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Basic Fibroblast Growth Factor: An Autocrine Growth Factor for Epiphyseal Growth Plate Chondrocytes

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Abstract Basic fibroblast growth factor (bFGF) is a permissive mitogen for cultured chondrocytes and has been localized in the specific zones of the epiphyseal growth plate. In this study, we demonstrate that bFGF present in cartilage originates from within the cellular constituents of this tissue. Utilizing reverse transcription coupled to the polymerase chain reaction (PCR), bFGF mRNA was found in extracts of cartilage tissue. Immunocytochemical studies revealed that bFGF was present intracellularly in freshly isolated proliferative chondrocytes and in the extracellular matrix (ECM) after 24 h of culture. Western blot analysis of protein extracts from isolated proliferative chondrocytes identified a bFGF immunoreactive species with a molecular weight of approximately 18 kDa. In situ hybridization confirmed the presence of bFGF mRNA in freshly isolated proliferative chondrocytes. The bFGF in the ECM seemed to be sequestered and not available for biological activity, since these cells still required exogenous bFGF for cell proliferation. This sequestered bFGF could be released to stimulate cell proliferation when cultures were treated with plasmin, a proteolytic enzyme. These data support the hypothesis that bFGF is synthesized by chondrocytes and functions as an autocrine/paracrine mitogen via its deposition into the ECM with subsequent release from the ECM of cartilage being a critical step in biological activity. In addition, the study provides further evidence that locally produced bFGF plays an important role in normal growth and development of cartilage tissue. © 1996 Wiley-Liss, Inc.

Key words: bFGF, extracellular matrix, in situ hybridization, RT-PCR, immunocytochemistry, cell proliferation, Western blotting

Longitudinal bone growth is regulated by the cellular activities of the epiphyseal growth plate cartilage, which in turn is influenced by various systemic hormones and local factors of paracrine and autocrine origin [Hunziker, 1994]. It is the complex interaction of these substances on chondrocytes in vivo that determines final bone growth. Evidence is rapidly accumulating that basic fibroblastic growth factor (bFGF) is an important growth factor in longitudinal bone growth. Sasse et al. [1992] reported the presence of bFGF and bFGF mRNA in fetal bovine rib growth plate cartilage. In a study localizing immunoreactive bFGF in the avian epiphyseal growth plate [Twal et al., 1994], bFGF was found in the resting-proliferative zones of this tissue while the amount of bFGF was greatly diminished in the adjacent prehypertrophic zone. Intense intracellular immunostaining was also present in the hypertrophic calcifying zone. Baron et al. [1994] showed that the infusion of bFGF into the epiphyseal growth plate of young rabbits greatly accelerated vascular and bone cell invasion from the adjacent metaphyseal bone. Moreover, it has been discovered that achondroplasia, the most common form of dwarfism in humans, results from point mutations in the transmembrane domain of FGF receptor-3 [Shiang et al., 1994; Rousseau et al., 1994]. Finally, Coffin et al. [1995] have reported that overexpression of bFGF in mice caused a variety of skeletal malformations, including shortening and flattening of the long bones.

bFGF is a member of a peptide family that exhibits a variety of biological activities [Burgess and Maciag, 1989] and has a high affinity for heparin [Klagsbrun and Edelmen, 1989]. Basic FGF is not a circulating hormone, but rather, is a locally active autocrine or paracrine factor. This multifunctional peptide has been purified from a wide variety of tissues including various cartilages of human, chick and bovine origin [Lobb et al., 1986]. With cultured chondro-

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cytes, bFGF can act as both a mitogen [Gospodarowicz and Mescher, 1977] and a morphogen [Kato and Gospodarowicz, 1984, 1985]. Furthermore, it has been reported that bFGF is also a key regulator in the terminal differentiation of chondrocytes in vitro [Kato and Iwamoto, 1990]. In addition, Logan et al. [1991] recently showed that ovine fetal growth plate chondrocytes can produce bFGF in vitro.

Although bFGF lacks a signal peptide for secretion [Abraham et al., 1986; Mignatti et al., 1992], it has been found outside the cell [Vlodavsky et al., 1991]. For example, bFGF is associated with the extracellular matrix (ECM) of endothelial cells [Bashkin et al., 1989; Vigny et al., 1988; Folkman et al., 1988; Morton et al., 1989; Yamada et al., 1989]. In the ECM and on the surface of endothelial cells, bFGF is associated with heparan sulfate proteoglycans (HSPG) and can be released by treatment with heparitinase, plasmin, or phospholipase C [Saksela and Rifkin, 1990; Bashkin et al., 1992].

The purpose of this investigation was to clarify the conflicting observations that although bFGF is present in cartilage tissue [Twal et al., 1994], exogenous bFGF is required for mitogenicity in cultured chondrocytes [Rosselot et al., 1994]. The current research was conducted (1) to determine whether the bFGF in the cartilage matrix originated from the chondrocytes or from an external tissue source, and (2) to examine the utilization of bFGF by chondrocytes. These results provide new insight into the relationship between bFGF and avian cartilage chondrocytes and contribute to an understanding of its role in regulating cartilage metabolism and skeletal growth.

MATERIALS AND METHODS

Dulbecco's modified Eagle's medium (DMEM), Ham's F12 powdered medium, hyaluronidase, collagenase (type II), rabbit antibovine basic fibroblast growth factor (bFGF) (1–24) IgG, goat antirabbit IgG conjugated to horseradish peroxidase, goat antirabbit IgG conjugated to fluorescein isothiocyanate (FITC), and plasmin were purchased from Sigma Chemical Company (St. Louis, MO). Recombinant human bFGF and acidic FGF (aFGF) were obtained from Amgen Biologicals (R&D Systems, Minneapolis, MN). Fetal bovine serum (FBS) and newborn calf serum (NCS) were purchased from Atlanta Biologicals (Norcross, GA). Trypsin was purchased from Worthington Biochemical Corporation (Freehold, NJ). The radioactive precursor methyl-^{3}H-thymidine (2.0 Ci/mmol) was purchased from ICN Radiochemicals (Irvine, CA). Plasmid pSP73 containing the chicken bFGF cDNA was obtained from Dr. Harold Moses (Department of Cell Biology, Vanderbilt University School of Medicine, Nashville, TN). Plasmid pDLRII containing the chicken type II collagen cDNA was obtained from Dr. Phoebe Leboy (Department of Biochemistry, University of Pennsylvania School of Dental Medicine, Philadelphia).

Cell Culture

Chondrocytes were isolated from the proximal (mainly proliferative) zones of the tibial epiphyseal growth plates of 4-week-old male Peterson × Arbor Acre chickens as previously described by Rosselot et al. [1992]. The cells were resuspended in DMEM supplemented with 5% NCS and plated at a final density of 1.1 or 4.4 × 10^5 cells/cm^2 and incubated overnight at 37°C under an atmosphere of 5% CO_2 and 95% air. Following overnight incubation, the cells were washed with phosphate-buffered saline (PBS) and exposed to different treatments.

Immunofluorescence

The procedure for the immunocytochemical detection of bFGF utilized the protocol of Kalcheim and Neufeld [1990] as modified by Twal et al. [1994]. Chondrocytes were plated at 4.4 × 10^5 cells/cm^2 on glass coverslips in 24-well plates and grown in DMEM supplemented with 5% NCS. At different days of culture, cells were fixed for 1.5 h in 0.1 M sodium cacodylate buffer solution containing 2% paraformaldehyde, 0.5% glutaraldehyde, and 8% sucrose (2% PGS) at room temperature (RT). Cells were then rinsed twice with 0.1 M cacodylate buffer containing 8% sucrose at RT (each for 2 h) and once overnight at 4°C [Akisaka and Gay, 1985]. Alternatively, cells were permeabilized with 0.5% Triton X-100 at 4°C for 20–30 min before being fixed with 2% PGS. Cells were then stored at −20°C until use.

Cells were blocked with 10% goat serum in PBS for 1 h at RT to minimize nonspecific binding followed by overnight incubation with a 1:200 dilution of rabbit anti-bFGF IgG in 0.5% goat serum in PBS at 4°C. Cells were subsequently incubated with goat antirabbit IgG antibody conjugated to fluorescein isothiocyanate (FITC) (1:40) in 0.5% goat serum for 1 h and

then washed with 0.5% goat serum at RT. To prevent autofluorescence and to serve as a counterstain, cells were rinsed with 0.003% Evans blue in PBS for 10 min. After rinsing with 0.5% goat serum, coverslips were mounted on slides with 10% glycerol in PBS. Cells were examined using a Leitz Ortholux microscope (Leitz GMBH, Wetzlar, Germany) equipped with epifluorescence optics using a wide-band, blue, high-intensity filter system (H2 filter cube: 455-490-nm excitation range, 510-nm emission, 515-nm suppression) and photographed using Kodak Ektachrome 400 color slide film (Eastman Kodak, Rochester, NY). Control cells were generated by substitution of primary antibody with nonimmune purified rabbit IgG at the same dilution.

In Situ Hybridization

A 229-bp *Eco*RI, *Hin*DIII fragment of pSP73 containing chicken bFGF cDNA was isolated by agarose gel electrophoresis followed by electroelution with an Elutrap (Schleicher & Schuell, Keene, NH). An 800-bp *Pst*I, *Bam*HI fragment of pDLRII containing chicken collagen II cDNA was isolated in a similar fashion. Linearized pBluescript was also isolated for use as a negative control. Restriction fragments of bFGF and collagen II cDNA, as well as linearized pBluescript were labeled with digoxigenin according to the manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN).

Freshly isolated chondrocytes were plated at 4.4×10^5 cells/cm^2 on glass coverslips in 24-well culture dishes and grown in DMEM supplemented with 5% NCS. At 2 h after plating, cells were fixed with 4% paraformaldehyde in 0.1M $NaPO_4$ (pH 7.4) for 20 min at RT. Cells were then permeabilized with 0.5% Triton X-100 for 20 min at RT. Cells were then stored at −20°C until use.

In situ hybridization was carried out by the method of Zheng et al. [1993]. Hybridization was detected by using alkaline phosphatase-conjugated digoxigenin antibodies and the substrates nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolylphosphate. Hybridization was indicated by the formation of a blue precipitate. Cells were observed and photographed using a Nikon TMS inverted phase-contrast microscope and Kodak Ektachrome 160T film (Eastman Kodak, Rochester, NY). Collagen II served as a positive control, while pBluescript was used as a negative control. As an additional negative control, cells were treated with RNase prior to hybridization with all probes.

Reverse Transcription Polymerase Chain Reaction

For the preparation of RNA all glassware was baked and all solutions were treated with diethyl pyrocarbonate. Epiphyseal growth plate tissue was homogenized in a guanidinium isothiocyanate solution with a Brinkmann polytron. RNA was purified by centrifugation in a cesium trifluoroacetate density gradient by the method of Smale and Sasse [1992].

Two oligonucleotides were synthesized (Midland Certified Reagent, Midland, TX) based on the published sequence of chicken bFGF [Zuniga et al., 1993]. The two 18mers synthesized were primer A: 5′-ACGGCGTCCGCGAGAAGA-3′ (corresponding to approximately amino acids 54–59) and primer B: 5′-GTCCAGGTCCAGTTTTTG-3′ (corresponding to approximately amino acids 140–145).

RNA was reverse transcribed using primer B according to the manufacturers protocol (Promega, Madison, WI). PCR reaction mixtures contained 200 ng of reverse transcribed RNA, 50 pmol of both primer A and B, 50 μM of each of the 4 deoxynucleoside triphosphates, 1.25 mM $MgCl_2$, and 2.5 units of Taq polymerase (Perkin Elmer, Foster City, CA) in 100 μl of 10 mM Tris (pH 9.0 at 25°C), 50 mM KCl, 0.1% Triton X-100. Thirty cycles of denaturation at 95°C for 15 s, annealing at 56°C for 30 s, followed by 30 s of extension at 72°C was performed. PCR products were visualized by electrophoresis in a 1.5% agarose gel, followed by staining with ethidium bromide. The identity of the PCR product was confirmed by size and Southern hybridization.

Extraction of bFGF from Freshly Isolated Chondrocytes

Chondrocytes were isolated from the proliferative zone of the avian epiphyseal growth plate as described by Rosselot et al. [1992]. The pellet containing 4.0×10^7 cells was resuspended in 100 μl lysate buffer containing 10 mM Tris pH 7.5, 35 μg/ml phenylmethanesulfonyl fluoride, 0.7 μg/ml pepstatin, 0.5 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM EDTA, 0.5% Triton X-100. After incubation for 10 min at 4°C, the suspension was frozen quickly in liquid nitrogen and then sonicated. This was repeated for 3 times and the suspension was then aspirated into a 1-ml syringe and repeatedly forced through

a 26½-gauge needle until the cells were lysed. The extract was then centrifuged at 14,000 rpm for 10 min at room temperature and the supernatant (crude extract) was collected.

SDS–PAGE and Western Blot Analysis

The presence of bFGF in protein extracts from freshly isolated chondrocytes was determined by Western blot analysis. Protein extracts were electrophoresed on a 12% polyacrylamide gel and electroblotted onto nitrocellulose according to the manufacturer's instructions (BioRad, Hercules, CA). The blot was blocked for 1 h in 5% nonfat dry milk and then incubated with a 1:1,500 dilution of rabbit anti-bFGF IgG (Sigma, St. Louis, MO). Antibody binding was visualized by utilizing antirabbit IgG conjugated to horse radish peroxidase, ECL detection reagents (Amersham, Arlington Heights, IL) and exposing the blot to Hyper film (Amersham, Arlington Heights, IL).

Cell Proliferation Assay

[^{3}H]thymidine incorporation has been shown to be a good indicator of chondrocyte proliferation by Rosselot et al. [1994]. This procedure was performed according to the protocol of Madsen et al. [1983] as modified by Rosselot et al. [1992]. Freshly prepared cells were plated into 96-well plates at 1.1×10^5 cells/cm^2 and incubated overnight in DMEM supplemented with 5% NCS. The next day, the cells were rinsed with PBS and treated with various levels of bFGF (0.1–4 ng/well) in 200 μl/well DMEM supplemented with 5% FBS. The media was removed and the same treatments were added again at 48 h in culture. The cells were pulse-labeled with [^{3}H]thymidine (a final activity of 1 μCi/well) during the last 18 h of the 3-day culture period. For determinations of [^{3}H]thymidine uptake, the cells were directly filtered onto a glass microfiber filter (EPM 2000; Whatman International, Maidstone, England) using a Brandel cell harvester (model M12V, Brandel, Rockville, MD). Radioactivity was determined by a liquid scintillation counter (Rackbeta 1209, Pharmacia LKB Nuclear, Gaithersburg, MD).

Mitogenic Effect of Plasmin on Chondrocytes

Chondrocytes were plated at 1.1×10^5 cells/cm^2 in 96-well plates and incubated overnight in DMEM media supplemented with 5% NCS. After 24-h incubation, cells were washed with PBS and then exposed to different concentrations of heparitinase (0.03–2.5 U/well), heparinase (0.03–5 U/well), phosphotidylinositol phospholipase C (0.1–0.4 U/well), heparin (0.2–2 U/well), heparan sulfate (HS) (0.2–2 U/well), chondroitinase ABC (0.03–3 U/well), or plasmin (10–80 μg/well) in 50 μl DMEM for 1 h at 37°C. Then 150-μl DMEM containing 6.67% FBS and 30 μg/well aprotinin was added to yield a final serum concentration as 5% FBS. After 48-h incubation, the cells were pulse-labeled with [^{3}H]thymidine. In a separate experiment, 25 μl anti-bFGF IgG or 25 μl nonimmune IgG was added along with the plasmin in 50 μl DMEM for 1 h at 37°C. Then the same procedure described above was performed.

RESULTS

Immunocytochemical Staining

Cells incubated for 2 h in DMEM containing 5% NCS adhered to coverslips and are referred to as freshly isolated cells. These cells were compared to other cells cultured for ≥ 24 h. Freshly isolated cells showed strong bFGF immunofluorescence intracellularly (Fig. 1A) and cells cultured for 24 h showed strong bFGF immunofluorescence both in cells and the ECM (Fig. 1B).

When examined with phase-contrast microscopy, freshly isolated chondrocytes and those cultured for 24 h were small and round in appearance (Fig. 1C). After prolonged culture (9 days), the cells increased in size and became polygonal in shape (Fig. 1D). The large polygonal cells had visibly less immunostaining than freshly isolated cells (Fig. 1E). Controls generated by substitution of primary antibody with non-immune rabbit IgG did not show any staining (Fig. 1F).

Western Blot Analysis

Western blot analysis of protein extracts from freshly isolated chondrocytes revealed an immunoreactive band of approximately M_r 18 kDa (Fig. 2, lanes 3, 4, 5). No cross-reactivity was observed with aFGF, which shares 55% sequence identity with bFGF (Fig. 2, lane 2).

Identification of Basic FGF mRNA in Tissue and in Cultured Chondrocytes

Reverse transcription of RNA isolated from growth plate tissue followed by PCR with primers specific to bFGF revealed the presence of bFGF mRNA (Fig. 3). In situ hybridization indi-

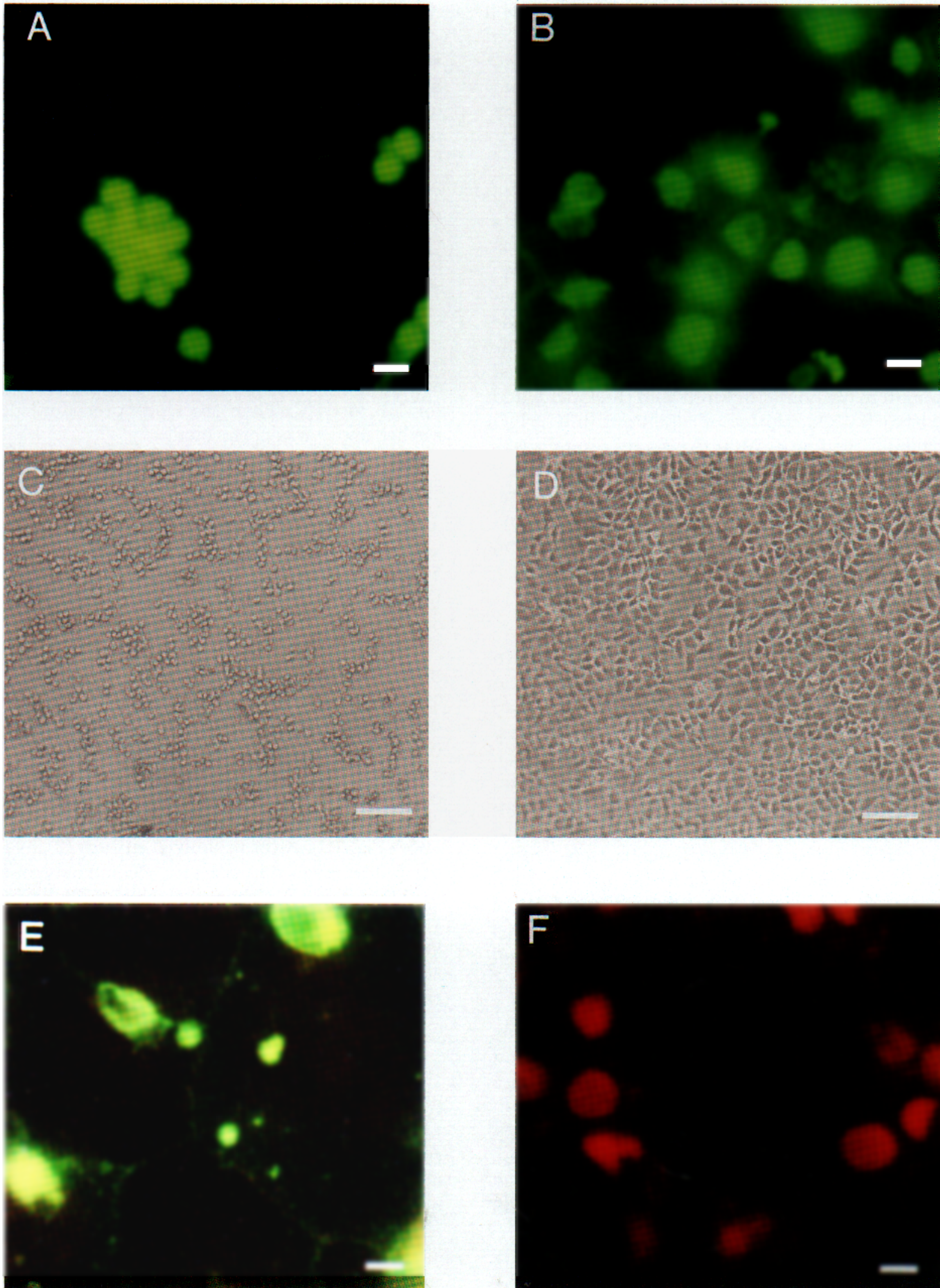


Fig. 1. Immunostaining for bFGF in freshly isolated proliferative zone chondrocytes (**A**) and chondrocytes cultured for 24 h in 5% NCS (**B**). Phase-contrast microscopy of freshly isolated proliferative zone chondrocytes (**C**) and chondrocytes cultured for 9 days in the presence of 5% NCS (**D**). Immunostaining for bFGF in chondrocytes cultured for 9 days in 5% NCS (**E**). Immunostaining in chondrocytes incubated for 24 h in 5% NCS when nonimmune rabbit IgG is substituted for rabbit anti-bFGF IgG (**F**). Bar = 10 μm (A, B, E, F); 100 μm (C, D).

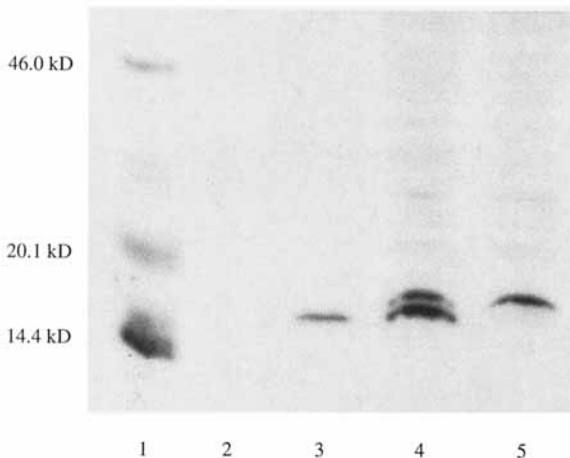


Fig. 2. Western blotting of protein extracts from freshly isolated proliferative zone chondrocytes reveals an immunoreactive band of 18 kDa. *lane 1,* molecular weight markers, *lane 2,* 10 ng aFGF; *lane 3,* 10 ng rh-bFGF, *lane 4,* 10 ng rh-bFGF + 50 μg of protein extract; *lane 5,* 50 μg of protein extract.

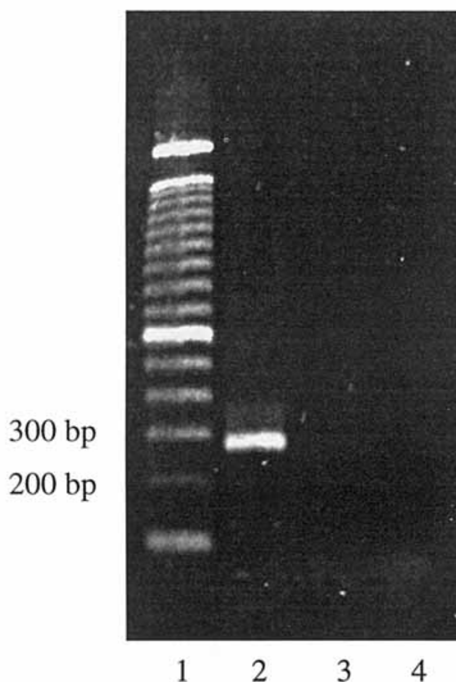


Fig. 3. RT-PCR of RNA isolated from the avian epiphyseal growth plate indicates that chondrocytes synthesize bFGF mRNA in vivo. *Lane 1,* 100 bp DNA ladder; *lane 2,* 279 bp bFGF PCR product; *lane 3,* PCR of RNA that was not reverse transcribed; *lane 4,* RT-PCR with no RNA.

cated that bFGF mRNA was present in freshly isolated chondrocytes (Fig. 4A). Treatment with RNase A significantly reduced the signal of bFGF (Fig. 4B). Negative controls produced by probing with digoxigenin-labeled pBluescript showed no staining (Fig. 4C). Strong staining was seen in chondrocytes probed for type II collagen, the characteristic collagen of proliferative chondrocytes (not shown).

Effect of Exogenous bFGF on Cell Proliferation

Exogenous bFGF stimulated [^{3}H]thymidine incorporation by chondrocytes in a dose-dependent manner at 0.1–4 ng/well ($P < 0.05$) (Fig. 5). It should be noted that the mitogenic response to exogenous bFGF was obtained in the presence of 5% FBS, confirming the observations of Rosselot et al. [1994] that other substances such as serum or growth factors had to be present for bFGF to elicit mitogenicity. Furthermore, the experiment (Fig. 5) was initiated with cells that had been cultured for 24 h and that contained substantial amounts of bFGF (Fig. 1B). We have also observed that bFGF accelerates the changes in cell morphology (Fig. 1D) and time to confluency, suggesting bFGF acts as a morphogen.

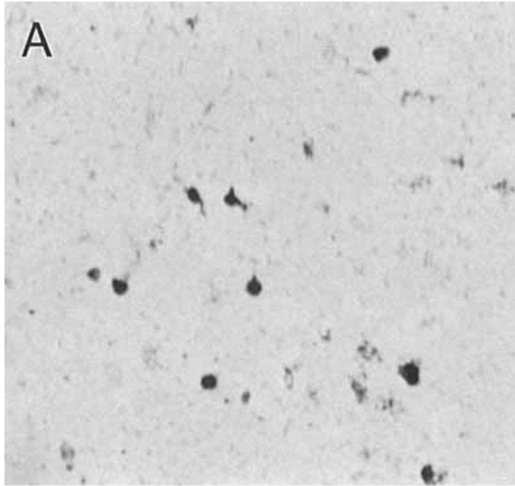
Mitogenic Effect of Plasmin on Chondrocytes

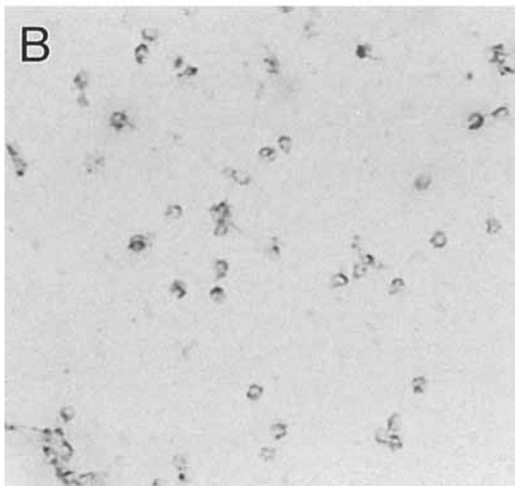
Heparitinase, heparinase, phosphotidylinositol phospholipase C, heparin, HS, chondroitinase ABC, and plasmin were tested for their ability to promote cell proliferation by releasing bFGF from the ECM or the cell surface of cultured chondrocytes. Control cells were incubated under the same conditions, except that test substances were omitted. After 48 h in culture, the extent of cell proliferation was evaluated using the [^{3}H]thymidine uptake assay.

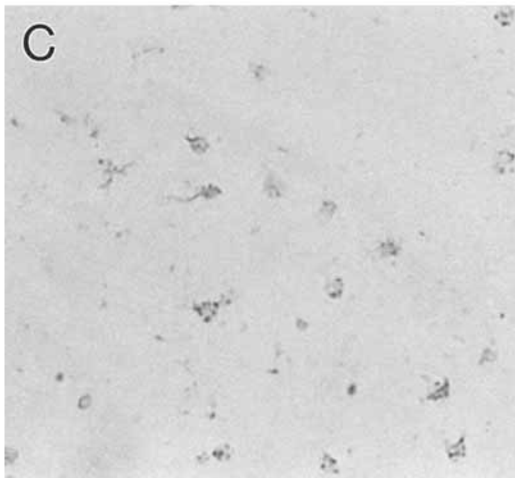
Only plasmin increased the proliferation of cultured chondrocytes. The mitogenic effect plasmin induced by the proteolytic enzyme treatment increased as a function of the amount of added (10–30 μg/well), with 30 μg/well plasmin resulting in cell proliferation that was about 9 fold higher ($P < 0.05$), as compared to control cultures not incubated with the enzyme (Fig. 6). In subsequent experiments, up to 80 μg plasmin/well was added, with no plateau being reached (data not shown).

Plasmin Treated Cells Released Biologically Active bFGF

Anti-bFGF IgG or nonimmune IgG was added to cultures during treatment with plasmin. The results showed the mitogenic activity released from plasmin treated cells was significantly inhibited by the addition of rabbit anti-bFGF (Fig. 7). By contrast, nonimmune rabbit IgG had no significant effect on this mitogenic activity (Fig.







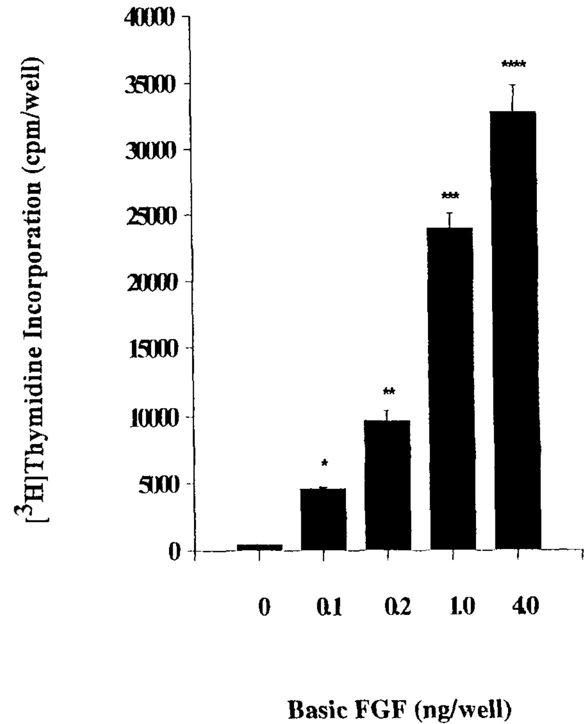


Fig. 5. Despite the presence of endogenous bFGF in cultured chondrocytes, exogenous bFGF is a potent mitogen in the presence of 5% FBS. Cells were cultured in 96-well plates (3.5×10^4 cells/well) in media supplemented with 5% FBS containing increasing levels of bFGF. [^{3}H]thymidine uptake was measured after pulse-labeling the cells during the last 18 h of a 3-day culture period. Values plotted are mean ±SEM ($P < 0.05$) (n = 3).

7), indicating that at least a portion of the mitogenic activity resulting from the plasmin treatment was due to the release of bioactive bFGF from the ECM.

DISCUSSION

The observation by Twal et al. [1994] that bFGF was present in the ECM surrounding the proliferative zone chondrocytes led to the question of whether this bFGF was produced endogenously or originated in another tissue. Using immunocytochemical methods and Western blotting analysis, it has been demonstrated that

Fig. 4. In situ hybridization confirms the presence of bFGF mRNA in freshly isolated proliferative zone chondrocytes. Cells were (**A**) probed with digoxigenin-labeled bFGF cDNA, (**B**) treated with RNase A and probed with digoxigenin-labeled bFGF cDNA, and (**C**) probed with digoxigenin-labeled pBluescript. ×400.

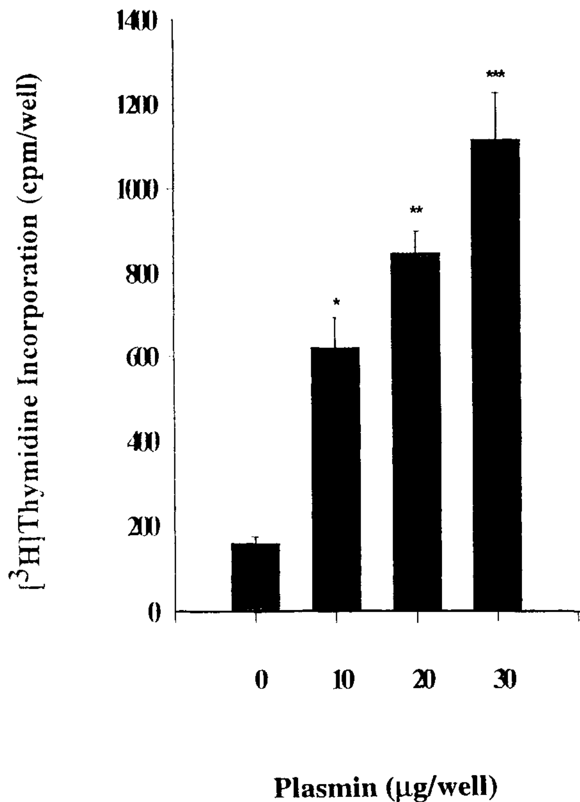


Fig. 6. Treatment of cultured chondrocytes with the protease plasmin stimulates cell proliferation. Cells were cultured in 96-well plates (3.5 × 10^4 cells/well) in media supplemented with 5% FBS containing increasing levels of plasmin. [^{3}H]thymidine uptake was measured after pulse labeling the cells during the last 18 h of a 2-day culture period. Values plotted are mean ±SEM ($P < 0.05$) (n = 2).

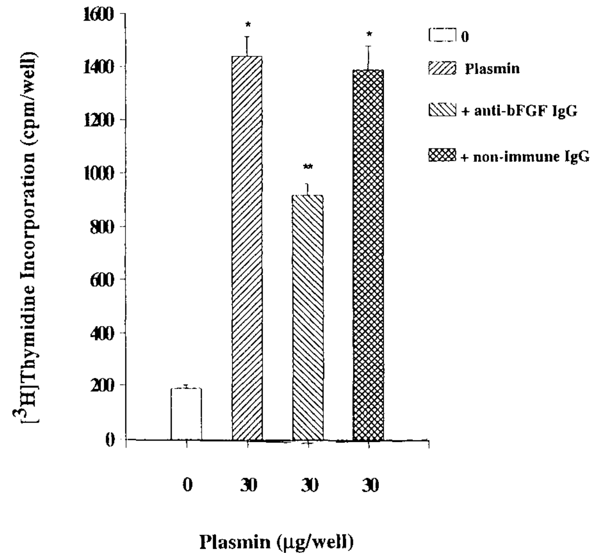


Fig. 7. Anti-bFGF significantly reduces the mitogenic effect of plasmin on cultured chondrocytes. Cells were treated with 30 μg/well plasmin alone or in the presence of rabbit anti-bFGF IgG or nonimmune rabbit-IgG. [^{3}H]thymidine uptake was measured after pulse labeling the cells during the last 18 h of a two day culture period. Values plotted are mean ±SEM ($P < 0.05$) (n = 2).

bFGF is present in freshly isolated chondrocytes, suggesting that bFGF in the cartilage originates within the chondrocytes of this tissue rather than as a paracrine factor originating from another type of cell.

The detection of bFGF mRNA expression by reverse transcription-polymerase chain reaction (RT-PCR) in tissue and by in situ hybridization analysis of isolated cells provides further evidence that chondrocytes have the capability of synthesizing bFGF. Similar results have been reported with cultured ovine fetal chondrocytes [Logan et al., 1991] and fetal bovine cartilage [Sasse et al., 1992].

The observation that bFGF is present intracellularly in the freshly isolated proliferative zone chondrocytes and in the ECM after 24 h in culture is consistent with biochemical and immunocytochemical evidence that despite the lack of a classic signal peptide [Abraham et al., 1986; Jaye et al., 1986], bFGF is exported out of the cell presumably following a novel route of secretion [Vlodavsky et al., 1987; Folkman et al., 1988; Sato and Rifkin, 1988; Bashkin et al., 1989]. However, the molecular mechanisms regulating its cellular release and subsequent matrix deposition remain unknown [Moscatelli, 1988; Rogelj et al., 1989; Vlodavsky et al., 1987].

In the current immunocytochemical study, it was shown that the small freshly isolated chondrocytes differentiated into large polygonal cells after 9 days in culture in the presence of 5% NCS. These cells had significantly decreased immunostaining for bFGF. The decrease in bFGF staining could be due to downregulation associated with differentiation, consistent with the study of Kato and Iwamoto [1990]. These investigators reported that bFGF inhibited the terminal differentiation of rabbit growth plate chondrocytes. Our results are also consistent with the observation made by Twal et al. [1994], who found bFGF present in the resting and proliferative zones, while staining was diminished in the prehypertrophic zone.

The observation that isolated chondrocytes require bFGF to proliferate is surprising, in light of the evidence that the same chondrocytes can produce bFGF and contain large amounts of bFGF both intracellularly and extracellularly after 24 h in culture. This suggests that this

bFGF must be inactive or unavailable. This observation is in agreement with reports for other types of cells. For example, the bFGF synthesized by cultured endothelial cells was detected in their ECM [Vlodavsky et al., 1987], and was not detected in the media conditioned by cells known to synthesize bFGF [Schwiegerer et al., 1987]. Furthermore, the bFGF present in the ECM of many tissues is found in concentrations that often exceed that necessary for the maximal stimulation of target cell growth in vitro [Barraclough et al., 1990; Ke et al., 1993]. Therefore, a key step for regulating the bioavailability of bFGF is the release of sequestered bFGF.

The release of sequestered bFGF has been studied in other cells such as endothelial cells by many investigators. There are at least three types of molecules which have been found to sequester bFGF: syndecan, a heparan sulfate (HS) proteoglycan attached to a transmembrane protein core, which can also act as a low-affinity receptor [Bernfield et al., 1992]; glypican, which is HS attached to the cell surface with a glycosylphosphatidylinositol anchor [David et al., 1990]; and perlecan, a heparan sulfate proteoglycan (HSPG) found in the ECM [Murdoch et al., 1992]. It has been reported that the protein core of the HSPGs is sensitive to degradation by wide-spectrum serine proteinases such as trypsin [Rapraeger and Bernfield, 1985]. Plasmin is widely distributed enzyme with a substrate specificity much like that of trypsin [Saksela, 1985]. Alternatively, both HS-degrading enzymes (heparinase, heparitinase) and heparin or heparin-like molecules also can release endogenous and exogenous bFGF from the ECM and cell surface of endothelial cells [Saksela and Rifkin, 1990; Bashkin et al., 1989]. In addition, bFGF associated with glycosyl-phosphotidylinositol anchored endothelial cell surface is released by glycosyl-phosphotidylinositol phospholipase C [Brunner et al., 1991; Bashkin et al., 1992].

Consistent with some reports, this current study shows that bFGF present in extracellular structures of chondrocytes can be released by the physiologically relevant protease, plasmin. The conclusive evidence for release of endogenous bFGF was provided by the substantial inhibition of the plasmin released growth-promoting activity specifically by bFGF antibodies but not by nonimmune IgG. Thus, the release of bFGF from the ECM of chondrocytes by plasmin treatment may be part of a general mechanism by which bFGF is made available to cellular receptors. On the other hand, inconsistent with other reports, heparitinase, heparinase, glycosyl-phosphotidylinositol phospholipase C, heparin, HS, and chondroitinase ABC failed to release bFGF from the ECM or the cell surface in this study, indicating that bFGF may be sequestered in the ECM of chondrocytes by a different mechanism than it is in the ECM of endothelial cells.

In summary, the results of the current study suggest that the bFGF present in the ECM of cultured proliferative zone chondrocytes may function in an autocrine/paracrine manner in controlling cartilage chondrocyte mitogenesis. Evidence for this conclusion is provided by the following observations: (1) chondrocytes can synthesize both bFGF mRNA and protein in culture as well secrete bFGF; (2) exogenously added bFGF is mitogenic for growth plate chondrocytes in the presence of serum; and (3) endogenously produced bFGF is mitogenic when released from the ECM by treatment with plasmin.

Based on the observations presented, the following model is proposed. In the proliferative zone of the growth plate, chondrocytes synthesize bFGF which is exported out of the cells and is subsequently deposited into the ECM, where it is initially unavailable to stimulate these cells. bFGF is released from the ECM by some mechanism as yet unknown (perhaps by a protease such as plasmin) and interacts with both low- and high-affinity bFGF receptors on the chondrocyte cell surface [Iwamoto et al., 1991; Trippel et al., 1992] to elicit mitogenic activity in an autocrine/paracrine manner.

It should be noted that our observations and discussion have dealt with the role of bFGF in the proliferative zone of the epiphseal growth plate. This peptide has also been localized in the hypertrophic zone [Twal et al., 1994], where it could play an important role in cartilage angiogenesis. This is consistent with the observation of Baron et al. [1994] that direct infusion of bFGF into the epiphseal growth plate accelerated vascular invasion of the growth plate. Although it is clear that bFGF is needed for normal endochondral bone formation, the details of its varied roles in different zones of the growth plate are not fully known at this time.

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