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Effects of basic fibroblast growth factor on osteoblast-related gene expression in the process of medullary bone formation induced in rat femur

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Abstract The effects of basic fibroblast growth factor (bFGF) on osteogenic differentiation in-vivo were investigated using a rat bone marrow ablation model. bFGF was infused directly into rat femora for 6 days after bone marrow ablation. The contralateral femur was infused with vehicle only and used as control. Bone formation was induced in the rat femoral cavity, and the gene expression of osteoblast markers was examined. Treatment with bFGF at 50 and 100 ng/day significantly enhanced the mRNA levels of osteopontin compared with the levels in the control leg, with increases of 25% and 24%, respectively. In contrast, bFGF infusion at 50 ng/day provoked a significant (nearly 20%) inhibition of expression for type I collagen. Infusion of bFGF at a higher dose exhibited an inhibitory tendency for bFGF action on gene expression. There were no significant changes in alkaline phosphatase and osteocalcin mRNA levels in response to any dose of bFGF. The findings presented here suggest that bFGF modulates osteogenic differentiation in-vivo and may play an important role in the process of bone remodeling.

Key words basic fibroblast growth factor · gene expression · bone formation

Introduction

The mineralized matrix of skeletal tissue is known to be a rich source of growth factors that could play an important role in bone formation [1,2]. In particular, basic fibroblast growth factor (bFGF), an 18-kD peptide, has recently received considerable attention by virtue of its potential use in clinical applications. bFGF has been suggested to be involved in organogenesis, vascularization, and wound healing, and it exerts a variety of biological actions [3–5]. A number of in-vivo studies concerning bFGF action in skeletal tissue have been reported. Systemic injection of bFGF has been reported to stimulate endosteal bone formation by increasing the number of preosteoblasts [6,7]. In addition, locally administered bFGF has been shown to enhance bone formation in rabbit ilium [8] and to accelerate the healing of fractures[9,10]. Recent studies have also shown that bFGF improves the healing of bony defects [11,12]. Based on in-vitro studies, bFGF has been described as a potent mitogen for mesenchymal cells and osteoblastic cells [13–15]. In addition, it has been shown that cultured bovine bone cells synthesize bFGF and store it in their extracellular matrix [1]. Therefore, it is believed that bFGF is one of the most important local regulators of bone remodeling, involving bone formation and bone resorption. In contrast, several investigators have suggested an inhibitory effect of bFGF on alkaline phosphatase activity in bone cells [13,16,17] and on osteoblast-related gene expression in an osteosarcoma cell line [18]. Thus, one question arises as to how the discrepancy between the inhibitory effects in vitro and the anabolic effects in-vivo can be explained. Most in-vivo studies of bFGF action are evaluated histologically and are insufficient to elucidate the involvement of bFGF in the bone-formation process, as, unlike the in-vitro studies, little has been described regarding the effects on gene expression for bone-matrix proteins. The aim of the present study was, therefore, to further elucidate the in-vivo effects of bFGF on osteogenic differentiation. We examined the effects of bFGF on mRNA expression for bone matrix proteins in-vivo by using a bone marrow ablation model.

Materials and methods

Animals

Male Wistar rats, 6 months of age, were supplied by the Animal Facility, Gerontology Research Center,

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National Institute on Aging (Baltimore, MD, USA). Throughout the experiment, animals were housed with a cycle of 12 h of light and 12 h of darkness, and fed ad libitum the standard National Institute of Health rat chow, consisting of 23.5% protein, 1.2% calcium, and 1.0% phosphate.

Bone marrow ablation

Femoral bone marrow was aspirated as described previously [19]. Briefly, rats were anesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg). A longitudinal incision was made across the medial aspect of the knee to expose the bone. A 1.6-mm hole was drilled through the femoral intercondylar space into the marrow cavity. The marrow was removed through a cannula inserted through the hole and attached to a vacuum line, followed by irrigation of the marrow cavity with saline. An Alzet minipump (ALZA, Palo Alto, CA, USA) containing bFGF solution was inserted subcutaneously on the back of the rat adjacent to the hip joint. A polyethylene catheter was used to deliver bFGF into the right femoral cavity. The left femur was infused with vehicle only and used as a control. The skin wound was closed with wound clips. Human recombinant bFGF (Bachem, Torrance, CA, USA) was infused at 20, 50, 100, or 500 ng/day for 6 days. Rats were killed on day 6 after the surgical procedure. Both femora were removed and stored at −80°C until processed. This protocol was approved by the Animal Care and Use Committee at the Gerontology Research Center, National Institute on Aging.

RNA preparation

Bones were pulverized with a mortar and pestle prechilled in dry ice. RNA was extracted from the bone as described previously [20]. Briefly, powdered bone was homogenized in a 6 M guanidine thiocyanate (Fluka Biochemika, Buchs, Switzerland) solution containing 1.5% N-laurosarcosine (Sigma, St. Louis, MO, USA), 67.5 mM potassium acetate, and 0.1% antiform A (Sigma). Debris was removed by centrifugation at 8000 *g* for 40 min, and the supernatants were loaded on a 5.6 M cesium chloride cushion. Total RNA was separated by density-gradient ultracentrifugation at 145 000 *g* for 18 h. The RNA pellet was dissolved in DEPC-treated H_2O, extracted with chloroform/isobutanol, and precipitated with 70% ethanol at −20°C. The purity of the RNA was monitored by the ratio of absorbances of the samples at 260 nm and 280 nm.

Northern analysis

Total RNA was denatured, separated on a 1% agarose gel (FMC, Rockland, ME, USA), and transferred to Gene Screen paper (New England Nuclear, Boston, MA, USA). Inserts of cDNA were released from the plasmids by restriction enzyme digestion and separated on a 0.75% agarose gel. The appropriate cDNA fragment was extracted from slices of the agarose gel with a Prep-A-Gene kit (Bio-Rad, Richmond, CA, USA). The cDNA probes used were rat alkaline phosphatase (0.6 kb; *Eco*RI) [21], rat procollagen (I) (1.3 kb; *Pst*I) [22], rat osteocalcin (0.5 kb; *Eco*RI) [23], and rat osteopontin (1.3 kb; *Eco*RI) [24]. ^{32}P-labeled probes were prepared with a multiprime labeling kit (Amersham, Arlington Heights, IL, USA). Blots were hybridized at 42°C for 24 h in 50% formamide. After hybridization, blots were washed with 2 × SSC (0.15 M NaCl and 0.015 M sodium citrate) and 0.1% sodium dodedyl sulfate (SDS) twice at room temperature, 1 × SSC and 0.1% SDS at 50°C, and 0.5 × SSC and 0.1% SDS at 60°C. The autoradiogram was exposed at −70°C with an intensifier screen. Resulting autoradiographs were scanned into a Macintosh computer, and the National Institutes of health (NIH) Image 1.56 image analysis program was used to calculate the density of each band. All values were normalized for RNA loading by densitometric analysis of ethidium bromide (EtBr)-stained 18 S rRNA bands in each gel prior to transfer.

Histology

Sections of rat femurs were surgically excised and immediately placed in neutral buffered formalin. Paraffin sections of 3–4 μm thickness were cut from formalin-fixed wax-embedded tissue and were stained with hematoxylin and eosin.

Statistics

Data values are shown as means ± SEM. Statistical analysis was performed using the Student's two-tailed paired or unpaired *t*-test for comparisons between two groups, using Statview software. Differences were considered significant if $P < 0.05$.

Results

Histological findings

The effects of bFGF infused locally into the femur were examined after marrow ablation. One femur was infused with vehicle only and served as the control, while the contralateral bone was infused with 50 ng/day of bFGF for 6 days. Figure 1 shows light microscopic findings of femurs infused with vehicle alone (Fig. 1A) or bFGF (Fig. 1B). The formation of primary cancellous

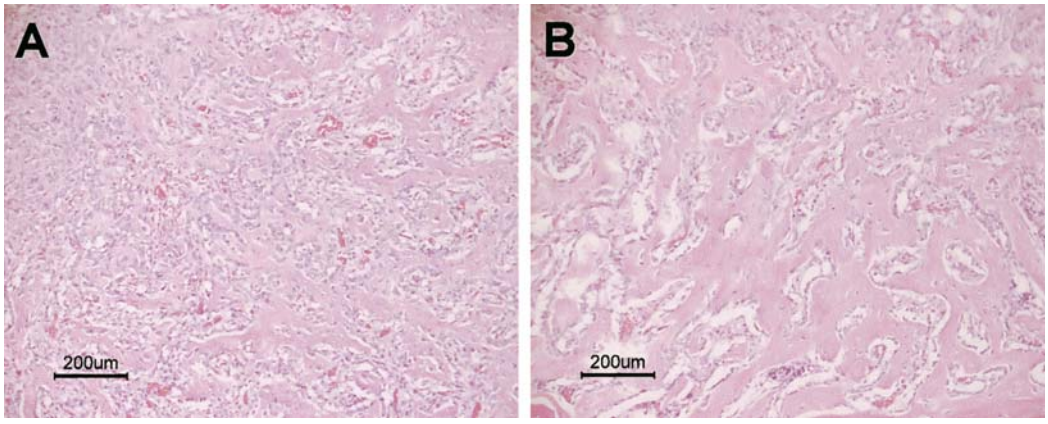

Fig. 1. Histology of rat femurs infused with vehicle or basic fibroblast growth factor (bFGF). Bone marrow was aspirated from both femurs. One leg was infused with 50 ng/day of bFGF for 6 days (**B**), and the contralateral leg was infused with vehicle only and served as the control (**A**). Specimens of harvested femurs were fixed, decalcified, embedded, and stained with hematoxylin and eosin

bone was found in the reactive tissue and endosteal surface and was accompanied by a lining of cuboidal osteoblastic cells. These findings were outstanding in the treated leg compared with the control leg. A few osteoclasts were identified by their morphology and located on the bone surface, but there was no difference in osteoclast numbers, suggesting that bone resorption was not enhanced by bFGF.

Effects of bFGF on the in-vivo gene expression of osteoblast markers

Total RNA was extracted from rat femurs, and the mRNA expression of osteoblast markers was analyzed by Northern hybridization. The results, shown in Fig. 2, demonstrate that infusion of bFGF caused stimulation of osteopontin gene expression and inhibition of type I collagen gene expression, although the alkaline phosphatase and osteocalcin genes were not affected. To quantitate the changes in mRNA levels in response to bFGF treatment, we analyzed the specific mRNA content by densitometric analysis of Northern blot analyses. Infusion of bFGF led to a significant increase in gene expression for osteopontin compared with the control leg ($P < 0.05$). In contrast, bFGF infusion provoked a significant inhibition in the gene expression for type I collagen ($P < 0.05$). Alkaline phosphatase mRNA levels exhibited a small increase that was not statistically significant. There were no significant changes in osteocalcin mRNA levels (Fig. 3).

Response of mRNA levels to various doses of bFGF

To compare the effects of various doses of bFGF, values for results were expressed as the ratio of the normalized

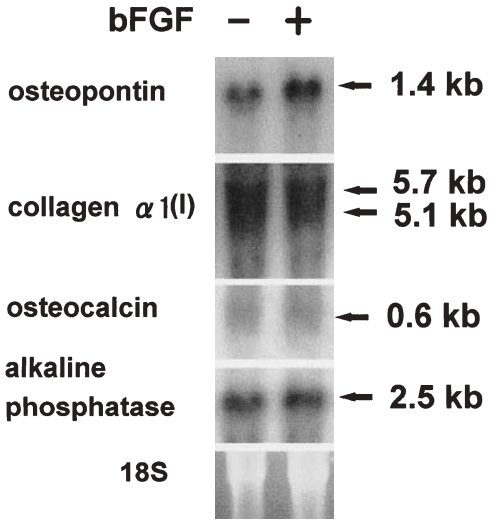

Fig. 2. Representative Northern blots of femoral mRNA after in-vivo bFGF treatment. One leg was infused with 50 ng/day of bFGF for 6 days, and the contralateral leg was infused with vehicle only and served as the control. Total RNA was prepared from the femurs and subjected to Northern blot analysis. Ethidium bromide staining of 18S ribosomal RNA in the same gel is shown at the bottom

mRNA level of the treated leg divided by the level of the control leg (Table 1). The infusion of various doses of bFGF resulted in a biphasic response. Treatment with bFGF at 50 and 100 ng/day significantly enhanced the mRNA levels of osteopontin, with increases of 25% and 24%, respectively. At 500 ng/day of bFGF, there were no significant stimulatory effects on osteopontin mRNA levels. Maximal inhibition (nearly 20%) of procollagen (I) mRNA was caused by 50 ng/day of bFGF. Infusion of bFGF at 100 and 500 ng/day pro-

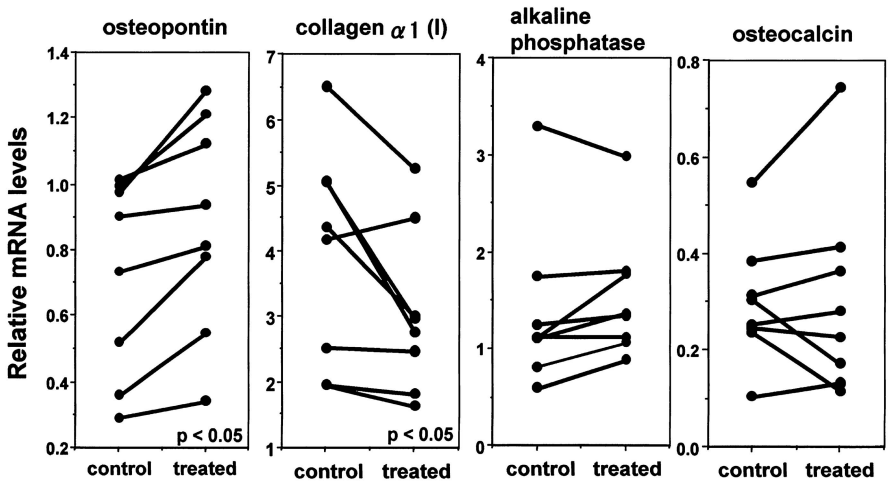

Fig. 3. Effect of bFGF infused locally on mRNA levels of osteoblast markers. One leg was infused with 50 ng/day of bFGF for 6 days, and the contralateral leg was infused with vehicle only and served as the control. Total RNA was prepared from the femur and subjected to Northern blot analysis. The level of each specific mRNA of Northern blots was quantitated by densitometric analysis after being normalized with 18S ribosomal RNA

Table 1. Effects of bFGF infused locally on mRNA levels of osteoblast markers

	Osteopontin	Procollagen (I)	ALPase	Osteocalcin
bFGF 20 ng/day ($n = 6$)	1.170 ± 0.387	1.003 ± 0.324	0.975 ± 0.237	0.949 ± 0.204
bFGF 50 ng/day ($n = 8$)	1.250 ± 0.184*	0.806 ± 0.189*	1.170 ± 0.239	1.020 ± 0.301
bFGF 100 ng/day ($n = 6$)	1.238 ± 0.404*	0.828 ± 0.253	0.854 ± 0.111	0.954 ± 0.068
bFGF 500 ng/day ($n = 7$)	1.103 ± 0.213	0.896 ± 0.27	0.921 ± 0.097	0.825 ± 0.257

Bone marrow was aspirated from both femurs. One leg was infused with various doses of basic fibroblast growth factor (bFGF) for 6 days, and the contralateral leg was infused with vehicle only and served as the control. Total RNA was prepared from the femur and subjected to Northern blot analysis. The level of each specific mRNA was quantitated by densitometric analysis after being normalized with 18S ribosomal RNA. Results are expressed as the ratio of the normalized mRNA value of the treated leg divided by that of the control leg. Values shown are means ± SD

ALPase, Alkaline phosphatase

* $P < 0.05$ vs control; paired t-test

duced an inhibitory trend that was not statistically significant. There were no significant changes in alkaline phosphatase and osteocalcin mRNA levels at any dose of bFGF.

Discussion

Although a number of studies have been reported regarding the in-vitro and in-vivo effects of bFGF on bone, the precise mechanism of its action remains unclear. To determine the effect of bFGF on osteoblast differentiation in vivo, we used a bone marrow ablation model. We have been using this model to examine the pattern of mRNA expression for bone-matrix proteins and growth factors in the in-vivo bone-formation process [19,25,26]. This injury induces the rapid activation of bone synthetic activity, the extensive deposition of trabecular bone, the initiation of resorption, and the reformation of marrow over a 3-week period [27,28]. Our results showed that mRNA for bone-matrix proteins was sequentially expressed in the process of bone deposition in the marrow cavity, with the peak increase in mRNA expression for these osteoblast markers being observed on day 6 when the maximum bone formation occurred [19]. The intramedullary bone-formation model lacks a cartilagous phase, unlike other in-vivo models such as the fracture-healing model or the bone mineral-matrix implantation model. Therefore, this model will provide a valuable means of assessing the process of osteogenic differentiation in-vivo.

After marrow ablation, bFGF was infused into femora for 6 days and gene expression for osteoblast-related markers was determined. In the present study, treatment with bFGF caused the stimulation of osteopontin gene expression and the inhibition of type I collagen gene expression, although alkaline phosphatase and the osteocalcin gene were not affected. Although a number of reports have documented an anabolic effect of bFGF on bone formation in in-vivo models, to our knowledge, the present study is the first to furnish evidence for bFGF action on osteoblast-related gene expression in vivo.

Previous in-vitro studies have also shown a stimulatory effect of bFGF on osteopontin gene expression. bFGF stimulates osteopontin mRNA expression in osteoblastic cells [18], as well as in bone marrow stromal cells [15], in a dose-responsive manner. The effects of

bFGF in the present study were statistically significant; however, the magnitude of the increase in gene expression was lower than that observed in in-vitro studies. This result is partly due to the possible secretion of endogenous growth factors during intramedullary bone formation, which may mediate this process and thus mask the effects of exogenous factors. One immunohistochemical study has identified an accumulation of the osteopontin-immunopositive matrix in response to direct injection of bFGF into rat bone marrow [29], confirming our present findings.

Although previous in-vivo studies have found a stimulatory effect of bFGF on bone formation, our present results show that bFGF inhibits the gene expression of type I collagen, a major bone matrix protein. Similar results have also been observed in in-vitro studies, demonstrating that bFGF inhibits collagen synthesis by a transcriptional mechanism and that the type I collagen gene promoter contains DNA sequences that mediate bFGF inhibition [18,30]. One study of the fracture-healing model has also found an inhibitory effect, in which acidic FGF injection stimulates cartilage enlargement but inhibits cartilage gene expression [31]. It is possible that the genetic expression of extracellular matrix proteins might not be accompanied by histologic changes, depending on the model used in the experiments, the method and timing of growth-factor administration, and the timing of histological or genetic change. It is also possible that the gene expression of type I collagen may not be associated with the phenotype development of osteoprogenitor cells. Because histomorphometric analysis was not carried out in our present study, additional experiments will be necessary to determine the relationship between histological events and the gene expression of extracellular matrix proteins in the bone marrow ablation model.

The maximum response of gene expression in the bone to bFGF was achieved at 100 ng/day and was then diminished at higher doses. However, such dose-response effects are common in growth-factor biology, and biphasic effects of bFGF have been documented previously. It has been found that bFGF enhances bone formation in demineralized bone matrix [32], bone grafts [33], and in a mandibular defect model [12] in a similar biphasic dose-response manner, possibly because bFGF influences cell differentiation in favor of fibrous-tissue formation at a higher dose. One possible mechanism of this biphasic bFGF effect may be explained by the downregulation of FGF receptors in response to FGF ligands [34]. A high dose of bFGF may induce a downregulation of cell-surface FGF receptors, resulting in reduced bFGF action.

Our present findings clearly show that bFGF modulates gene expression for the osteogenic phenotype in a biphasic manner for in-vivo bone formation. The process of bone formation is regulated through a complex of growth-factor networks, including that of bFGF. bFGF may affect other growth factors and their receptors, resulting in a modulation of their activities. Taken together, the characteristics of bFGF, including multiple functions and a narrow dose range, may reflect the complex regulation of bone formation through growth-factor networks. Our data also support the hypothesis that bFGF plays an important role in bone formation and resorption in the process of bone remodeling by modulating osteogenic differentiation. Further studies will be necessary to understand the role of bFGF in the bone-remodeling process to further explore its potential use in therapeutic applications.

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