Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 8091–8093, November 1988
Developmental Biology

Developmental changes in fibroblast growth factor in the chicken embryo limb bud

(mesodermal proliferation/extracellular matrix production/hyaluronate)

SYEDA I. MUNAIM*, MICHAEL KLAGSBRUN†, AND BRYAN P. TOOLE*

*Department of Anatomy and Cellular Biology, Tufts University Health Sciences Center, Boston, MA 02111; and †Departments of Surgery and Biological Chemistry, Children's Hospital and Harvard Medical School, Boston, MA 02115

Communicated by Jerome Gross, July 11, 1988

ABSTRACT **Cell proliferation is a major event during early limb development. Significant levels of growth factor activity, as measured by stimulation of DNA synthesis in mouse BALB/c 3T3 cells, were found in extracts of chicken embryo limb buds at early stages of development. Extracts from stage-18 limbs (3 days of incubation) were 2 to 3 times more potent than were extracts from older stages, namely 22–24 (4 days), 26 (5 days), and 28 (6 days). Basic fibroblast growth factor (bFGF) was measured specifically using an RIA, and the amounts of factor obtained corresponded to the activities measured by the 3T3 cell-growth assay. In addition, most growth factor in the extracts bound with high affinity to heparin-Sepharose columns. Western (immunologic) blotting and immunoprecipitation with an antibody specific for bFGF revealed a protein of identical size to bFGF—i.e., 18 kDa, in the extracts. Thus, a growth factor with the properties of bFGF is present in the early limb, and the level of this factor is highest when proliferation is a predominant cellular event in the developing limb. These and other data suggest that fibroblast growth factor is a key regulatory factor in embryonic growth and morphogenesis.**

The major cellular event occurring during the first stages of development of the chicken embryo limb is growth. Growth during the first several hours of limb development—i.e., stages 16–20 of embryonic development, is due to mesodermal cell proliferation and synthesis of extracellular matrix. Subsequently, specific regions of the limb mesoderm condense (beginning at stage 22) and then differentiate to cartilage or muscle (beginning at stage 25); other regions give rise to connective tissue. Evidence has been obtained indicating that factors that influence growth of embryonic limb mesoderm are produced by the ectoderm, especially the specialized region termed the apical ectodermal ridge (1–3). However the molecular nature of these ectodermal factors or other endogenous growth factors produced by the early-limb ectoderm or by the mesoderm itself is unknown. In this study we demonstrate the presence of basic fibroblast growth factor (bFGF) in the chicken early limb bud and show that its concentration is highest at the earliest stages of its development. In other studies (4, 5) we have shown that bFGF stimulates mesodermal cell proliferation and synthesis of hyaluronate, a major component of the extracellular matrix of the early limb (6, 7). Thus, bFGF may regulate growth in the early limb, both in relation to cell proliferation and extracellular matrix production.

MATERIALS AND METHODS

Cell Proliferation Assay. Limbs from the staged embryos (8) (four to nine dozen per stage) were dissected with sharpened fine-point forceps and collected in Hanks' balanced salt solution. They were centrifuged, resuspended in 0.01 M Tris/1 M NaCl buffer, pH 7.5, and sonicated on ice for 30 to 60 sec. The sonicate was centrifuged at 15,000 × *g* for 30 min, and the supernatant was diluted 1:5 with 0.01 M Tris, pH 7.5. Growth factor activity was determined by measuring the ability of these limb extracts to stimulate [^{3}H]thymidine incorporation into DNA of BALB/c 3T3 cells in culture as described (9). A unit of growth factor activity is defined as that amount required to stimulate half-maximal DNA synthesis in the mouse 3T3 cells.

Heparin-Sepharose Chromatography. Limb extracts prepared as described were diluted with 0.01 M Tris, pH 7.5, to give a final NaCl concentration of 0.1 M. Chromatography was done as described (10) with a 1- × 8-cm column equilibrated with 0.1 M NaCl/0.01 M Tris, pH 7.5. After application of the sample in the same buffer, a 100-ml gradient of 0.1 M to 3.0 M NaCl in the Tris buffer was applied at a flow rate of 1 ml/min. Two-milliliter fractions were collected and tested for growth factor activity in the 3T3 cell assay described in the previous section.

Western (Immunologic) Blot Analysis. Subsequent to sodium dodecyl sulfate/PAGE in 15% acrylamide gels (11), the proteins were transferred to nitrocellulose, treated with antibody to bFGF and then by biotinylated second antibody, streptavidin–biotinylated peroxidase complex, and the peroxidase substrate 4-chloro-1-naphthol, as described (12). The primary antibody used was made against a synthetic peptide comprising the 12 amino acids at the N-terminus of bFGF [bFGF (1–12)].

RIA for bFGF. Competitive RIA was done as described (12, 13). Briefly, 50 μl of a solution containing either serial dilutions of hepatoma-derived bFGF or chicken limb-bud extract was added to 100 μl of assay buffer (10 mM phosphate/150 mM sodium chloride/200 mM sodium acetate/25 mM EDTA/0.1% azide/0.1% human serum albumin/125 mM urea, pH 6.2) followed by 30 μl of antiserum raised against the N terminus of bFGF [bFGF (1–12)]. After 24 hr at 37°C, 20 μl of ^{125}I-labeled Tyr^{13}-bFGF (1–12) was added to each well and incubated at 4°C for 2 hr. To separate bound from free antigen, 50 μl of normal rabbit serum (diluted 1:50) was added to each tube followed by 50 μl of goat anti-rabbit serum (Pel-Freeze, diluted 1:12). The tubes were incubated at 4°C for 12 hr. After centrifugation for 30 min, 1900 × *g* at 4°C, the pellet was washed in assay buffer and counted in a Beckman 5500 γ counter. A standard curve was prepared using the dilutions of hepatoma FGF. The chicken limb extracts were analyzed in triplicate, and the concentrations of bFGF were obtained by reference to the standard curve. Units of bFGF activity in the chicken limb buds were calculated from the known activity of the standard hepatoma bFGF, which was 1 unit per 5 ng (14).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: FGF, fibroblast growth factor; bFGF, basic FGF; bFGF (1–12), 12 amino acids of the N terminus of bFGF.

Table 1. Growth factor activity at different stages of limb development

Stage	Growth factor activity, units/mg of protein
18	50 ± 4.0
22–24	30 ± 3.0
26	17 ± 0.8
28	26 ± 1.7

Growth factor activity was determined by measuring the ability of limb extracts to stimulate [^{3}H]thymidine incorporation into DNA of BALB/c 3T3 cells in culture as described. A unit of growth factor activity is defined as that amount required to stimulate half-maximal DNA synthesis in the mouse 3T3 cells. Results are expressed as means of four measurements (± SEM).

RESULTS

Effect of Limb Extracts on Cell Proliferation. To demonstrate growth-promoting activity in chicken embryo limb buds, we first examined the effect of extracts on stimulation of DNA synthesis in cultured 3T3 cells. Extracts were obtained of limbs dissected from chicken embryos that had been incubated in a humid atmosphere at 38.5°C for 3, 4, 5, and 6 days. The embryos were staged, and stages 18 (day 3), 22–24 (day 4), 26 (day 5), and 28 (day 6) were selected. The limbs were dissected, extracted, and tested as described. As shown in Table 1, the extracts at each stage contained significant amounts of activity. The extracts of stage-18 limbs, however, contained 2 to 3 times higher levels of activity than the older stages.

Heparin-Sepharose Chromatography of Limb Extracts. Various well-characterized growth factors differ markedly in their affinity of binding to heparin-conjugated Sepharose columns (15). Acidic FGF elutes at ≈1.0 M NaCl, and bFGF elutes between 1.5 M and 2.0 M NaCl, whereas other well-characterized factors elute at much lower ionic strengths (10). Extracts of the limb buds at all four stages tested—i.e., stages 18, 22–24, 26, and 28, were applied to heparin-Sepharose columns and eluted with a gradient of NaCl. Major peaks eluting with NaCl concentrations between 1.0 and 2.0 M were obtained consistently at all four stages. Examples of stages 22–24 and 26 are shown in Fig. 1. In some cases an additional peak eluting at 0.5 M NaCl was obtained. In all cases 80% of the activity eluted at NaCl concentrations of 1.0 to 2.0 M. These data suggested that the major factor detected may be FGF.

Identification of bFGF by Western Blot. To determine the molecular mass of the FGF-like factor in extracts of the stage-18 limb buds, Western blots were performed with an antibody raised against an N-terminal bFGF peptide. A single band was seen with a molecular mass of 18 kDa comigrating with a standard preparation of hepatoma cell-derived bFGF (Fig. 2). This result was further confirmed by immunoprecipitation of the bFGF from extracts of limb buds that had been incubated in culture with [^{35}S]methionine followed by SDS/PAGE and fluorography (data not shown).

RIA of bFGF in Limb Extracts. Because the data presented above suggest that the growth-promoting activity of the limb extracts has bFGF-like properties, the amount of bFGF in the extracts was measured by RIA (Table 2). The amounts of

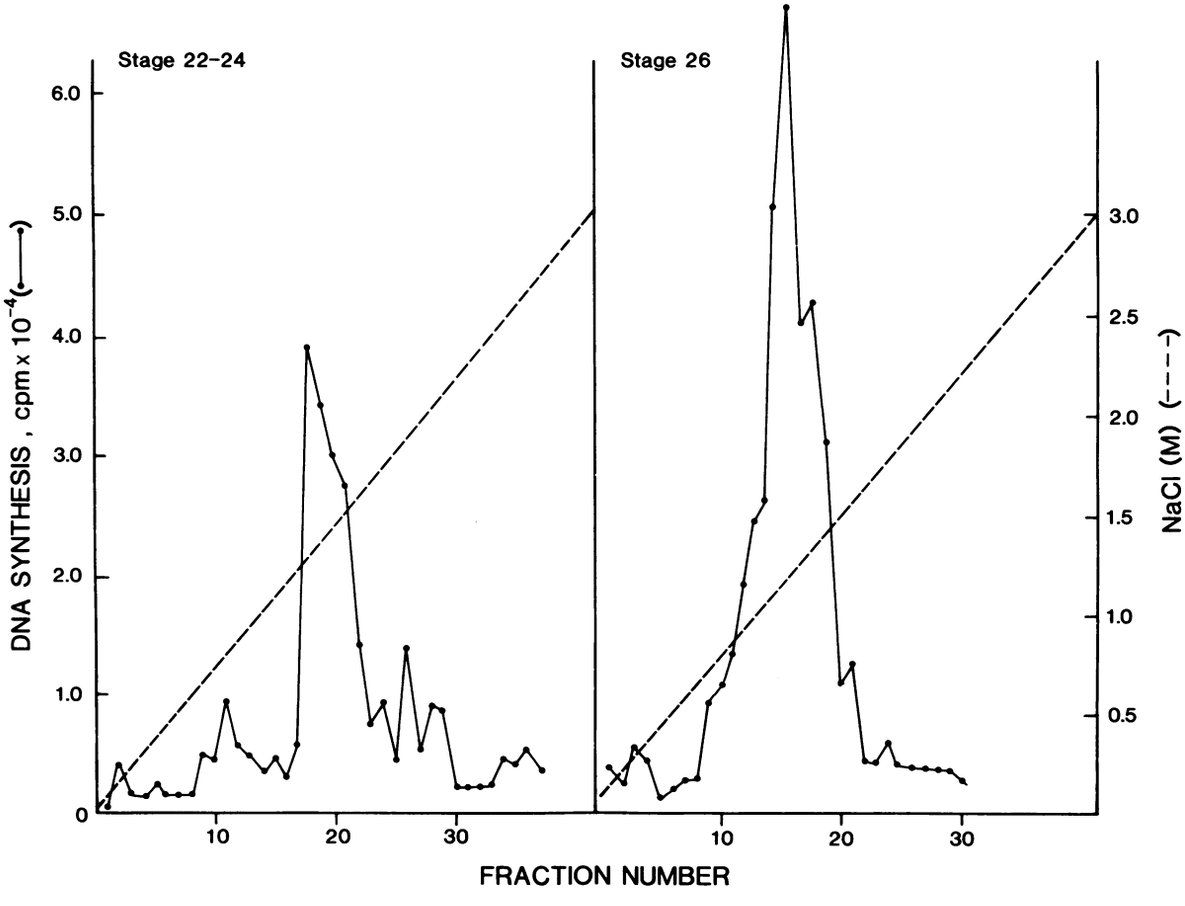

FIG. 1. Chromatography of stage 22–24 and 26 chicken limb-bud extracts on heparin-Sepharose. Limb extracts were prepared and chromatographed, and the fractions were tested as described. Approximately twice as much protein was used for chromatography of the stage-26 extract (40 mg) as the stage 22–24 extract (18 mg). Similar chromatographic profiles to these were obtained for extracts of stage-18 and stage-28 limbs.

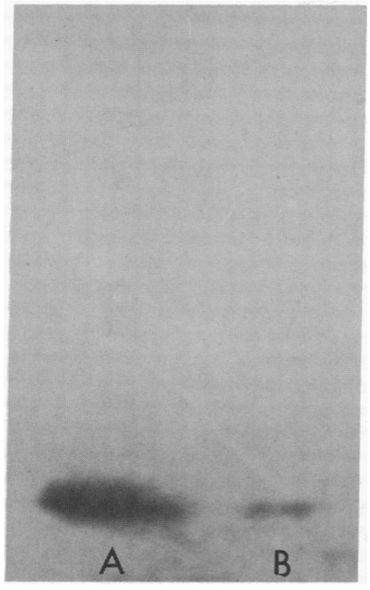

FIG. 2. Identification of bFGF in stage-18 limb-bud extracts by transfer (Western) blot. Lane A, purified hepatoma-derived bFGF (14); lane B, stage-18 limb-bud extract.

bFGF so obtained show a temporal pattern consistent with that of the total units of growth factor activity (Table 1)—i.e., the level of bFGF is higher in the stage-18 limb-bud extracts than in the extracts of later-stage limb buds.

DISCUSSION

The results indicate that a major growth-promoting activity in the embryonic limb is closely related, if not identical, to bFGF. Moreover, the level of bFGF is highest at the stage of limb development when proliferation is a prominent cellular activity and decreases at the stages of development when cell condensation and differentiation occur. These findings are consistent with other studies in which exogenous FGF has been found to stimulate proliferation and suppress differentiation of myoblasts (16) and chondrocytes (5), two major cell types in the developing limb. Two other important cellular activities that occur in the early limb bud at maximum bFGF activity are the development of an extensive capillary network (17) and the immigration of muscle cell precursors (18). FGF is a potent angiogenic factor (19) and stimulates cell motility (20) and thereby may be involved in these processes as well.

Table 2. RIA of bFGF at different stages of limb development

Stage	bFGF, ng/mg of protein	bFGF, units/mg of protein
18	324 ± 2.5	65 ± 0.6
22–24	85 ± 2.5	17 ± 0.5
26	63 ± 2.8	13 ± 0.5
28	107 ± 2.0	21 ± 0.4

Competitive RIA was done as described. Units of bFGF activity in the chicken limb buds were calculated from the known activity of a standard hepatoma bFGF.

Several studies have pointed toward an interrelationship between regulation of cell proliferation and interactions of cells with their surrounding extracellular matrix (21). A major component of the early-limb matrix is hyaluronate, which is thought to be important in limb morphogenesis (6, 7). Changes in synthesis of hyaluronate in the limb bud correlate with the changes in bFGF level seen here; both are high when the mesodermal cells are actively proliferating, and both decrease during the stages of cellular condensation and differentiation (6, 7). bFGF stimulates proliferation of limb mesodermal cells and hyaluronate synthesis by these cells (4), suggesting that bFGF regulates growth in the early limb both in relation to cell proliferation and extracellular matrix production. In view of these results and the work of others with different embryonic systems—e.g., mesoderm induction in *Xenopus* embryos (22), angiogenesis in the embryonic kidney (23), proliferation of embryonic myoblasts (16), and neurite outgrowth (24), FGF may be a key regulatory factor in embryonic growth and morphogenesis.

The authors thank Dr. David Hamerman for helpful discussions. This work was supported by grants from the National Institutes of Health to B.P.T. (DE 05838 and HD 23681) and M.K. (CA 37392).

1. Saunders, J. W. (1948) *J. Exp. Zool.* **108,** 363–404.
2. Cooke, J. C. & Summerbell, D. (1980) *Nature (London)* **287,** 697–701.
3. Reiter, R. S. & Solursh, M. (1982) *Dev. Biol.* **93,** 28–35.
4. Munaim, S. I., Klagsbrun, M. & Toole, B. P. (1987) *J. Cell Biol.* **105,** 275a (abstr.).
5. Hamerman, D., Sasse, J. & Klagsbrun, M. (1986) *J. Cell. Physiol.* **127,** 317–322.
6. Knudson, C. B. & Toole, B. P. (1985) *Dev. Biol.* **112,** 308–318.
7. Toole, B. P. (1981) in *Cell Biology of the Extracellular Matrix,* ed. Hay, E. D. (Plenum, New York), pp. 259–294.
8. Hamburger, V. & Hamilton, H. L. (1951) *J. Morphol.* **88,** 49–82.
9. Klagsbrun, M., Langer, R., Levenson, R., Smith, S. & Lillehei, C. (1977) *Exp. Cell. Res.* **105,** 99–108.
10. Klagsbrun, M. & Shing, Y. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 805–809.
11. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
12. Wadzinski, M. G., Folkman, J., Sasse, J., Devey, K., Ingber, D. & Klagsbrun, M. (1987) *Clin. Physiol. Biochem.* **5,** 200–209.
13. Baird, A., Bohlen, P., Ling, N. & Guillemin, R. (1985) *Regul. Pept.* **10,** 309–317.
14. Klagsbrun, M., Sasse, J., Sullivan, R. & Smith, J. A. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 2448–2452.
15. Shing, Y., Folkman, J., Sullivan, R., Butterfield, C., Murray, J. & Klagsbrun, M. (1984) *Science* **223,** 1296–1298.
16. Olwin, B. B. & Hauschka, S. D. (1986) *Biochemistry* **25,** 3487–3492.
17. Caplan, A. I. (1985) *Cell Differ.* **16,** 1–11.
18. Chevallier, A., Kieny, M. & Mauger, A. (1977) *J. Embryol. Exp. Morphol.* **41,** 245–258.
19. Folkman, J. & Klagsbrun, M. (1987) *Science* **235,** 442–447.
20. Herman, I. M. & D'Amore, P. A. (1984) *J. Muscle Res. Cell Motil.* **5,** 697–709.
21. Gospodarowicz, D., Neufeld, G. & Schweigerer, L. (1986) *Cell Differ.* **19,** 1–17.
22. Slack, J. M., Darlington, B. G., Heath, J. K. & Godsave, S. F. (1987) *Nature (London)* **326,** 197–200.
23. Risau, W. & Ekblom, P. (1986) *J. Cell Biol.* **103,** 1101–1107.
24. Neufeld, G., Gospodarowicz, D., Dodge, L. & Fujii, D. K. (1987) *J. Cell. Physiol.* **131,** 131–140.