Journal of Cellular Biochemistry 30:79–85 (1986)
Perspectives in Inflammation, Neoplasia, and Vascular Cell Biology 97–103

Immunoreactive Fibroblast Growth Factor (FGF) in a Transplantable Chondrosarcoma: Inhibition of Tumor Growth by Antibodies to FGF

Andrew Baird, Pierre Mormède, and Peter Böhlen

Laboratories for Neuroendocrinology, The Salk Institute for Biological Studies, La Jolla, California 92037

Using a radioimmunoassay for bovine pituitary fibroblast growth factor (FGF), we have established the presence of the immunoreactive mitogen in extracts of a transplantable mouse chondrosarcoma. Both neutral and acidic extracts of the tumor contain an immunoreactive FGF (ir-FGF) that cross-reacts in a parallel and dose-dependent fashion in the radioimmunoassay. The ir-FGF is retained on heparin-Sepharose affinity columns and can be detected in the same molecular weight forms as rat pituitary FGF. Mice (C57/Bl) inoculated with the tumor (10 mg, im) show a decreased rate of tumor growth when passively immunized with the antiserum to FGF. The results establish the presence of FGF in this tumor and implicate its role in the etiology of its development.

Key words: fibroblast growth factor (FGF), chondrosarcoma-derived growth factor, immunoreactive mitogen

Fibroblast growth factor (FGF) has recently been purified to homogeneity from extracts of the bovine pituitary [1] and shown to be identical to the species present in brain [2]. Partial sequence analysis of this mitogen provided enough sequence information to raise highly specific antibodies that are capable of immunoneutralizing FGF activity in vitro [1]. The radioimmunoassay (RIA) developed with the antisera provided the first demonstration that the mitogen is widely distributed in many tissues [2–6] and, coupled to the powerful technique of heparin-affinity chromatography [7], enabled the isolation and partial characterization of FGF from adrenal, corpus luteum [Gospodarowicz et al, in preparation], and kidney, retina, and liver [Baird et al, in preparation].

Chondrosarcoma-derived growth factor shows several structural and biological characteristics that make it similar to FGF. In particular, both are cationic proteins with molecular weights of 16,000–18,000 Daltons and have strong affinities in

Received May 28, 1985; accepted September 4, 1985.

© **1986 Alan R. Liss, Inc.**

heparin-Sepharose affinity columns [2–7]. Moreover, both mitogens share similar mitotic activities in vitro and possess angiogenic activity in vivo [1,7–11]. It was for these reasons that we tested the hypothesis that the transplantable mouse chondrosarcoma EHS contains an FGF-like protein that is responsible, at least in part, for the angiogenic activity contained in this tumor. In an effort to establish whether the chondrosarcoma-derived FGF is involved in the progression of this tumor, we investigated the effect of antibodies to FGF on tumor growth.

MATERIALS AND METHODS

Tissue Extractions

The transplantable mouse chondrosarcoma EHS was the generous gift of Dr. Denis Gospodarowicz (University of California at San Francisco). Female C57/Bl mice were inoculated with the equivalent of 10 mg of tumor by injection of the cell suspensions into the right thigh muscle. Tumors (mean weight 1.6 gm) were excised 20–35 days later and frozen in liquid nitrogen. Acid extracts of tissues were prepared by extraction in 5 volumes of 0.15 M $(NH_4)_2SO_4$, pH 4.5, homogenization, and readjustment of the pH to 4.5. After mixing for 2 hr, the solution was centrifuged at 48,000g. Neutral extracts were prepared by homogenizing in 0.15 $(NH_4)_2SO_4$ at pH 7. The amounts of FGF immunoreactivity (ir-FGF) in these extracts were measured by RIA.

Purification of FGF

The ir-FGF detected in the extracts was tested for capacity to bind to heparin-Sepharose affinity columns. The extracts were loaded onto columns of heparin-Sepharose (0.5 $\times$ 2 cm) that had been prepared according to the specifications of the manufacturer (Pharmacia, Piscataway, NJ). The columns were washed with 5 ml of 0.15 NaCl in 10 mM Tris buffer and subsequently washed with step gradients of 0.6, 1.1, 2, and 3 M NaCl in Tris buffer (pH 7.4). The amounts of ir-FGF in the column fractions were measured by RIA.

Gel permeation column chromatography of the neutral tumor extract was performed by loading the extract on a bed of Sephacryl S300 (V_t = 230 ml) that was prepared in 0.067 M phosphate buffer [5]. Ir-FGF in the column fractions was measured by RIA [12].

Radioimmunoassay for FGF

Amino-terminal antibodies to FGF were obtained from the immunization of rabbits against a conjugated synthetic replicate of the amino terminal of FGF. The hapten, $[Tyr^{10}]$-FGF(1–10)OH, was radioiodinated by the method of Greenwood et al [13] as described [12], and antiserum from RB-716-B8 was used at a dilution of 1:10,000 under the RIA conditions of this laboratory. The antiserum recognizes the synthetic hapten and native bovine FGF on an equimolar basis [1,12].

Inoculation of C57/Bl mice

Sera from normal rabbits (NRS), from hyperimmune rabbits (anti-PG), or from rabbits immunized against the synthetic amino terminal of FGF (anti-FGF) were precipitated with ammonium sulfate four times and dialyzed extensively against borate-buffered saline. The retentate was reconstituted to a volume that gave 50%

binding of radioiodinated [Tyr^{10}]-FGF(1–10)OH at a dilution of 1:7,500. The NRS and hyperimmune sera were diluted accordingly. The anti-PG serum was prepared by immunizing rabbits with a peptide (PG) conjugated to BSA via a bis-benzodiazotized linkage. PG is a nonsense peptide related to the carboxyl terminal of prepro growth hormone-releasing factor with the sequence Tyr-Val-Ala-Leu-Leu-Gln-Lys-His-Arg-Asn-Ser-Gln-Gly-OH. The antisera are highly specific for the free carboxyl terminal (Gly-OH) of the peptide and do not cross-react (< .01%) with any carboxyl-truncated forms of the peptide. The antibodies also have no endogenous ligand in rat, mouse, bovine, or human tissues as assessed by immunohistochemical staining or radioimmunoassay.

C57/Bl mice were treated with 100 μl of the protein preparation (ip) daily for 2 days prior to the inoculation with tumor cells. On the third day, the chondrosarcoma was prepared for inoculation by physical dispersion using extensive trituration. After dilution of the cell suspension in DMEM, the cells (10 mg equivalent) were administered (im) to the right thigh of each mouse. This dose of chondrosarcoma was previously shown to induce significant tumors in all animals in 3 weeks. On the following day, all animals received the antibody preparation, and this treatment was repeated every second day for the duration of the experiment.

Tumor size was quantitated after dissection of the right leg by carefully excising the tumor free of muscle and weighing all of the abnormal tissue obtained. Blood was simultaneously collected and the titer of anti-FGF antibodies in the treated mice was quantitated by binding to radioiodinated [Tyr^{10}]FGF(1–10). Mean titer at the end of these experiments was 1:500.

RESULTS

Partial Characterization of Immunoreactive FGF in Extracts of the Chondrosarcoma

Both acidic (pH 4.5) and neutral (pH 7) extracts of the chondrosarcoma show a parallel and dose-dependent cross-reactivity in the RIA for FGF. The neutral extract shows a five-fold higher yield of ir-FGF than its acidic counterpart (Fig. 1). Heparin-Sepharose affinity chromatography of the acidic extract (Fig. 2) revealed that the ir-FGF contained in the extract is strongly bound to the column. As much as 2–3 M NaCl was required to elute the immunoreactivity from the column. When a similar experiment was done with the neutral tumor extract, although there is quantitatively more ir-FGF in the extract, no immunoreactivity was detected in the column fractions (results not shown). All the ir-FGF in the neutral extract was found in the unretained fraction. Gel permeation chromatography of the neutral extract revealed what might be the reason for this apparent discrepancy. Sephacryl S300 column chromatography of the ir-FGF contained in the neutral extract showed that the ir-FGF in the neutral extract had an estimated molecular weight of 70,000 Daltons (Fig. 3A). In an effort to determine whether this observation was characteristic of the tumor, we also examined the molecular form of the mitogen in a neutral extract of rat anterior pituitary (Fig. 3B). In this case, the ir-FGF also had an estimated molecular weight of 70,000 Daltons, indicating that neutral extraction was the same in both tissues. Acidic extraction of pituitary [1,2] and enzymatic digestion [7,8] of these tumors, however, result in the detection of a 16,000-Dalton molecule, which, as shown here and elsewhere [7], binds strongly to heparin-Sepharose affinity columns.

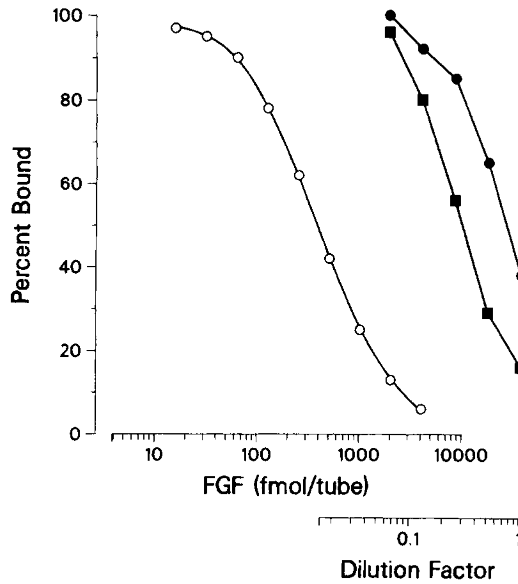

Fig. 1. Cross-reactivity of the chondrosarcoma-derived FGF. Aliquots of the neutral (closed squares) and acid (closed circles) extracts were tested after serial dilution for their cross-reactivity in an RIA for FGF (open circles). A dilution factor of 1 corresponds to an initial 20 mg of tumor (starting wet weight).

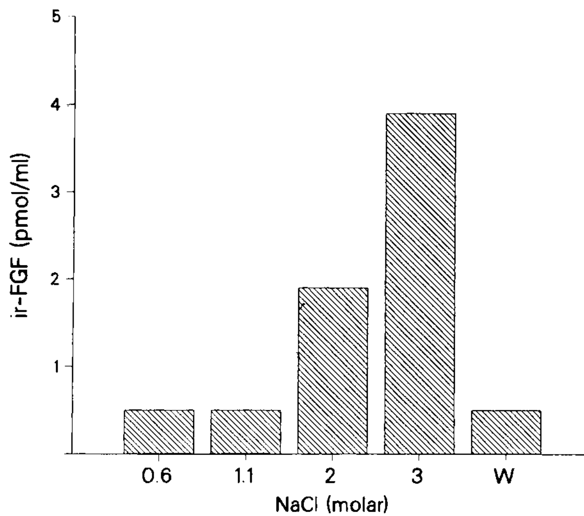

Fig. 2. Heparin-Sepharose affinity chromatography of the acidic chondrosarcoma extract. Aliquots of the column fractions were tested for the presence of ir-FGF by RIA. An identical experiment with the neutral extract failed to demonstrate retention of the high-molecular-weight FGF (Fig. 3).

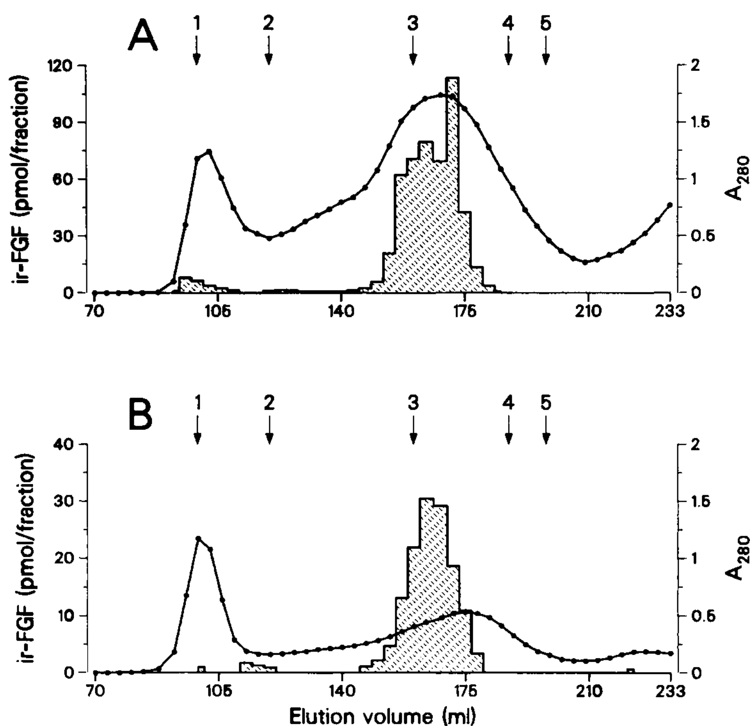

Fig. 3. Gel permeation chromatography of the neutral chondrosarcoma extract (A) and rat pituitary extract (B). The samples were extracted as described in the text and loaded and eluted from the Sephacryl S300 equilibrated with a 0.067 M phosphate buffer. Aliquots of the column fractions were tested for the presence of ir-FGF by RIA. The column was calibrated with 1, blue dextran, Mr = 2,000,000; 2, fibrinogen, Mr = 400,000; 3, bovine serum albumin, Mr = 67,000; 4, chymotrypsinogen, Mr = 25,000; and 5, cytochrome C, Mr = 12,400.

Inhibition of Tumor Growth by Antibodies to FGF

To investigate the possibility that FGF is actively involved in the etiology of the tumor development, we examined the effect of passive immunization on the development of the chondrosarcoma in vivo. Mice were treated with IgG prepared from normal rabbit serum (NRS), anti-FGF serum (anti-FGF), or control hyperimmune serum (anti-PG) (Fig. 4). On days 21 (Fig. 4A) and 28 (Fig. 4B) of the experiment, the tumors were harvested and weighed. Treatment with antibodies to FGF had a significant effect on the development of the tumors in both cases ($P < .01$ and $P < 0.05$ respectively). That was reflected in a 52% reduction in the tumor sizes of animals treated with anti-FGF antibodies. Treatment of animals with control hyperimmune serum (Fig. 4C) had no effect on tumor weight. In no instance was there evidence for tumor regression or necrosis.

DISCUSSION

The results presented here establish the presence of FGF in extracts of the transplantable chondrosarcoma (EHS). The capacity of antibodies to FGF to inhibit

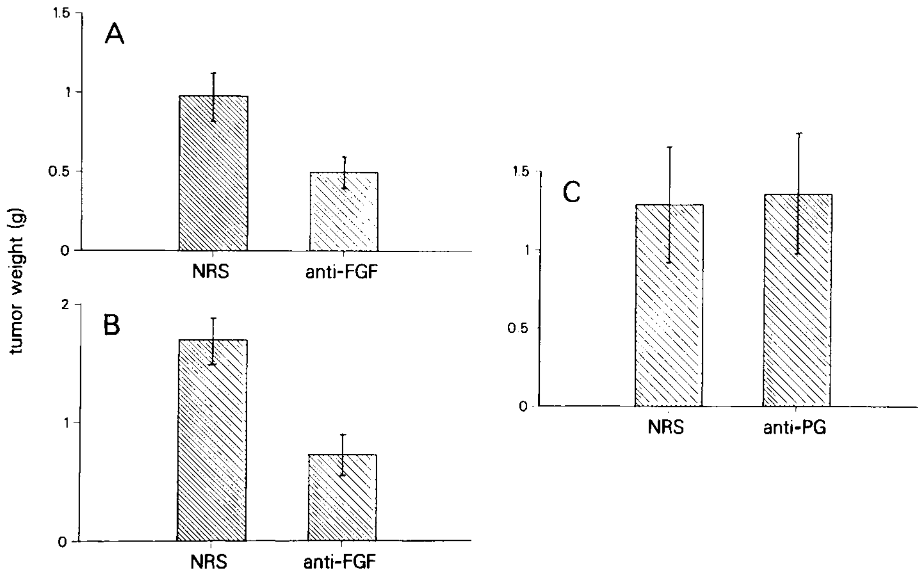

Fig. 4. Tumor weight was determined after 21 days (A) and 28 days (B) of treatment with IgG prepared from either normal rabbit serum (NRS) or anti-FGF serum (anti-FGF). Some mice were treated with hyperimmune serum (anti-PG) and sacrificed 28 days after receiving the tumor inoculation (C).

its development strongly implicates the growth factor in the growth of the tumor in vivo. Whether the ir-FGF is the same as the tumor growth factor described by Shing et al [7] and Azizkhan et al [8] remains to be established. Clearly, they have many common characteristics including their cationic nature, molecular weights, retention on heparin-Sepharose affinity columns, target cell populations, and angiogenic activities [2,7–11]. The structural characterization of each mitogen should clarify this relationship.

It is particularly interesting to note that, as described by Mormède et al [5] in other tissues, the major form of ir-FGF in neutral extracts has an estimated molecular weight of 70,000 Daltons. In contrast, acid extracts yield a 16,000-Dalton molecule [1,2,7,8]. Although at first this might seem a fundamental difference between FGF and the chondrosarcoma-derived mitogen, pituitaries extracted at neutral pH also show the presence of the 70-kD molecule with this extraction procedure. This might account, at least in part, for the many differences in the literature [1-7,14] regarding the molecular weights of endothelial cell growth factors (ECGFs). The possibility that 70-kD FGF generates a smaller molecule in acid extracts is supported by the observation of Maciag et al [14], who demonstrated the ability of acid to yield low-molecular-weight ECGFs from the high-molecular-weight mitogens that are extracted at neutral pH. Furthermore, purification of FGF from tissues such as liver, kidney, and corpus luteum [manuscripts in preparation] has led to the isolation of several microheterogeneous forms of FGF that appear to be generated by the extraction procedure.

How the relative affinities of the 70-kD and 16-kD FGF for heparin will correlate with their biological activities remains to be determined. Clearly, if there is no interaction between the 70-kD form of FGF and heparin as the results presented here suggest, then the generation of the heparin binding form (ie, 16-kD) will determine the physiological role of heparin in the modulation of FGF activity. It will be of particular importance then to determine the exact relationship (ie, precurser, aggregation, etc) between the higher-molecular-weight form of FGF and the lower-molecular-weight species.

The observation that antibodies to FGF can inhibit growth of the chondrosarcoma suggests that this mitogen is involved in its development. At the present time, however, it is not clear whether tumor growth is inhibited via an inhibition of neovascularization or through removal of the trophic stimulus for tumor cell growth. FGF is a potent mitogen for chondrocytes as well as endothelial cells [10,11], so it might act at one or both target cells. Furthermore, there was no evidence for necrosis or tumor regression in any of the treated mice, suggesting that the antibodies were inhibiting new tumor growth and not the survival of existing tumor tissue. Further studies with the purified mitogen and these antibodies should clarify the role of FGF in this experimental model.

ACKNOWLEDGMENTS

The authors wish to thank Robert Klepper and Terri Durkin for expert technical assistance. This research was supported by grants from the NIH (AM-18811 and HD-09690) and from the Robert J. Kleberg and Helen C. Kleberg Foundation. Pierre Mormède is a visiting scientist from the Institut National de la Recherche Agronomique (France) and is a recipient of a NATO fellowship.

REFERENCES

1. Böhlen P, Baird A, Esch F, Ling N, Gospodarowicz D:Proc Natl Acad Sci USA 81:5364–5368, 1984.
2. Gospodarowicz D, Cheng S, Lui GM, Baird A, Böhlen P: Proc Natl Acad Sci USA 81:6963–6967, 1984.
3. Baird A, Mormède P, Böhlen P: J Cell Biochem 9A[Suppl]:351, 1985.
4. Baird A, Mormède P, Böhlen P: Biochem Biophys Res Commun 126:358–364, 1985.
5. Mormède P, Baird A, Pigeon P: Biochem Biophys Res Commun 128:1108–1113, 1985.
6. Esch F, Baird A, Ling N, Ueno N, Böhlen P, Gospodarowicz D, Guillemin R: Proc Natl Acad Sci USA 82:6507–6511, 1985.
7. Shing Y, Folkman K, Sullivan R, Butterfield C, Murray J, Klagsbrun M: Science 223:1296, 1984.
8. Azizkhan J, Sullivan R, Azizkhan R, Zetter BR, Klagsbrun M: Cancer Res 43:3281–3286, 1983.
9. Klagsbrun M, Folkman J, Lund D, Shing Y: J Cell Biochem 9A[Suppl]:335, 1985.
10. Gospodarowicz D, Massoglia S, Cheng J, Liu GM, Böhlen P: J Cell Physiol 22:323–332, 1985.
11. Gospodarowicz D, Bialecki H, Thakral T: Exp Eye Res 18:501–514, 1981.
12. Baird A, Böhlen P, Ling N, Guillemin R: Reg Peptides 10:309–317, 1985.
13. Greenwood FD, Hunter WH, Glover JS: J Biochem 89:114–123, 1963.
14. Maciag T, Hoover GA, Weinstein R: J Biol Chem 257:5333–5336. 1982.