. Cell Physiol.* **140**, 75.

Harper, J. W. and Lobb, R. R. (1988) *Biochemistry* **27**, 671.

Kan, M., Wang, F., Xu, J., Crabb, J. W., Hou, J. and Mckeehan, W. L. (1993) *Science* **259**, 1918.

Kaplow, J. M., Bellot, F., Crumley, G., Dionne, C. A. and Jaye, A. (1990) *Biochem. Biophys. Res. Commun.* **172**, 107.

Klagsbrun, M. (1989) *Prog. Growth Factor Res.* **1**, 207.

Klagsbrun, M. and Shing, Y. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 805.

Lee, M. K. and Lander, A. D. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 2768.

Miki, T., Bottaro, D. P., Fleming, T. P., Smith, C. L., Burgess, W. L., Chan, A. M. L. and Aaronson, S. A. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 246.

Moscatelli, D. (1987) *J. Cell Physiol.* **131**, 123.

Ornitz, D. M. and Leder, P. (1992) *J. Biol. Chem.* **267**, 16305.

Ornitz, D. M., Yayon, A., Flanagan, J. G., Svahn, C. M., Levi, E. and Leder, P. (1992) *Mol. Cell. Biol.* **12**, 240.

Saksela, O., Moscatelli, D., Sommer, A. and Rifkin, D. B. (1988) *J. Cell Biol.* **107**, 743.

Shing, Y., Folkman, J., Sullivan, R., Butterfield, C., Murray, J. and Klagsbrum, M. (1984) *Science* **223**, 1296.

Walicke, P. A., Feige, J. J. and Baird, A. (1989) *J. Biol. Chem.* **264**, 4120.

Yayon, A., Klagsbrun, M., Esko, J. D., Leder, P. and Ornitz, D. M. (1991) *Cell* **64**, 841.

Zhang, J., Cousens, L. S., Barr, P. J. and Sprang, S. R. (1991) *Proc. Natl. Acad. Sci. USA* **83**, 3446.

Zhu, X., Komiya, H., Chirino, A., Faham, S., Fox, G. M., Arakawa, T., Hsu, B. T. and Rees, D. C. (1991) *Science* **251**, 90.