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Stimulation of bone formation by intraosseous injection of basic fibroblast growth factor in ovariectomised rats

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Summary. *The effect on intraosseous bone formation of a single local injection of recombinant human basic fibroblast growth factor into trabecular bones was examined in ovariectomised osteoporotic rats. Fibroblast growth factor (400 μg), or the vehicle alone, was injected into the ilium at 16 weeks after ovariectomy or a simulated operation. Bone mineral density in the ovariectomised rats increased to a level similar to the latter at 2 weeks and reached a maximum at 8 weeks. After 8 weeks, BMD decreased slowly and the value at 24 weeks was still higher than that in the ovariectomised rats. Fibroblast growth factor stimulated osteoid formation in the first 2 weeks, bone volume reaching a peak at 8 weeks. From 8 to 12 weeks, bone resorption increased, resulting in decreases in bone volume to the levels of the group with simulated operations at 24 weeks. Structural analysis at 8 and 24 weeks showed that ovariectomy decreased the continuity of trabeculae and the injection of fibroblast growth factor restored it to levels higher than, or equal to, those who had the simulated operation. The present study demonstrated that intraosseous fibroblast growth factor given to ovariectomised rats restored bone volume and quality to the levels of the rats who had a simulated operation only.*

Résumé. *L'effet d'une unique injection locale dans les os trabeculaires de facteur de croissance de base de fibroblaste humain recombinant (bFGF) sur la formation intra-osseuse a été examiné sur des rats ostéoporotiques ovariectomisés. L'injection bFGF (400 μg) ou véhicule seul (V) a été pratiquée dans l'ilion 16 semaines après ovariectomie (OVX) ou pseudo-opération (Sham). A 2 semaines, la densité osseuse moyenne des OVX+bFGF avait augmenté jusqu'à un niveau similaire aux Sham+V pour atteindre son maximum à 8 semaines, soit 19% de plus que les Sham+V. Au-delà de 8 semaines, la densité moyenne a décliné lentement, conservant à 24 semaines une valeur supérieure aux OVX+V. Le bFGF a stimulé la formation ostéoïde des 2 premières semaines. Le volume d'os minéralisé a atteint son sommet à 8 semaines. Entre 8 et 12 semaines, la résorption osseuse a été facilitée, conduisant à des baisses du volume osseux jusqu'aux niveaux Sham+V 24 semaines. Les analyses structurelles à 8 et 24 semaines ont montré que l'injection bFGF ramène à des niveaux égaux ou supérieurs à Sham+V la connectivité des trabeculae réduite par l'OVX. La présente étude établit qu'une application intraosseuse de bFGF après ovariectomie ramène le volume et la qualité osseuse au niveau des rats pseudo-opérés.*

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Introduction

Basic fibroblast growth factor (bFGF, FGF-2) is a potent mitogen for a variety of cells of mesodermal and ectodermal origin [6, 20, 24]. In skeletal tissues, bFGF is produced by cells of osteoblastic lineage, accumulated in bone matrix, and acts as an autocrine/paracrine factor for bone cells [1, 5, 7, 9,

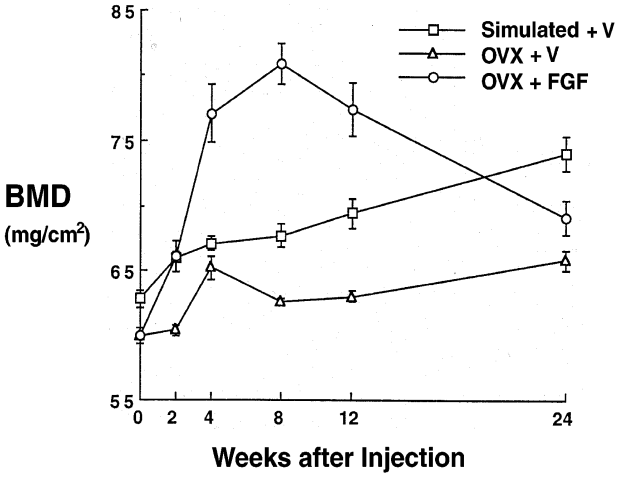

Fig. 1. Time course of the effects of bFGF on bone mineral density (BMD) of iliac trabecular bone. *Symbols* indicate means and vertebral *lines* indicate the standard error (SEM) for 7 animals/group

22]. bFGF shows variable regulations of proliferation and differentiation of cells of osteoblastic lineage so that is modulates bone formation in vitro [1, 8], and is also reported to stimulate bone resorption in bone organ cultures [13, 23].

Recently it has been reported that bFGF shows anabolic effects on bone formation in vivo. We have recently demonstrated that a single injection at fracture sites facilitates fracture healing [12]. Aspenberg and his colleagues have reported that a local application of bFGF increased bone yield in implanted demineralised bone matrix [25]. Using young and old rats, two separate groups have shown that daily systemic administration of bFGF caused increased endosteal bone formation [14, 17], and these effects were seen as early as 1 to 3 weeks whether the injection was either local or systematic.

Some patients with osteoporosis need a rapid increase in bone strength as, for example, those with hip fracture whose other hip is at risk. The present drugs used for osteoporosis work slowly over months or years. If a local injection of bFGF increases bone strength rapidly, it might lead to a new approach to the treatment of osteoporosis.

Bone strength depends not only on the quantity of bone represented by bone volume, but also on the quality of its structure. Since some trabeculae are perforated in osteoporotic patients [11, 16], restoration of their continuity may give rise to an increase in the scaffold on which bone cells could make new bone.

The present study was undertaken to investigate the effects of single local injection of bFGF on bone quantity and quality in the iliac trabeculae of ovariectomised osteoporotic rats.

Materials and methods

Animals and bFGF injection

Fourteen-week-old female Wistar rats were purchased from Shizuoka Laboratory Animal Co, Shizuoka, Japan. They had either an ovariectomy or a simulated operation under general anaesthesia. After both operations, the rats were kept in individual cages with standard food, containing 1.15% Ca and 0.88% phosphate (Oriental Yeast Co Ltd, Japan), and water was freely available. At 16 weeks, a 26G bone marrow puncture needle was inserted into the cancellous bone of the

Table 1. Structural analyses of trabecular bone

	8 weeks			24 weeks		
	Simulated+V	OVX+V	OVX+bFGF	Simulated+V	OVX+V	OVX+bFGF
Node/free end ratio	0.88 ± 0.10[a]	0.28 ± 0.07[c]	4.25 ± 1.05[c, e]	1.10 ± 0.20	0.29 ± 0.08[b]	1.16 ± 0.29[d]
% Strut length (% of total strut length)						
Node to node	25.2 ± 3.6	9.4 ± 3.1[b]	12.2 ± 4.4	21.5 ± 3.9	11.8 ± 5.0	29.2 ± 4.8
Node to free end	26.7 ± 4.7	27.5 ± 3.3	11.5 ± 3.1[b, d]	29.5 ± 5.2	25.7 ± 5.2	20.3 ± 3.0
Node to loop	12.1 ± 2.7	8.3 ± 5.7	23.1 ± 6.7	18.4 ± 4.8	3.6 ± 2.6	13.0 ± 4.2
Free end to free end	5.9 ± 1.9	8.8 ± 2.8	1.1 ± 0.5	2.2 ± 1.0	6.6 ± 1.9	4.0 ± 1.9
Cortex to node	10.5 ± 2.8	19.3 ± 5.4	31.2 ± 4.1[c]	14.5 ± 3.0	13.4 ± 2.3	22.5 ± 7.0
Cortex to free end	9.9 ± 1.7	22.9 ± 7.5	4.0 ± 2.2[d]	4.7 ± 0.1	29.0 ± 11.1[b]	5.4 ± 2.0[d]
Cortex to cortex	9.8 ± 6.9	3.8 ± 3.7	17.0 ± 4.8	9.2 ± 4.0	9.9 ± 3.5	5.6 ± 2.9

[a] Data are expressed by mean ± SEM for 7 samples for each group
Significantly different from simulated+V
[b] $P < 0.05$
[c] $P < 0.01$
Significantly different from OVX+V
[d] $P < 0.05$
[e] $P < 0.01$

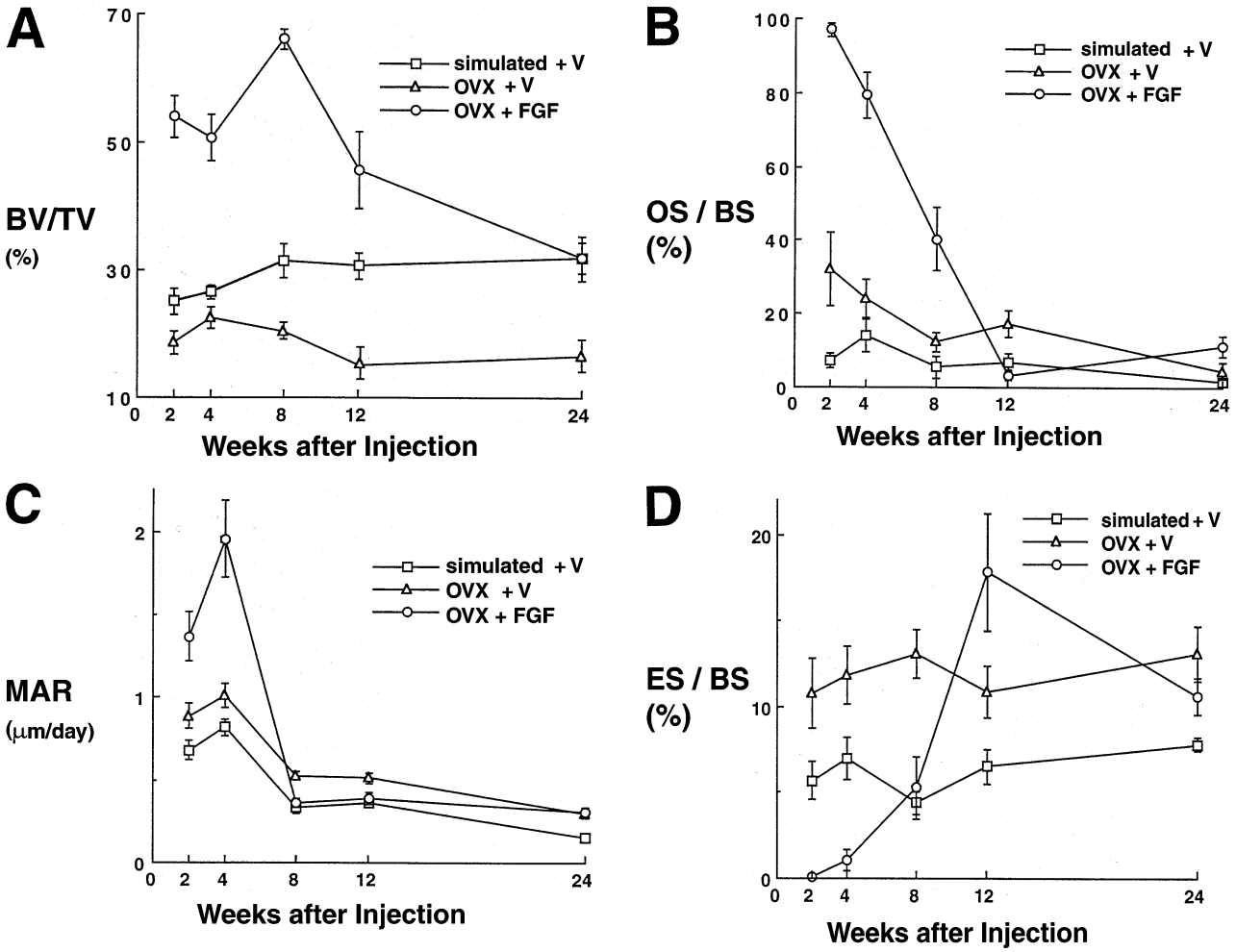

Fig. 2. Time course of the effects of bFGF on histomorphometric parameters of iliac trabecular bone. *Symbols* indicate means and vertebral *lines* indicate the standard error (SEM) for 7 animals/group. **A** bone volume/tissue volume (BV/TV). **B** osteoid surface/bone surface (OS/BS), **C** mineral apposition rate (MAR), **D** eroded surface/bone surface (ES/BS)

right iliac crest to a depth of 3 mm under general anaesthesia. 400 μg of recombinant human bFGF (Scios Nova, Mountain View, CA) in 50 μl aqueous saline solution, or the vehicle (V) alone, was injected through the needle into the ilium of the ovariectomised (OVX) animals (OVX+bFGF and OVX+V, respectively). The vehicle alone was injected into the animals who had a simulated operation (simulated+V). The iliac bones were harvested at 0, 2, 4, 8, 12 and 24 weeks after these procedures.

Measurement of bone mineral density (BMD)

BMD of the whole area, 2 cm long, from the iliac crest, was measured by dual energy X-ray absorptiometry (DEXA) using a bone mineral analyser (Dichroma Scan DCS-600R, Aloka Co., Tokyo, Japan).

Histomorphometric analysis

The iliac bone removed was stained in Villanueva bone solution and embedded in methyl methacrylate; 5 μm sections were cut sagittally at the point where the bFGF or vehicle had been applied, and then stained with Villanueva Goldner. The specimens were analysed using a microscope with a video camera connected to an image analysis system (SP-1000, Olympus, Tokyo, Japan). Areas and perimeters of cancellous bone of rectangular areas, 0.3×3.0 mm in size, at 4 mm from the iliac crest were measured at 10× magnification.

To measure mineral apposition rate (MAR) all rats were injected subcutaneously with calcein (Wako Pure Chemical Industries Ltd, Japan) at 2 to 12 weeks before harvesting; tetracycline hydrochloride (Sigma), 20 mg/kg body weight, was given 2 days previously. Double fluorescent labelling of calcein and tetracycline was analysed with a fluorescent microscope using an image analysis system (SP-1000, Olympus, Japan).

Structural analyses of trabecular bone

The two-dimensional trabecular bone structure of the iliac bone which had been removed at 8 and 24 weeks after the injection were analysed by Garrahan et al's method [3, 4]. The

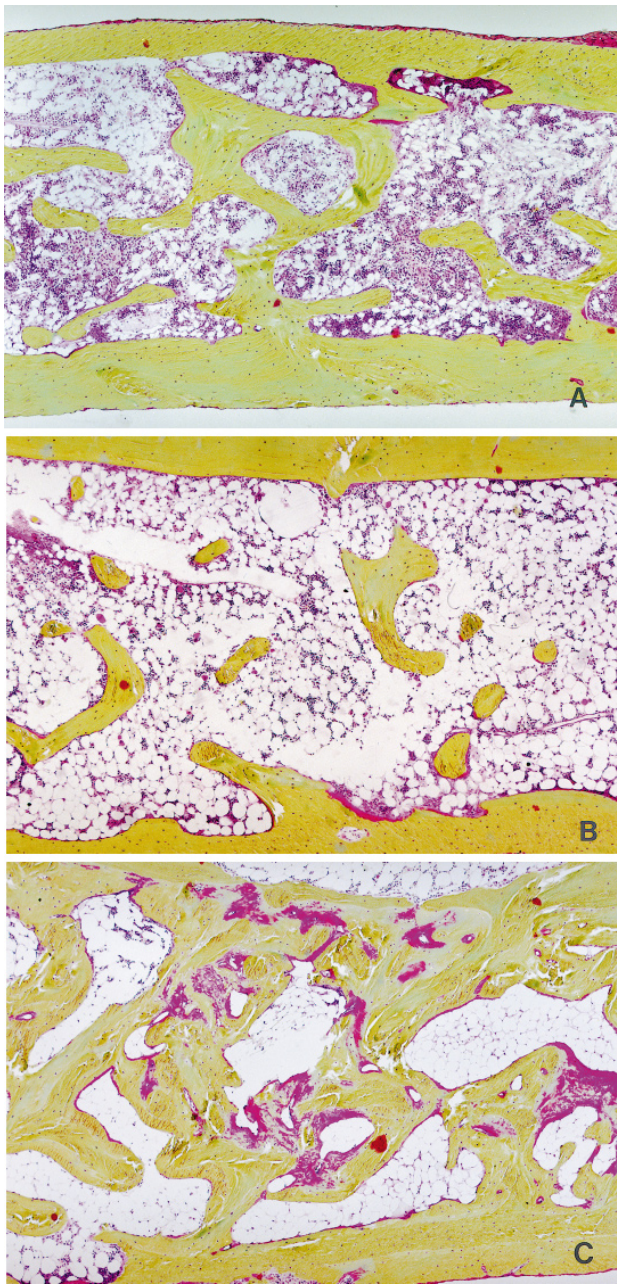

Fig. 3. Histological appearances of trabecular bones of simulated+V (**A**), OVX+V (**B**), and OVX+bFGF (**C**) rats at 8 weeks. (Villanueva Goldner stain, X15)

specimens were assessed for the continuity of trabecular bone represented by the ratio of nodes to free ends and the percentage length of different types of strut.

Statistical analyses

Data were analysed by one-way analysis of variance. When the test indicated significance, the differences between the groups was determined by Dunnett's test. The test of significance was performed at the 95% confidence interval compared to the control group.

Results

Ovariectomy was effective because the mean uterine weights of the OVX rats were significantly lower than in the rats who had simulated operations (1/6–1/7, $P < 0.01$) at 16 weeks after operation (data not shown).

The BMD of the OVX+bFGF rats increased rapidly up to 4 weeks and reached maximum at 8 weeks; it reached a level similar to the simulated+V rats at 2 weeks, at 8 weeks, it was 19% higher than that of the simulated+V rats and 29% higher than that of OVX+V. After 8 weeks, the BMD in OVX+bFGF decreased slowly, the value at 24 weeks being lower than that of simulated+V, but still higher than that in OVX+V (Fig. 1).

Figure 2 shows the effect of bFGF on the histomorphometric parameters of trabecular bone. At 2 weeks, bFGF significantly increased the bone volume ratio (BV/TV) and mean trabecular thickness (MTT) (data not shown) of OVX rats to much higher levels than simulated+V. The effect of bFGF reached a maximum of about twice that of simulated+V rats at 8 weeks, and then decreased slowly until 24 weeks when the levels were similar to simulated+V. The values of OVX+bFGF rats at 24 weeks were still higher than those of OVX+V rats (Fig. 2A).

The most marked effect of bFGF on osteoid formation occurred at an early stage (Fig. 2B). bFGF markedly increased osteoid volume (OV/BV) (data not shown) and the osteoid surface (OS/BS) at 2 weeks. Both these parameters for osteoid formation decreased up to 24 weeks, although the osteoid volume of the OVX+bFGF rats was still significantly higher than that of simulated+V and OVX+V rats. Figure 3 shows the histology of trabecular bone in simulated+V, OVX+V, and OVX+bFGF rats at 8 weeks. Injection of bFGF stimulated trabecular bone formation in OVX rats to levels similar to that found in simulated+V rats, and osteoid formation to higher levels than simulated+V rats.

Mineral apposition rates (MAR) of OVX+bFGF rats were significantly higher than those of simulated+V and OVX+V rats at 2 weeks, and further increased up to 4 weeks. The value decreased rapidly to levels similar to simulated+V and OVX+V rats for up to 8 weeks, and thereafter changed little (Fig. 2C).

The parameter for bone resorption, the eroded surface ratio (ES/BS), was much lower in OVX-bFGF rats at 2 and 4 weeks than in the other two groups. However, it then increased rapidly up to 12 weeks (60% higher than OVX+V rats at

12 weeks), and decreased to levels similar to the other two groups at 24 weeks (Fig. 2D).

Table 1 shows the structural analysis of trabecular bone at 8 weeks and 24 weeks. At both OVX decreased node/free end ratios significantly, and injection of bFGF restored the ratio to levels higher than 8 weeks or equal to 24 weeks in those of simulated+V rats. Because node to node, node to loop, and cortex to node length ratios are increased, while node to free, free end to free end, and cortex to free end length ratios are decreased by the injection of bFGF, bFGF also increased the continuity of trabecular bone.

Discussion

The present histological studies over a 24 week period demonstrated that a single local injection of bFGF into the ilium of ovariectomised rats restored bone volume and bone quality to the levels of the rats with simulated operations.

As we have reported previously [18], bFGF induced the proliferation of undifferentiated mesenchymal cells around the trabeculae immediately after the injection. In the early phase, especially during the first 2 weeks, bFGF strongly stimulated osteoid formation which was followed by mineral apposition with a maximal effect at 2 to 4 weeks. Mineralised bone volume reached a peak at 8 weeks, which was significantly higher than that in the rats with simulated operations. From 8 to 12 weeks, bone resorption, presumably due to physiological remodelling, was markedly increased in the bFGF-treated group, resulting in decreases in bone volume to the level of the rats with simulated operations at 24 weeks. Continuity of trabecular bone was increased by bFGF injection to the levels found in the rats with simulated operations at both 8 and 24 weeks.

Previous studies have shown that bFGF is a more potent mitogen for fibroblasts and pre-osteoblasts than for differentiated osteoblasts [15]. It has also been shown to inhibit bone cell differentiation or matrix synthesis [1, 2, 10, 21]. Thus, the stimulatory effect of bFGF on bone formation in the present study appears to be mainly due to the stimulation of mesenchymal cell proliferation, and these cells then differentiate into precursor cells, or support this differentiation. In addition, we and others have reported that bFGF induces transforming growth factor (TGF)-β gene expression at the fracture site and in cultured osteoblastic cells [12, 19]. Thus, bFGF may act as a factor initiating the cascade of events for new bone formation by enhancing the release of additional factors such as TGF-β or bone morphogenetic proteins.

A single injection of bFGF into the cancellous bone of the ilium in ovariectomised rats increased both the quantity and quality of bone very rapidly.

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