Journal of Cellular Biochemistry 61:152–166 (1996)

Acidic Fibroblast Growth Factor Inhibits Osteoblast Differentiation In Vitro: Altered Expression of Collagenase, Cell Growth–Related, and Mineralization-Associated Genes

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Abstract Fibroblast growth factors (FGF) are osteoblast mitogens, but their effects on bone formation are not clearly understood. Most in vitro studies examining the effects of FGFs on osteoblasts have been performed only during the initial proliferative stage of osteoblast culture. In these studies, we examined the consequential effect of acidic FGF in cultures of rat fetal diploid osteoblasts that undergo a developmental differentiation program producing a mineralized bone-like matrix. During the initial growth period (days 1–10), addition of acidic FGF (100 μg/ml) to actively proliferating cells increased ($P < 0.05$) ^{3}H-thymidine uptake (2,515 ± 137, mean ± SEM vs. 5,884 ± 818 cpm/10^4 cells). During the second stage of maturation (days 10–15), osteoblasts form multilayered nodules of cells and accumulate matrix, followed by mineralization (stage 3, days 16–29). Addition of acidic FGF to the osteoblast cultures from days 7 to 15 completely blocked nodule formation. Furthermore, addition of acidic FGF after nodule formation (days 14–29) inhibited matrix mineralization, which was associated with a marked increase in collagenase gene expression, and resulted in a progressive change in the morphology of the nodules, with only a few remnants of nonmineralized nodules present by day 29. Histochemical and biochemical analyses revealed a decrease in alkaline phosphatase and mineral content, confirming the acidic FGF–induced inhibition of nodule and matrix formation. To identify mechanisms contributing to these changes, we examined expression of cell growth and bone phenotypic markers. Addition of acidic FGF during the proliferative phase (days 7–8) enhanced histone H4, osteopontin, type I collagen, and TGF-β mRNA levels, which are coupled to proliferating osteoblasts, and blocked the normal developmental increase in alkaline phosphatase and osteocalcin gene expression and calcium accumulation. Addition of acidic FGF to the cultures during matrix maturation (days 14–15) reactivated H4, osteopontin, type I collagen, and TGF-β gene expression, and decreased alkaline phosphatase and osteocalcin gene expression. In an in vivo experiment, rats were treated with up to 60 μg/kg/day acidic FGF intravenously for 30 days. Proliferation of osteoblasts and deposition of bone occurred in the marrow space of the diaphysis of the femur in a dose-related fashion. The metaphyseal areas were unaffected by treatment. In conclusion, our data suggest that acidic FGF is a potent mitogen for early stage osteoblasts which leads to modifications in the formation of the extracellular matrix; increases in TGF-β and collagenase are functionally implicated in abrogating competency for nodule formation. Persistence of proliferation prevented expression of alkaline phosphatase and osteocalcin, also contributing to the block in the progression of the osteoblast developmental sequence.

Key words: acidic FGF, osteoblast differentiation, collagenase, osteopontin, osteocalcin

Acidic fibroblast growth factor (acidic FGF) is a 16 kDa mitogen originally isolated from brain [Thomas et al., 1984]. Acidic FGF is one of the heparin-binding family of growth factors that affects proliferation and differentiation of various cell types derived from the embryonic mesoderm or neuroectoderm [Gospodarowicz et al., 1987; Folkman and Klagsbrun, 1987]. The discovery of acidic FGF in the extracellular matrix in association with heparin sulfate proteoglycans suggests that it may act as a local regulator for cell growth and differentiation during embryogenesis, angiogenesis, and tissue repair [Folkman and Klagsbrun, 1987; Gospodarowicz et al., 1983; Vlodavsky et al., 1987].

Received September 6, 1995; accepted October 18, 1995.

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Normal bone tissue has been reported to contain both acidic FGF and basic fibroblast growth factor (basic FGF) stored in the extracellular matrix [Vlodavsky et al., 1987; Hauschka et al., 1986; Globus et al., 1989; Guenther et al., 1986]. FGF-like substances including endothelial cell–conditioned medium, partially purified FGF, and purified FGF have been shown to stimulate DNA synthesis and modulate osteoblast phenotype markers including type I collagen, osteocalcin, and alkaline phosphatase in calvaria-derived osteoblasts [Canalis and Raisz, 1980; Rodan et al., 1987, 1989; Nicolas et al., 1990; McCarthy et al., 1989; Canalis et al., 1988; Globus et al., 1988; Canalis and Lian, 1988; Hurley et al., 1993; Noda and Vogel, 1989]. While several in vitro studies indicate decreased osteoblast activity [Hurley et al., 1993; Rodan et al., 1989; Canalis et al., 1988] in response to basic FGF, repeated intravenous administration of basic FGF to rats stimulates endosteal bone formation [Mayahara et al., 1993; Nakamura et al., 1995] and dramatic hyperostosis in long or spongious bone [Mazue et al., 1992]. These reports suggest that FGFs may function as an autocrine or paracrine regulator of bone growth and differentiation. To date, most in vitro studies addressing acidic FGF effects on osteoblast activities were carried out during the initial short periods of osteoblast culture (proliferative stage). It is now known from studies of fetal rat calvaria-derived osteoblasts that modifications of the growth period by hormones [Owen et al., 1991; Breen et al., 1994; Bellows et al., 1987] or growth factors (e.g., TGF-β1) [Breen et al., 1994; Harris et al., 1994; Antosz et al., 1989] affect the subsequent periods of osteoblast differentiation and bone tissue–like formation. Little is known, however, about selective effects of acidic FGF on osteoblast proliferation and differentiation during the stages of sequential development of the osteoblast phenotype.

Osteoblasts isolated from the calvaria of 21 day fetal rat pups can be grown in vitro to develop into mature osteoblasts producing mineralized nodules consisting of multiple layers of cells within an organized bone tissue–like mineralized extracellular matrix [Bellows et al., 1986]. The developmental progression of the osteoblast phenotype is characterized by a temporal sequence of expression for the genes encoding growth-related proteins and osteoblast phenotypic markers [Owen et al., 1990; Stein et al., 1990; Stein and Lian, 1993]. The pattern of gene expression defines three distinct periods that promote signaling mechanisms for osteoblast maturation. Initially, there is a period of active proliferation during which cell growth–related genes are actively expressed and maximal levels of histone, type I collagen, and transforming growth factor-β1 (TGF-β) mRNA are observed. With the downregulation of proliferation, a period of matrix maturation occurs when cells organize into bone nodules, alkaline phosphatase gene is maximally expressed, and the extracellular matrix is rendered competent for mineralization. Finally, during the last period, expression of osteocalcin and osteopontin becomes significantly elevated with the onset of extracellular matrix mineralization. During this period, apoptosis occurs [Lynch et al., 1994] and collagenase mRNA levels are elevated [Shalhoub et al., 1992], contributing to maintenance of tissue organization.

Using this experimental model, we have examined the mechanism by which addition of acidic FGF to cells during the proliferative and matrix maturation periods modulates cell growth and subsequent cell differentiation. We analyzed the acute and chronic effects of acidic FGF as related to the stage of osteoblast differentiation on changes in morphology, DNA synthesis, expression of cell growth and differentiated associated genes, and their related products characteristic of osteoblast differentiation. Our results clearly demonstrated that acidic FGF enhances cell proliferation of early osteoblasts and inhibits nodule formation and the subsequent mineralization by inducing expression of cell growth–related genes and the collagenase gene associated with degradation of the extracellular matrix. The mitogenic effect contributes to suppression of postproliferative phenotypic genes (e.g., alkaline phos phatase and osteocalcin), which are necessary for the progressive development of bone tissue. This uncoupling of the growth/differentiation relationship in vitro is reflected by a striking increase in the amount of newly formed bone in vivo. After the chronic intravenous administration of acidic FGF for 30 days (60 μg/kg/day), the bone marrow cavity was occupied by bone trabeculae originating from proliferating endosteal surface and marrow stroma–derived cells.

MATERIALS AND METHODS

Cell Culture

Calvaria from 21 day fetal rats were isolated and subjected to sequential digestions of 20,

20, and 90 min at 37°C in 1 mg/ml collagenase P (Boehringer-Mannheim, Indianapolis, IN)/0.25% trypsin (Gibco, Grand Island, NY) as previously described [Owen et al., 1990]. Cells from the first two digests were discarded and cells released from the third digest were plated in minimal essential medium (MEM, Gibco) supplemented with 10% fetal calf serum (FCS) in 100 mm dishes (Corning, Corning, NY) at a density of 7×10^5 cells/dish. At confluence (day 5), cells were released by trypsin digestion, counted, and subcultivated in MEM supplemented with 10% FCS in 100 mm dishes or six-well dishes at a density of 7×10^5 or 5×10^5 cells/dish, respectively. At confluence (day 2), cells received media supplemented with 10% FCS and 25 μg/ml ascorbic acid. On the following feeding day, MEM was changed to BGJb medium (Gibco, Grand Island, NY) supplemented with 10% FCS, 50 μg/ml ascorbic acid, and 10 mM β-glycerol phosphate. In some experiments, dexamethasone was added at a concentration of 10^{-7}M at the first two feedings (day 2 and 5) to promote cell differentiation [Bellows et al., 1987; Shalhoub et al., 1992].

Cells were incubated with human recombinant acidic FGF 100 ng/ml at various times and for variable lengths of time (from Merck, Sharp, and Dohme, West Point, PA; stock solution: 1 mg/ml acidic FGF in 0.33 mg/ml heparin with 0.15 mM EDTA in phosphate buffer saline, PBS). Control cells were incubated with vehicle alone at these different time points during osteoblast differentiation. The various protocols are discussed in the Results.

Histochemical Analysis

Cells grown in six-well plates were rinsed twice with PBS and fixed for 10 min in 2% paraformaldehyde. Cells were then rinsed and stored at 4°C in 0.1 M cacodylate buffer. Alkaline phosphatase was detected after incubating the cells for 30 min at room temperature with shaking in 10 mM Tris HCl, pH 8.4, containing 20 mg/ml disodium naphthol AS-MX phosphate and 40 mg/ml fast red TR salt (Sigma Chemical Co., St. Louis, MO). Mineral was assayed by von Kossa staining of cultures [30 min in 3% AgNO3] [Clark, 1981].

Thymidine Uptake and Autoradiography

The rate of DNA synthesis at each indicated point was measured by the addition of ^{3}H-thymidine 5 uCi/ml (Amersham, Arlington Heights, IL) to the cells growing in six-well plates for 1 hr. Cells were rinsed with PBS and incubated with 5% trichloroacetic acid (TCA) for 5 min × 2. Cells were then solubilized in 0.5 ml 10% SDS, and counted in a scintillation counter following addition of 4 ml scintillation fluid.

For in situ autoradiography, ^{3}H-thymidine 20 uCi/ml was added to the cells growing on tissue culture cover slips (Nunc, Inc., Naperville, IL) in six-well plates for 2 hr. Cells were then rinsed twice in ice-cold PBS and fixed in absolute methanol (−20 C). Autoradiography was performed using Kodak NTB-2 emulsion (Eastman Kodak Company, Rochester, NY) as described by the manufacturer. Exposures were for 5–7 days at 4 C.

Biochemical Determinations

For calcium content, cell layers were hydrolyzed in 6N HCl under vacuum at 110 C for 24 hr. Aliquots of the hydrolysates were diluted in sodium diluent 0.27N [Na^+] pH 2.2. Aliquots were analyzed by a calorimetric assay using a Sigma Diagnostics Calcium Kit 587-A. For alkaline phosphatase activity, cell layers were harvested by scraping in appropriate buffers. Alkaline phosphatase activity was determined spectrophotometrically by measuring the amount of p-nitrophenol formed at 37 C after 30 min [Lowry et al., 1954]. Since the majority of synthesized osteocalcin is secreted into the medium, aliquots of the medium were simultaneously analyzed for secreted osteocalcin by radioimmunoassay as previously described [Owen et al., 1990; Gunberg et al., 1984]. For all biochemical determinations, the value represents the mean ± SEM of three independent samples.

RNA Isolation and Analysis

Following acidic FGF treatment, cells were washed twice with ice-cold PBS and total cellular RNA was isolated by the acid guanidinium-thiocyanate-phenol-chloroform extraction method of Chomczynski and Sacchi [Chomczynski, Sacchi, 1987] using materials obtained from Cinna/Biotecx Lab Inc (Houston, TX). Total cellular RNA concentration was quantified by UV absorption at 260 nm. For northern blot analysis, equal amounts of RNA samples were fractionated by electrophoresis in 1.2% agarose gel containing 2.2 M formaldehyde and were blotted on Duralose-UV filters (Stratagene, CA) and filters were crosslinked by UV. After prehybridization for 6 h at 42 C in a solution containing 10% dextran sulfate, 50% deionized formamide, 1% SDS, 200 ug/ml denatured salmon sperm DNA

and 1 M NaCl, the filters were hybridized to ^{32}P-labelled cDNA probes for 12 hr at 42 C. The type I collagen probe is a cDNA plasmid containing 1.6 kb of pro-α1(I) collagen-specific sequences inserted into the PstI site of pBR322 [Genovese et al., 1984]. The osteocalcin gene probe is a genomic 3.4-kb fragment isolated from the EcoRI/BamHI site of pUC19 plasmid pOC3.4 [Lian et al., 1989]. The alkaline phosphatase probe is a 2.4 kb insert isolated from the EcoRI site of a bluescript plasmid pRAP54 [Noda, and Rodan, 1987]. The osteopontin gene probe is a 1.3-kb fragment prepared from a cDNA library [Oldberg et al., 1995]. The TGF-β probe was an EcoRI cDNA insert from the plasmid pMurB2 [Derynck et al., 1986]. The rat collagenase cDNA was used as probe [Quinn et al., 1990]. The rat H4 probe is a 2.3 kb cDNA insert [Grimes et al., 1987]. Blots were washed in 2 × SSC (1 × SSC = 0.15 M NaCl, 15 mM sodium citrate), 0.1% SDS three times at room temperature for 15 min, and then in 0.5 × SSC, 0.1% SDS for 30 min at 65°C. Filters were exposed to Kodak XAR-5 film at −70°C using a double-fluorescence intensifying screen. Quantification of the autoradiogram was performed by densitometry. The relative amounts of mRNA were normalized for ribosomal 18S RNA content.

In Vivo Effects of Acidic FGF on Bone

One hundred and twenty 37-day-old Sprague–Dawley Crl:CD (SD) BR rats, which were obtained from the Charles River Breeding Laboratories, Inc., Wilmington, MA, were studied. Animals were housed in individual cages in a balanced random allocation scheme in clean air, temperature-controlled rooms. Purina Certified Rodent Chow No. 5002 and drinking water was available ad libitum. Food was withdrawn at least 16 hr prior to necropsy. The rats weighed 125–179 g (males) or 109–150 g (females) at the time of study initiation. Treatment groups of 15 males and 15 females included a control group, which received the vehicle containing human serum albumin (0.7550 mg/ml, Albuminar-5, Armour Pharmaceuticals), heparin (0.225 mg/ml, Upjohn Co., beef lung 1,000 units/ml), and phosphate-buffered saline, and acidic FGF–treated groups received 0.6, 6.0, or 60.0 μg/kg/day acidic FGF in the vehicle. The dosing volume was 0.8 ml/kg administered as a bolus into the tail vein. The rats were killed by exsanguination while under ether anesthesia, and the right femur–tibia/fibula was removed from each rat, maintained for 2 days in 10% neutral-buffered formalin, and then decalcified in a 15% formic acid solution for 5 days. The bone samples were trimmed longitudinally through the knee joint, dehydrated via immersion in increasing grades of alcohol, cleared with xylene, and embedded in molten paraffin. Six micron sections of the paraffin-embedded tissue were cut with a microtome, and all sections were stained using the standard hematoxylin and eosin method.

RESULTS

Effects of Acidic FGF on Cell Proliferation at Different Stages of Osteoblast Development

Primary osteoblasts isolated from fetal rat calvaria passaged after confluency and grown in culture over a 29 day period develop a collagenous extracellular matrix which supports the ordered deposition of mineral resulting in the three-dimensional characteristics of bone nodules. To determine the effect of acidic FGF on cell proliferation at different stages of osteoblast development, cells were pulsed with ^{3}H-thymidine for uptake analysis and autoradiography after incubating cells in the presence or absence of acidic FGF (100 ng/ml) for 24 hr on days 2, 14, or 28. Addition of acidic FGF to actively proliferating cells (day 2) resulted in a 134% (2,515 ± 137 vs. 5,884 ± 818 cpm/10^4 cells; $P < 0.05$) increase in ^{3}H-thymidine uptake and a marked increase in ^{3}H-thymidine-positive cells (Fig. 1). When cells were treated with acidic FGF during matrix maturation and mineralization periods (days 14 and 28, respectively), there was a smaller but significant increase in ^{3}H-thymidine uptake (88% and 38%, respectively) and cell number (29% and 19%). The increase in ^{3}H-thymidine uptake during the postproliferative stages was far less than that observed during the proliferative period. In control cultures, ^{3}H-thymidine-labeled cells decrease and are primarily restricted to cells in the periphery of the nodules during the matrix maturation and mineralization periods. In acidic FGF–treated cultures, however, the increase in ^{3}H-thymidine-labeled cells was observed primarily in the internodular regions.

Effects of Acidic FGF on Nodule Morphology and Biochemical Parameters

The effects of acidic FGF on bone-like nodule formation were observed by morphologic examination of the cells treated continuously with acidic FGF (100 ng/ml) from day 7 and from day

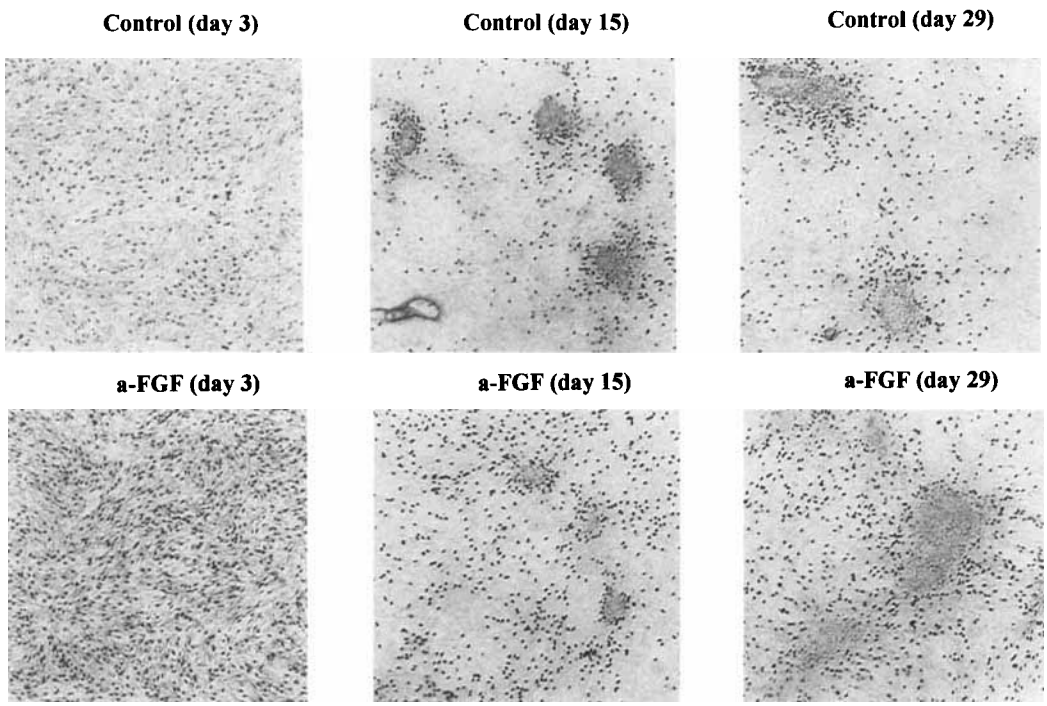


Fig. 1. Autoradiography of ^{3}H-thymidine incorporation by control and acidic FGF (100 ng/ml)–treated rat osteoblasts at different stages of growth and differentiation. Rat osteoblast cultures were treated with acidic FGF on days 2 (active proliferation), 14 (matrix maturation), and 28 (mineralization) for 24 hr. Then control and acidic FGF–treated cultures were labeled with 20 μCi/ml ^{3}H-thymidine for 2 hr. Cells were fixed and exposed for 5 days after applying the photographic emulsion. Photographed at 10× after counterstaining with Toluidine blue.

14. In control cultures, osteoblasts started to form multilayered nodules on day 12 and mineralized nodules by day 21. Addition of acidic FGF to the cultures before multilayered nodule formation (days 7–14) prevented nodules from developing, and the cultures remained nodule free throughout the remainder of the 29 day culture period (pictures not shown). Cells treated with acidic FGF after nodule formation (days 14–29) showed progressive changes in the morphology of the nodules. Initially, cells at the periphery of the nodules exhibited an elongated spread shape and merged with the internodular cells. Due to progressive blending of the nodule-associated cells with surrounding cells, the margins of the nodules became blurred. Nodule size was noticeably reduced after 7 days exposure to acidic FGF. By day 29, only cells at the central portion of many nodules remained, forming a crescent-like remnant (Fig. 2, bottom). Numerous mineralized nodules had developed on day 29 in the control cultures but most of the nodules had disappeared in cultures exposed to FGF from days 14 to 29, and the remnants consisted of nonmineralized matrix. Thus, acidic FGF added to cultures in which the nodules had already formed, blocked the further progression of nodule development, and caused the regression of well formed nodules.

To further confirm the effect of acidic FGF on bone-like nodule formation and mineralization, biochemical markers of bone phenotype were measured. Alkaline phosphatase is a bone phenotype marker which precedes the onset of mineralization. Addition of acidic FGF to osteoblast cultures from day 7 to 15 resulted in significantly ($P < 0.05$) lower levels of alkaline phosphatase activity (Fig. 3, top). In control cultures, alkaline phosphatase activity increased by 195% from day 15 to 29. Acidic FGF added to osteoblast cultures from days 7 to 15 and days 7 to 29 completely blocked this rise in alkaline phosphatase activity. Before mineralization (day 15), calcium content in control or acidic FGF–treated cultures was low. In control cultures, calcium levels increased significantly ($P < 0.05$) from day 15 to day 29. Addition of acidic FGF from days 7 to 29 completely blocked the normal developmental increase in calcium concentrations (see Fig. 3, bottom).

Osteocalcin, a well established marker of the mature osteoblast phenotype, is transcription-

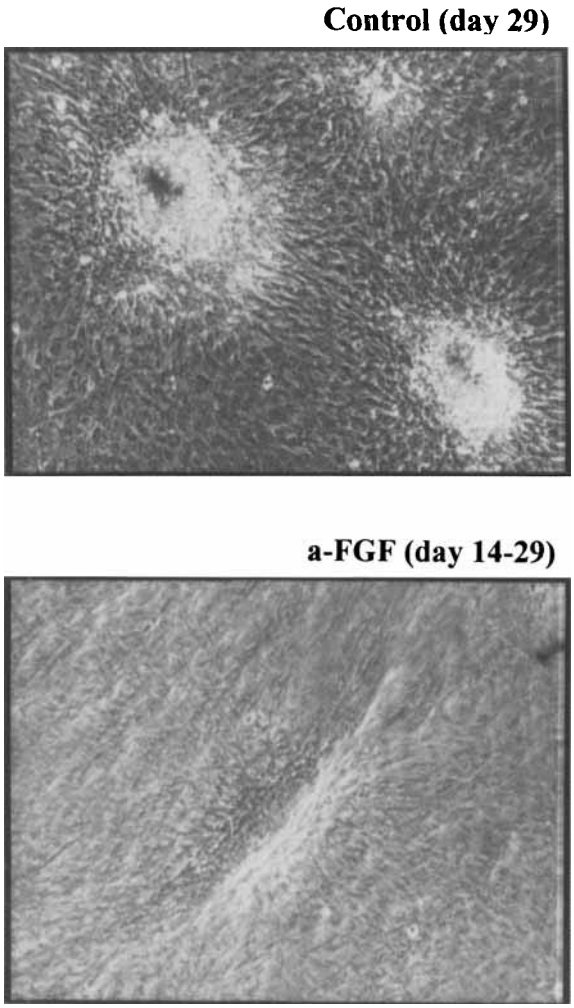


Fig. 2. Morphologic features of nodule formation and mineralization in control and acidic FGF–treated cultures on day 29. Rat osteoblasts were cultured as described in Materials and Methods until multilayered nodules were formed. On day 14, cultures were treated with or without acidic FGF (100 ng/ml) continuously throughout the rest of the experiment. The representative phase-contrast pictures show numerous mineralized nodules in the control cultures and few nonmineralized crescent-like remnants in acidic FGF–treated cultures.

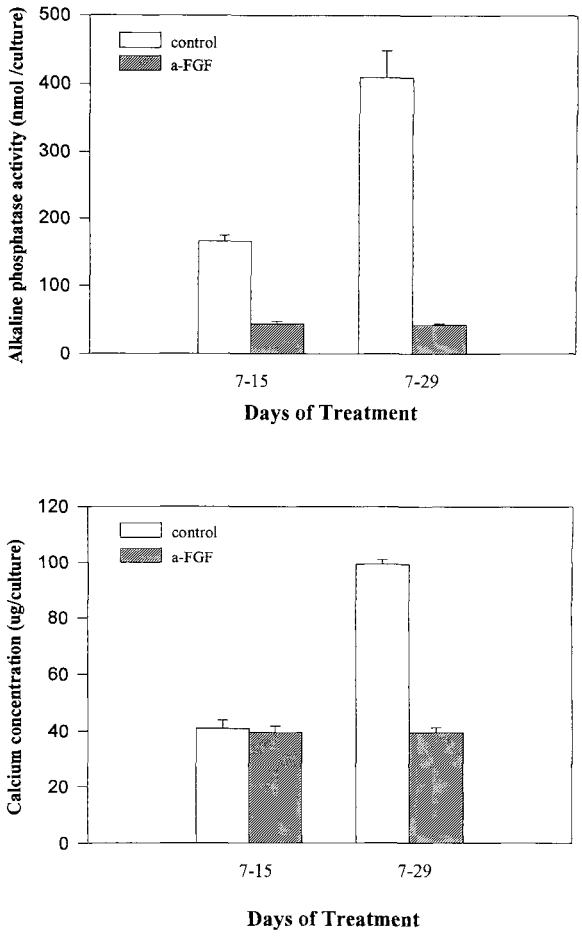


Fig. 3. Effect of acidic FGF on alkaline phosphatase activity (**top**) and calcium concentration (**bottom**) in rat osteoblast cultures. Rat osteoblasts were cultured in the presence or absence of acidic FGF (100 ng/ml) continuously from day 7 and harvested on days 15 and 29, respectively. The amount of p-nitrophenol released/30 min was expressed as alkaline phosphatase activity. The data are presented as mean nmol/culture + SEM of n = 3 cultures. The calcium concentrations were determined by colorimetric method and expressed as mean μg/culture + SEM of n = 3 cultures.

ally regulated postproliferatively in a coordinate manner with the ordered deposition of mineral in the extracellular matrix [Owen et al., 1990]. In control cultures, osteocalcin increased progressively after day 14 and reached peak values on day 26 (see Fig. 4). Addition of acidic FGF (10 or 100 ng/ml) from days 7 to 26 completely blocked the developmental rise in osteocalcin concentrations (Fig. 4). Similarly, acidic FGF (100 ng/ml) added to the osteoblast cultures after nodule formation (days 14–26) resulted in the maintenance of osteocalcin levels at the day 14 level.

The inhibitory effect of acidic FGF on the differentiation of the osteoblast phenotype is clearly indicated by histochemical analysis of alkaline phosphatase and mineral content (Figs. 5, 6). In control cultures, both alkaline phosphatase and silver stains increased from day 15 (Fig. 5, top) to day 29 (Fig. 6, top). In osteoblast cultures exposed to acidic FGF from days 7 to 29, there was little alkaline phosphatase or silver staining on day 29. These findings are consistent with the absence of the bone-like nodules by morphology. Delaying the addition of acidic FGF to day 14, after formation of the multilayered nodules, resulted in a decrease in alkaline

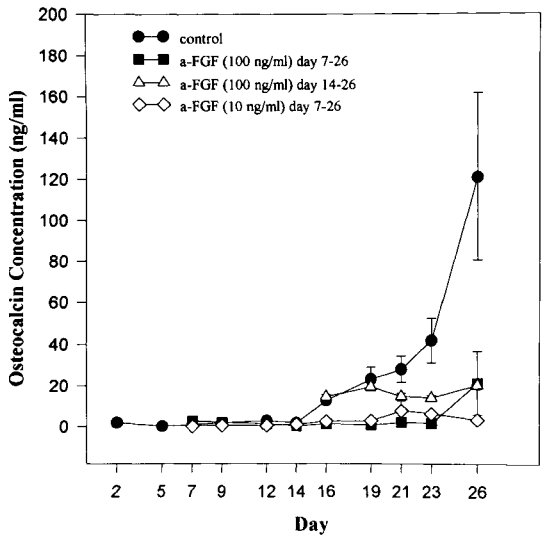


Fig. 4. Effect of acidic FGF on osteocalcin synthesis in rat osteoblast cultures. The amount of secreted osteocalcin throughout the development of the osteoblast phenotype was measured by radioimmunoassay of media. Cells were treated with 10 or 100 ng/ml acidic FGF continuously from day 7, or treated with acidic FGF 100 ng/ml continuously from day 14. The data were expressed as mean ng osteocalcin + SEM synthesized in a 48 hr period per ml of media of n = 3 wells.

phosphatase and silver stains compared with control cultures. These studies demonstrate that acidic FGF not only inhibits osteoprogenitor differentiation to the mature osteoblast phenotype, but also has the ability to modify the ongoing course of osteoblast differentiation.

Modification of Gene Expression Induced by Acidic FGF

To examine the mechanisms involved in the acidic FGF–induced potentiation of osteoblast proliferation and inhibition or modification of nodule formation and mineralization, we examined the effects of acidic FGF on the expression of osteoblast genes during the proliferative (day 8) and matrix maturation (day 15) periods. As illustrated in Figure 7, the expression of osteopontin, type I collagen, and TGF-β genes in control cultures decreased from day 8 (proliferating period) to day 15 (matrix maturation period). Cells treated with acidic FGF on day 7 for 24 hr enhanced histone H4, osteopontin, type I collagen, and TGF-β gene expression. Stimulation of the cells with acidic FGF from days 7 to 15 increased osteopontin and type I collagen mRNA levels severalfold, but the elevated histone H4 or TGF-β mRNA levels observed on day 8 were not sustained into the matrix maturation period. In control cultures, the expression of osteocalcin and alkaline phosphatase increased from days 7 to 15. Addition of acidic FGF to osteoblast cultures from days 7 to 15 blocked the increases in alkaline phosphatase and osteocalcin gene expression (Fig. 7). To determine if the inhibition of alkaline phosphatase and osteocalcin gene expression was secondary to either the growth stimulatory effect or the absence of bone nodule formation and osteoblast differentiation, acidic FGF was added to the cultures on day 14 after osteoblast maturation was initiated. Cells treated with acidic FGF on day 14 for 24 hr reactivated expression of the genes associated with proliferating osteoblasts: histone H4, osteopontin, type I collagen, and TGF-β, which were normally downregulated during this matrix maturation stage. In control cultures, there is an increase in alkaline phosphatase and osteocalcin gene expression on day 15. Addition of acidic FGF to the cultures on day 14 for 24 hr decreased the expression of the alkaline phosphatase (fourfold) and osteocalcin (threefold) genes.

Together, the effects of acidic FGF on expression of the histone, osteopontin, and TGF-β genes, reflecting osteoblast proliferation, and osteocalcin and alkaline phosphatase, reflecting differentiation, are compatible with the results obtained from the changes in ^{3}H-thymidine uptake, morphology, histochemical, and biochemical examinations. However, the very significant increase in collagen mRNA by acidic FGF (greater than 20-fold in the continuously treated cultures) was inconsistent with the apparent loss of matrix and the striking regression of nodule size in cultures exposed to acidic FGF from days 7 to 15. We, therefore, examined expression of the matrix-degrading enzyme, collagenase, a product of osteoblasts [Meikle et al., 1991, 1992; Sakamoto and Sakamoto, 1984; Partridge et al., 1987]. Figure 8 illustrates the temporal expression of collagenase in control cultures during the growth and initial matrix maturation periods. On day 8, extremely low or nondetectable levels of collagenase mRNA are present, which increase slightly on day 15. Treatment of the cultures with acidic FGF for either 24 hr (days 7–8 or day 14–15) or continously from days 7 to 15 resulted in a marked, 20-fold increase in collagenase mRNA.

Effect of In Vivo Administration of Acidic FGF on Bone Development

We have largely observed an inhibition of osteoblast differentiation and mineralization formation in vitro as a result of increased cell

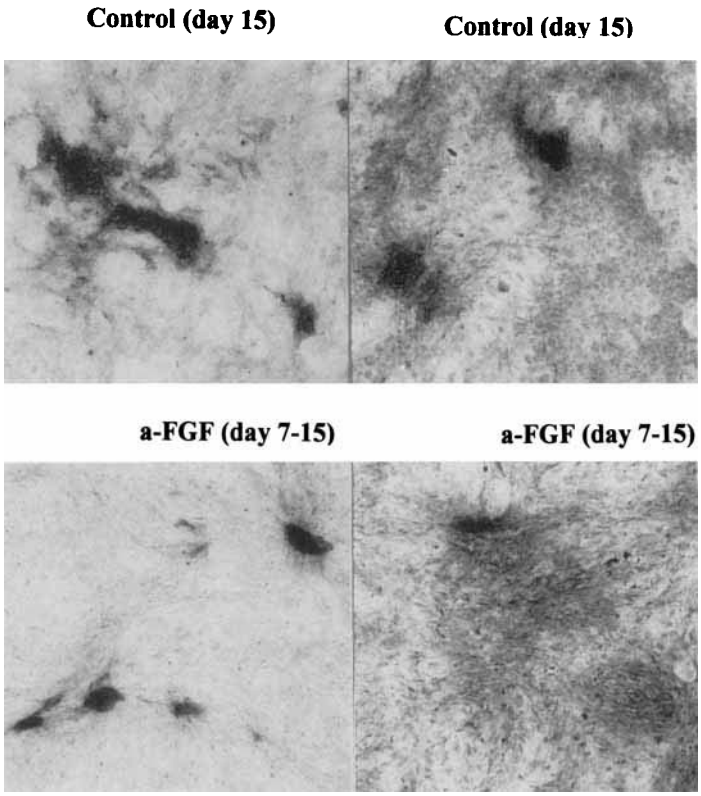


Fig. 5. Morphologic features of alkaline phosphatase and von Kossa silver stains in control and acidic FGF–treated cultures. Rat osteoblasts were treated with or without 100 ng/ml acidic FGF continuously from day 7 to day 15. Cells were then fixed and stained for alkaline phosphatase and mineral, respectively. There is a marked decrease in alkaline phosphatase–positive cells and mineral content in acidic FGF–treated cultures. Photographed at 10× after counterstaining with Toluidine blue.

proliferation and activation of collagenase contributing to matrix degradation. These findings are in contrast to the known affects of FGFs observed in vivo leading to increased bone formation. We, therefore, investigated the in vivo effects of acidic FGF to clarify the relationship of our in vitro findings to potential anabolic effects of acidic FGF.

Experiments were carried out for 30 days at three dosages of acidic FGF. No histologic lesions were present in the bones from the control or the 0.6 μg/kg/day acidic FGF–treated rats. In the bones from the rats treated with 6.0 and 60.0 μg/kg/day acidic FGF, varying amounts of active bone deposition were taking place in the diaphyseal marrow space of 14 of 30 and 26 of 30 rats, respectively. In the 60.0 μg/kg/day acidic FGF–treated rats, the lesion was especially pronounced in three of 30 animals, where a significant proportion of the hematopoietic elements in the marrow space were displaced by the presence of cancelous bone in various stages of deposition and maturation. The overall picture was much like normal intramembranous bone formation via matrix deposition with subsequent mineralization and appositional growth (Fig. 9). There were areas containing mature bony spicules composed of slender lamellar trabeculae that ramified and anastomosed, and incorporated osteoclast-containing lacunae; osteogenic cells were present on portions of their surface. In other areas, an active, dense osteoblast population was the most prominent feature; the morphology of the osteogenic cells suggested a highly active physiologic state: large polymorphous vesiculated nuclei with prominent nucleoli and, at sites where the population of cells was congruous, indistinct cytoplasmic borders (Fig. 10). Irregularly shaped deposits of matrix material were commonly interspersed amongst the population of active osteoblasts within the marrow cavity. This feature suggested differentiation of marrow stroma progenitor cells to osteoblast in acidic FGF–treated animals. The metaphyseal/epiphyseal regions of the bone were histologically normal in control and acidic FGF–treated animals in this study. There were no clinical signs (such as evidence of pain or impaired mobility) that correlated with the acidic FGF–induced histologic changes in the bone.

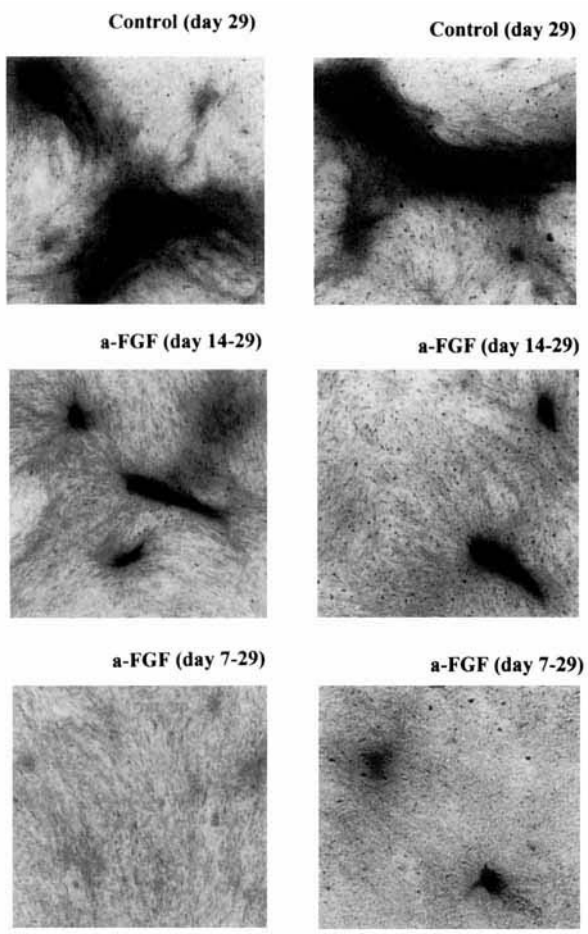


Fig. 6. Morphologic features of alkaline phosphatase (**left**) and mineralization (von Kossa silver stain, **right**) in control and acidic FGF–treated cultures. Rat osteoblasts were treated with or without acidic FGF (100 ng/ml) continuously from days 7 to 15 (treated before nodule formation) and from days 14 to 29 (treated after nodule formation), respectively. Cells were then fixed and stained for alkaline phosphatase and mineral (von Kossa silver stain), respectively. There is a marked decrease in alkaline phosphatase–positive cells and mineral content in cultures treated with acidic FGF before and after nodule formation. Photographed at 10× after counterstaining with Toluidine blue.

DISCUSSION

The present studies demonstrate that growth and the ordered developmental sequence of osteoblast differentiation, reflected by a temporal expression of genes and related gene products, are affected by acidic FGF. Acidic FGF disrupts the well documented progression of events required for maturation of the bone cell phenotype that occurs concomitant with development of organized bone tissue [Owen et al., 1990; Stein and Lian, 1993]. Our studies suggest that several mechanisms contribute to this inhibitory effect and that the effect of acidic FGF is dependent upon the stage of osteoblast maturation. The findings indicate that (1) acidic FGF induces differentiation of marrow progenitors to osteoblasts in vivo leading to formation of bone trabeculae in the marrow cavity space of the diaphyses; (2) in vitro acidic FGF results in a sustained mitogenic effect on proliferating and immature osteoblasts, which blocks expression of postproliferative genes (e.g., alkaline phosphatase) necessary for matrix maturation; (3) acidic FGF induces TGF-β, another growth mediator that inhibits osteoblast differentiation in vitro [Breen et al., 1994; Harris et al., 1994]; and (4) induction of collagenase by acidic FGF results in degradation of the matrix that supports mineralization of bone-forming nodules. These in vitro events all contribute to inhibition of the osteoblast developmental sequence.

Maximal stimulation of cell growth by acidic FGF was found during the proliferative phase; ^{3}H-thymidine incorporation, histone H4 mRNA levels, and DNA concentration were increased. These results are consistent with the potent mitogenic effect of acidic FGF [Gospodarowicz et al., 1987; Rodan et al., 1987; Nicolas et al., 1990]. Addition of acidic FGF to the osteoblast cultures during matrix maturation and mineralization periods had only a slight stimulatory effect, which was restricted to the internodular regions and not to mature osteoblasts/osteocytes within the mineralized matrix of the nodule. These results are consistent with our in vivo findings, which showed no changes in the mature bone of the metaphysis.

The expression of several genes necessary for phenotype development are coupled to proliferation. Osteopontin, a 60 kDa acidic glycoprotein [Oldberg et al., 1995] with several putative calcium-binding sites, exhibits a biphasic pattern of expression during the osteoblast developmental sequence and during the proliferative and mineralization periods [Owen et al., 1990; Stein et al., 1990; Stein and Lian, 1993]. In the present study, we found that acidic FGF increased osteopontin gene expression during the proliferative (day 7) and matrix maturation (day 14) periods, associated with the rise in histone H4 mRNA levels and ^{3}H-thymidine uptake. This result supports the concept that the rise of osteopontin mRNA levels induced by acidic FGF is important or related to cell proliferation and not necessarily coupled with mineralization of bone-like nodules later in the developmental sequence.

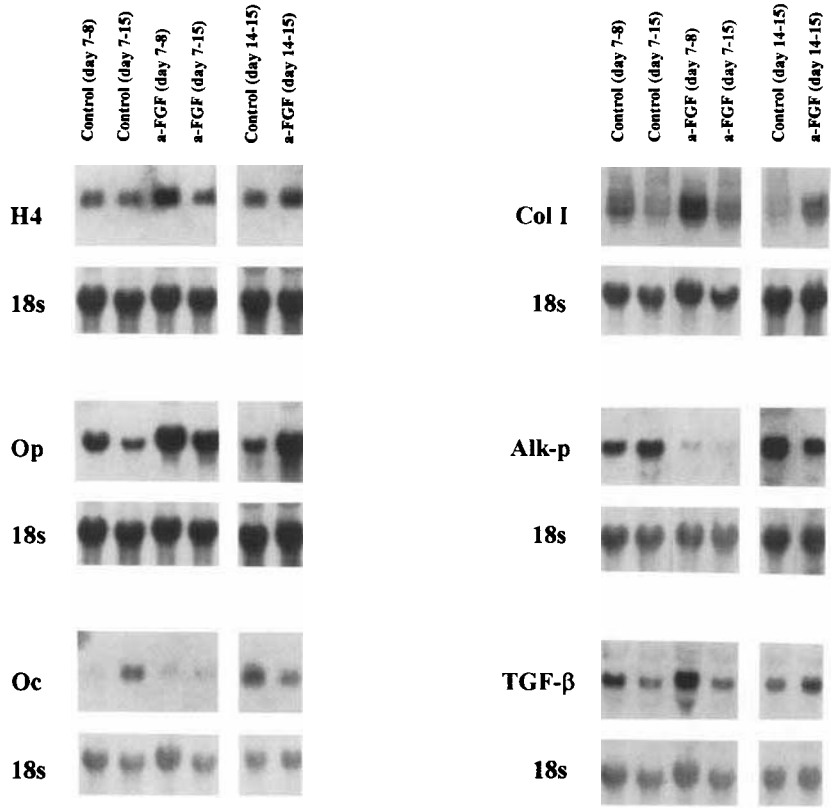


Fig. 7. Northern blot analysis of histone H4, osteopontin, type I collagen, TGF-β, osteocalcin, and alkaline phosphatase gene expression in control and acidic FGF–treated cultures during proliferative and matrix maturation stages. Cells were treated with or without acidic FGF (100 ng/ml) on day 7 and on day 14 for 24 h, or continuously from days 7 to 15. Total RNA 10 or 15 μg of each sample was subjected to Northern blot analysis. The results are presented as one representative blot of three experiments of similar results.

Expression and ordered accumulation of type I collagen gene during the proliferative/matrix maturation periods are required for mineralization and development of the osteoblast phenotype [Owen et al., 1990; Stein et al., 1990; Stein and Lian, 1993]. Pulse or continuous stimulation of cells with acidic FGF increased type I collagen gene expression during the matrix maturation period. These results are supported by other studies that demonstrate that acidic FGF stimulates collagen synthesis in bone-derived cultures [McCarthy et al., 1989]. However, the increase in collagen induced by the acidic FGF does not contribute to competency of the matrix for subsequent osteoblast differentiation. This inconsistency may be explained by our findings of an increase in TGF-β and collagenase gene expression by acidic FGF. This finding is supported by the report that basic FGF enhances TGF-β gene expression in osteoblast-like cells [Noda and Vogel, 1989]. TGF-β is also a potent mitogen in these rat calvaria-derived cell cultures [Breen et al., 1994], and blocks formation of mineralized bone nodules, as reported by several groups [Breen et al., 1994; Harris et al., 1994; Antosz et al., 1989]. The upregulation of TGF-β by acidic FGF may, therefore, exert an additional effect on cell proliferation and contribute to the inhibition of osteoblast differentiation. Further, we found that acidic FGF increased collagenase gene expression, which could result in the inhibition of nodule formation in the proliferative period and regression of the formed nodules when acidic FGF is added in the postproliferative period. Thus, although collagen mRNA levels are upregulated, the early and sustained induction of collagenase by continuous acidic FGF treatment prevented the accumulation of nodule-associated collagen, requisite for mineralization. These findings are corroborated by studies that show that FGF increases collagenase expression in fibroblasts (hMRC-5 cells) [Edwards et al., 1987] and recently in rat osteoblasts [Gabbitas and Canalis, 1995]. The

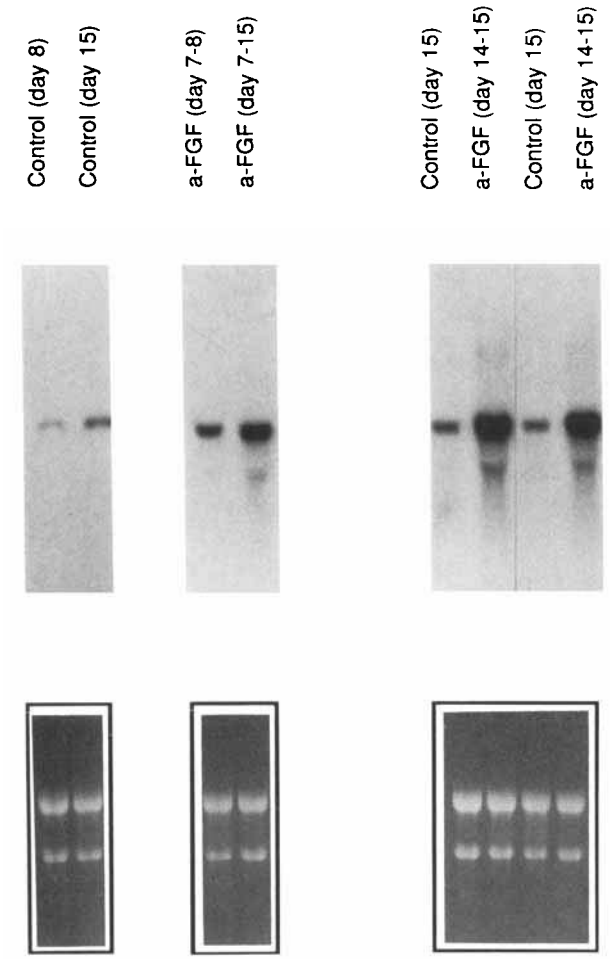


Fig. 8. Northern blot analysis of collagenase gene expression in control and acidic FGF–treated cultures. Cells were treated with or without acidic FGF (100 ng/ml) on day 7 and on day 14 for 24 h, or continuously from days 7 to 15. The results were presented as one representative blot of three experiments of similar results.

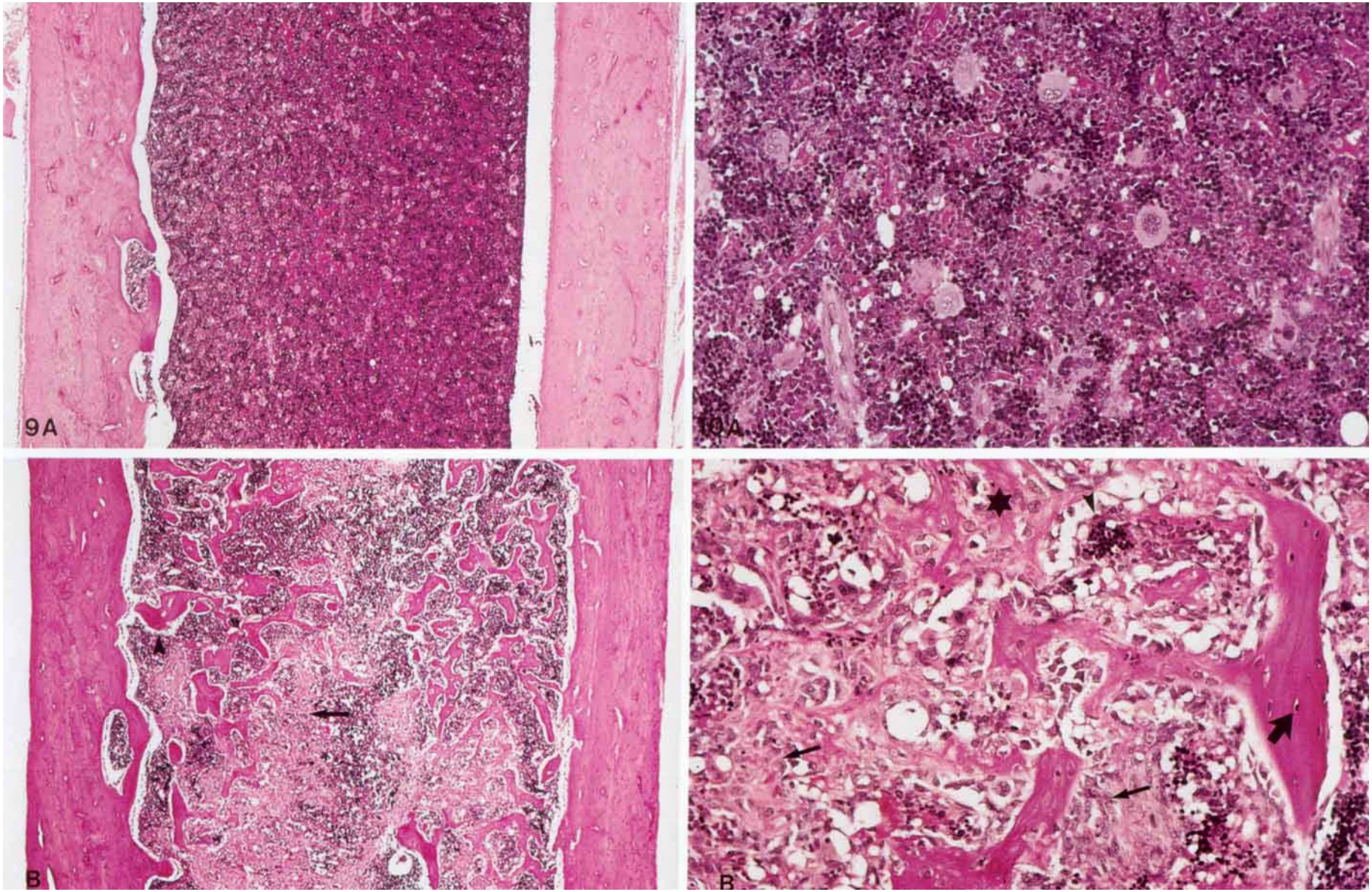
induction of collagenase by acidic FGF may relate to earlier observations that FGF promotes resorption of cultured rat long bones [Simmons and Raisz, 1991]. Stimulation of collagenase synthesis by bone resorbing agents has been well documented [Heath et al., 1984; Meikle et al., 1992; Jeffrey and Peck, 1988; Delaisse et al., 1988]. Thus, prolonged FGF treatment could modulate bone turnover by both inhibiting osteoblast differentiation and stimulating bone resorption through its induction of collagenase. In fibroblasts, the induction of collagenase by FGF is blocked by TGF-β; thus, the elevation in TGF-β by FGF that we and others have observed in osteoblasts may serve to regulate and/or balance collagenase production [Delaisse et al., 1988; Noda and Vogel, 1989].

The inhibited expression of other bone-related genes also contributes to the block in osteoblast differentiation in cultures exposed to acidic FGF. Alkaline phosphatase mRNA and enzyme activity normally precede mineralization of osteoblast cultures, and osteocalcin expression rises with the increase in total mineral accumulation in the cultures [Owen et al., 1990; Stein et al., 1990; Shalhoub et al., 1992]. A reciprocal relationship between cell growth and expression of alkaline phosphatase and osteocalcin has been well documented; thus the dramatic downregulation of these genes in acidic FGF–treated cultures is expected with growth stimulation [Nicolas et al., 1990; McCarthy et al., 1989]. Our results clearly demonstrate that acidic FGF inhibits expression of these genes at the early phase of osteoblast development. Thus, the downregulation of alkaline phosphatase contributes to decreased mineralization and, together with the absence of mineralized nodule formation, osteocalcin synthesis and calcium levels are decreased.

Acidic FGF has a marked effect on bone formation in vivo. It is worthy of note that the lesion observed in the in vivo study was limited to the shaft of the bone, without any evident effect on the area of normal active bone deposition in the metaphyseal region. It may be that the osteogenic cell population in the metaphysis is maximally stimulated by endogenous growth factors, so that introduction of exogenous growth factors would not disturb the normal course of events in these areas. It has been reported that

Fig. 9. Diaphysis of the femur from a control rat (**A**). The marrow space is filled with hematopoietic elements and blood vessels. Diaphysis of the femur from a rat treated with 60 μg/kg/day of acidic FGF intravenously for 30 days (**B**). The appearance of the marrow space is in marked contrast to that seen in the control animal. Numerous bony spicules invade the marrow space, and are especially concentrated at the periphery (*arrowhead*). Areas of dense osteogenic cell populations and osteoid are more concentrated toward the center of the shaft (*arrow*). Cells were stained with H&E (50×).

Fig. 10. Representative area of marrow space in the diaphyseal shaft of the femur from a control rat (**A**). The entire space is filled with hematopoietic and vascular elements. Representative area of marrow space in the diaphyseal shaft of the femur from a rat treated with 60 μg/kg/day of acidic FGF intravenously for 30 days. (**A**) Mature bony spicules containing lacunae (*large arrow*) are interspersed with dense populations of osteoblasts (*small arrows*) and osteoid (*star*). Hematopoietic elements are largely displaced by the osteogenic process (*small arrowhead*). Cells were stained with H&E (257×).


9A
10A
B
B

intravenous administration of basic FGF to rats at doses of 0.1 mg/kg/day and greater for 2 weeks causes endosteal proliferation of bone in the diaphyseal region [Mayahara et al., 1993], and that basic FGF (1, 10, or 100 μg/kg/day) administered intravenously to rats for up to 4 weeks produces "dramatic hyperostosis" by day 10 in long or spongious bone [Mazue et al., 1992].

In the present studies, we have observed that there were various stages of bone deposition and maturation within the marrow tissue of the shaft in treated rats. Areas of more mature bony spicules adjacent to areas of dense osteoblastic activity and matrix deposition were observed. We have suggested that various focal progenitor cell populations may be susceptible to the proliferative and stimulatory (or suppressive) effects of growth factors at different times during their cell cycles, therefore continually providing susceptible populations, at least during the course of our 30 day study.

It is also of interest that in a repeat 1 month experiment in rats (6.0 and 60.0 μg/kg/day acidic FGF) followed by an 8 week recovery period, the incidence and severity of the bone lesion decreased significantly. Thus, bone formed in response to acidic FGF underwent continued remodeling and resorption without osteoblast activity following cessation of treatment. Regression of hyperostosis induced by intravenous treatment of rats with basic FGF has also been reported [Mazue et al., 1992]. These findings are consistent with findings that acidic and basic FGF induce bone resorption [Gospodarowicz et al., 1987; Simmons and Raisz, 1991].

The discrepancy between the in vitro observations of inhibition of osteoblast maturation and the in vivo findings of increased new bone formation can be explained. Acidic and basic FGF affects various cell types. Furthermore, our in vitro findings indicate selective effects of acidic FGF are related to the stage of osteoblast differentiation. As our osteoblasts are isolated from central calvarial bone, they do not represent a stem cell progenitor-like population as occurs in bone marrow. Acidic FGF appears to target such cells in vivo for proliferation and differentiation through osteoblast lineage. TGF-β, induced by FGF, also may mediate this effect [Nakamura et al., 1995]. In vivo, inhibitory effects on mature osteoblasts may be suppressed due to serum, local factors, and the cross talk between different cell types. Such intercellular communication may be limited in cultures of a selected cell population.

In conclusion, our data suggest that acidic FGF is a potent mitogen for osteoblasts derived from the fetal rat calvaria. The changes in the extracellular environment mediated by acidic FGF during the proliferative and matrix maturation stages of osteoblast cultures render the matrix incompetent for nodule formation and prevent cellular differentiation into mature osteoblasts. The modifications include altered expression of genes for cell growth, the extracellular matrix, and bone-related proteins. Acidic FGF additionally causes regression of developing bone nodules associated with, and potentially functionally related to, upregulation of collagenase and extracellular matrix degradation. These changes, together with the upregulation of collagenase and downregulation of the alkaline phosphatase and osteocalcin genes, result in an altered matrix composition that renders the cells unable to form the organized multilayered nodules, blocking mineralization of the matrix and, thereby, osteoblast differentiation.

ACKNOWLEDGMENTS

This work was supported in part by grants DK 18919 and GM32010 from the NIH, Bethesda, MD.

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