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Osteoporosis and Bone Regeneration

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1. Introduction

Majority of skeletal conditions generate or heal normally due to inherent capacity. However, compromised conditions such as induced by congenital or acquired diseases may sometimes lead to uncompleted development or regenerate. The quality and volume of bone is an important factor to be considered in orthopaedic and dental fields. We occasionally encounter difficult clinical cases because of insufficient bone. Bone is a tissue that is being constantly remodelled, and bone mass at any given time depends on the balance between the rate of osteoblastic bone formation and osteoclastic bone resorption. These cellular functions are controlled by various systemic and local factors.

Osteoporosis is a skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue with a consequent increase in bone fragility and susceptibility to fracture (1993). The term osteoporosis was first introduced in France and Germany during the last century, meaning “porous bone” and initially implied a histological diagnosis, but was later refined for bone which was normally mineralized but reduced in quantity. Estrogen deficiency in postmenopausal women elicits bone loss in the vertebrae and long bones resulting in bone fractures, and this condition is called postmenopausal osteoporosis (T.J. Wronski et al., 1985, 1988, 1989a).

To the anatomical site features, a bone defect repair rate is mainly dependent on the bone wound size (J.P. Schmitz & J.O. Hollinger, 1986). Theoretically, an experimental osseous injury performed to study repair mechanisms should be wide enough to preclude a spontaneous healing. In order to do this, the non-regeneration threshold of bone tissue was investigated in the studied models, inducing a so-called critical-sized defect (CSD). The CSD may be defined as “the smallest size intraosseous wound in a particular bone and species of animal that will not heal spontaneously during the lifetime of the animal” (J.P. Schmitz & J.O. Hollinger, 1986; J.O. Hollinger & J.C. Kleinschmidt, 1990). The CSD may be therefore considered the prototype of discontinuity defects, as a condition of failed osteogenesis for overcoming the threshold of physiologic repair processes.

Although autologous or allogous bone transplant is partially effective, a simple and effective method for bone augmentation and regeneration is clinically desired in the orthopedic and dental fields. In the 1960s, Urist proposed the existence of bone-inducing molecules, which he termed bone morphogenetic proteins (BMP) (M.R. Urist, 1965). In 1988, the cDNA of BMPs was characterized, and human recombinant BMP2 (rhBMP2) (H.D. Zegzula et al., 1997; D.L. Wheeler et al., 1998; R.D. Welch et al., 1998; J.R. Lieberman et al., 1999; J.L. Dragoo et al., 2003) and BMP7 (X. Chen et al., 2002; F.C. den Boer et al., 2003; M.M. Abu-Serriah et al., 2004) are currently available. However, large amounts of rhBMP2 or 7 are required for

use in clinical treatment. Although treatment with rhBMPs is effective, the extremely high cost of their clinical application is a barrier to their use. Development of an efficient carrier for BMPs or characterizing inhibitors of BMPs and blocking them may solve this problem in the future. Furthermore, fibroblast growth factor (FGF) family members also participate in regulating osteogenesis during fracture repair. FGF2 has been shown to be widely secreted around the fracture site in the processes of wound healing and bone regeneration; however, FGF4, which plays important roles in bone development during embryogenesis, has not yet been detected in such cases in postnatal ages.

In this chapter, several histological events concerning bone are demonstrated: first, an osteoporotic effect on mandibular bone is introduced; second, calvarial healing in the bone defect was demonstrated by GBR; and subsequently, it is shown that systemic and local deliveries of FGF4 and locally carried BMP2 can contribute to osteogenesis in animal experiments.

2. Experimental cases and discussion

2.1 Osteoporosis of mandible

2.1.1 Background

Bone is constantly remodelled, and bone mass at any given time is controlled by bone formation and bone resorption. These cellular events and functions are intimately associated with various systemic and local factors. Estrogen deficiency in postmenopausal women elicits bone loss in the vertebrae and long bones resulting in bone fractures, and this condition is called postmenopausal osteoporosis (T.J. Wronski et al., 1985, 1988, 1989a). Compared with the vertebrae and long bones, considerably less information is available on bone loss of the mandibles under estrogen deficient conditions. The bone mineral content of edentulous mandibles decreases with aging especially post menopause, but this is not evident in elderly men (E. Klemetti et al., 1993a, 1993b; C.W. Ulm et al., 1994; H. May et al., 1995). The ovariectomized (OVX) rat provides an experimental model of postmenopausal osteoporosis. It has been introduced that the bone mineral content and mechanical properties of the mandibles of OVX rats are similar to those of sham-operated rats; however, maxillary molar extraction causes bone loss from the mandibles of OVX rats (R.P. Elovic et al., 1994, 1995; E. Klemetti & P. Vainio, 1994). These previous studies indicate that estrogen deficiency somehow affects the mandible in both humans and experimental animals. To investigate precise region of the affected mandible, rats were ovariectomized and then subjected to longitudinal scanning for bone mineral density (BMD) measurement.

2.1.2 Materials and methods

Adult female Sprague-Dawley rats were bilaterally OVX- or sham-operated under Nembutal anaesthesia. The mandibles and femurs were dissected out after 3 months immersed in 70% ethanol. The uteri were also retrieved and weighed to check the effects of ovariectomy. Soft X-ray images of the mandibles and femurs were taken with a soft X-ray radiographic apparatus (SPOM50; Sofron, Tokyo, Japan). Total femoral BMD was initially measured with a DEXA (DCS-600R; Aloka, Tokyo, Japan), and then calculated. Total mandible BMD was measured using DEXA, after which the BMD of the incisal edge and condylar region of the mandible was measured. The trabecular and cortical BMD of the femurs were measured at cross sections 2 mm and 17 mm from the growth plate, respectively, perpendicular to the long axis with peripheral quantitative computed tomography (pQCT) (XCT 960 A; Stratec, Pforzheim, Germany) (Fig. 1). The positions are

trabecular and cortically rich regions, respectively. Also, the mandibles were scanned from the medial plane to the distal plane of the molar region with pQCT at interval of 0.5 mm as indicated in Fig. 1, following which the trabecular BMD and cortical BMD of the mandibular sections, excluding the incisor and molar, were measured using computer software.

2.1.3 Results

In OVX rats, radio opacity of the femur attenuated after OVX was observed by soft X-ray radiography, and both uterus weight (data not shown) and femoral total BMD in DEXA analysis (Fig. 3) decreased by 80.8 and 13.7%, respectively. Although the total bone mineral density and the incisor mineral density of the mandible of the ovariectomized rats were similar to those of the sham-operated rats, the bone mineral density of the condylar region in the ovariectomized rats had markedly decreased by 14%. In pQCT analysis, decrease of 30% at the 17-mm section in trabecular BMD of the femur was prominent in OVX rats whereas ovariectomy did not affect BMD of the cortical bone of the femur. The molar region of the mandible excluding the molar showed decrease of maximal 13% in trabecular bone mineral density at the eighth and its adjoining slices; on the hand, cortical BMD was not affected in any of the slices (data not shown). This study revealed regional differences in bone mineral density decrease in the mandible in ovariectomized rats.

Fig. 1. Areas and positions for BMD measurement (S. Kuroda et al., 2003).

External incisal ridge and mandibular condyle in the areas surrounded with rectangles (4x6 mm) and square (2x2 mm) were scanned by DEXA, respectively. The sections including molars at interval of 0.5 mm were scanned by pQCT. Trabecular and cortically rich regions of the femur were scanned at 2 mm and 17 mm from the growth plate, respectively.

Fig. 2. Soft X-ray Radiography of the femurs. Radio opacity decreased after OVX (S. Kuroda et al., 2003).

Fig. 3. (A) Total bone mineral density of femur by DEXA. (B) Trabecular and cortical bone mineral densities of femur at the sections shown in Fig. 1. Values are presented as Mean $\pm$ SD, n = 6. *p < 0.05 was regarded as statistically significant. (S. Kuroda et al., 2003)

Fig. 4. (A) The total mandible, the external part of incisor and the condylar region of mandible by DEXA. (B) Trabecular bone mineral densities of mandible at the sections shown in Fig. 1. Values are presented as Mean $\pm$ SD, n = 6. *p < 0.05 was regarded as statistically significant. (S. Kuroda et al., 2003)

2.2 Guided bone regeneration with a collagen membrane

2.2.1 Background

Guided Bone Regeneration (GBR) technique is clinically used to acquire the sufficient bone volume, which has been developed by Nyman and Dahlin (C. Dahlin et al., 1988, 1989, 1990). The concept of this technique is that the application of a membrane creates a secluded space to facilitate proliferation of angiogenic and osteogenic cells from the basal bone into the defect without interference by fibroblasts. The membranes for GBR are mainly divided into three types: expanded polytetrafluoroethylene (ePTFE), synthetic biodegradable polyesters and collagen. Collagen is a material of resorbable membranes, which has several advantages such as hemostatic function, allowance of an early wound stabilization, chemotactic properties to attract fibroblasts and facilitating nutrient transfer. Therefore, collagen membranes are currently the membrane of choice for most GBR procedures.

2.2.2 Materials and methods

To investigate the efficacy of the collagen membrane for enhancement of bone regeneration in rat parietal bone defects, two symmetrical full thickness bone defects (5 mm diameter) were created at the calvarial bone of adult male Wistar rats. The defects were covered with a collagen membrane (Koken Tissue Guide, Koken, Japan) for GBR for 1 to 12 weeks. And then the specimens of the bone defect along with surrounding bone and soft tissues were collected and denuded of the skin. The samples were subjected to X-ray imaging using a μ CT scanner (InspeXio; Shimadzu Science East Corporation, Tokyo, Japan) with a voxel size of 70 μ m/pixel. Tri/3D-Bon software (RATOC System Engineering Co. Ltd, Tokyo, Japan) to make a 3D reconstruction from the resulting set of scans, and were also analysed by DEXA to measure the bone morphology in the defect area.

After radiographical analyses, the samples were fixed in 10% neutralized formalin for 1 week, followed by decalcification in 10% EDTA for 4 weeks. After decalcification, an incision was made precisely through the midpoint of the bone defects to ensure that the microtome sections were made in the ROI and dehydrated in ascending grades of ethanol. The samples were consequently embedded in paraffin to allow for the preparation, staining with hematoxylin-eosin, and observation of 5- μ m-thick coronal sections under an optical microscope (BZ-8000; Keyence, Osaka).

2.2.3 Results

The swelling and scabbing at the incised area attenuated by 2 weeks. Alteration of the skeletal defects was visualized in the μ CT images over the period (Fig. 5). The opacity of newly formed bone was not found to reach the level of the surrounding host bone in the defects of the collagen group, showing incomplete healing at 4 weeks and bone recovery became abundant at 8 weeks after surgery (Fig. 5); and on the other hand, although new bone apposition was observed partially in the control defects, they acquired newly generated bone only along the defect rim with the similar opacity to that of the collagen covered defect and did not heal completely afterward.

Fig. 5. μ CT images of the parietal bone covered with the collagen membrane at 4 weeks (right) and without membrane at 8 weeks (left) after surgery.

Observation of the bone development process evenly and gradually replaced the collagen membrane. As shown in Fig. 6, bone regeneration as well as membrane absorption was evident in the collagen group, but the thickness of the new bone was significantly higher for the control group over the study period. Notably, osteogenesis was observed to occur primarily inside the collagen membrane.

Fig. 6. Photomicrographs of the calvarial defects at 2 and 4 weeks (Hematoxylin and eosin, original magnification $\times 4$).

The bone regenerate with the membrane was significantly prominent with BMC of newly formed bone in the defect at 8 and 12 weeks after surgery. Furthermore, the BMD of newly formed bone was significant at 12 weeks. These results indicate that covering the bone defects with collagen membranes has an ability to deliver the suitable space for bone regeneration and make the regenerated bone better quality (Fig. 7).

Fig. 7. (A) BMC of the defects by DEXA. (B) BMD of the defects by DEXA. *Statistically different from the uncovered control, $p < 0.05$.

2.3 Osteoconductive/osteoinductive proteins stimulate bone
2.3.1 Background

Gene expressions and productions of cytokines and growth factors in local regions that were traumatically or surgically injured are very crucial for tissue regeneration and engineering. However, cascades of the mechanisms and interactions of their roles have not been completely represented. Therefore, progress of such studies may lead to therapeutic aid.

Several FGF family members exert anabolic effects in bone when either systemically administered or locally applied (P. Aspenberg & L.S. Lohmander, 1989; H. Kawaguchi et al., 1994; T. Nakamura et al., 1995, 1998). FGF4 consists of 206 amino acid residues (M. Taira et al., 1987, T. Yoshida et al., 1987) and it has been reported that the FGF family plays a major role in the stimulation of cellular proliferation (M. Seno et al., 1990). In this study, the effects of rhFGF4s were clarified in mice after its systemic injection and in rat femurs after local administration.

In 1988, the cDNA of BMPs was characterized (J.M. Wozney et al., 1988). Gene therapy using genes of osteogenic proteins, such as BMPs, has been a focus of considerable attention (J. Fang et al., 1996; J. Bonadio et al., 1999, 2000; J.R. Lieberman et al., 1999; R.T. Franceschi et al., 2000; N. Abe et al., 2002; Y. Chen et al., 2002; J.L. Dragoo et al., 2003; C.H. Rundle et al., 2003; H. Tsuda et al., 2003; A.L. Bertone et al., 2004; I. Ono et al., 2004). When osteogenic genes are transferred to local cells, protein secretion begins and the stimulation of osteogenesis by the protein continues for a longer time than that seen in protein therapy. Experimental studies on osteogenic gene transfer have been emerging, and there are several gene transfer techniques used to stimulate bone regeneration: *ex vivo* (J.R. Lieberman et al., 1999; R.T. Franceschi et al., 2000; N. Abe et al., 2002; Y. Chen et al., 2002; J.L. Dragoo et al., 2003; C.H. Rundle et al., 2003; H. Tsuda et al., 2003; A.L. Bertone et al., 2004) and *in vivo* (J. Fang et al., 1996; J. Bonadio et al., 1999; J. Bonadio, 2000; K. Honma et al., 2001; H. Uusitalo et al., 2001; A. Sano et al., 2003; I. Ono et al., 2004) gene transfers and gene transfers with viral vector (J.R. Lieberman et al., 1999; R.T. Franceschi et al., 2000; H. Uusitalo et al., 2001; N. Abe et al., 2002; Y. Chen et al., 2002; J.L. Dragoo et al., 2003; C.H. Rundle et al., 2003; H. Tsuda et al., 2003; A.L. Bertone et al., 2004) or with nonviral vector (J. Bonadio et al., 1999; K. Honma et al., 2001; A. Sano et al., 2003; I. Ono et al., 2004). Here demonstrated is an *in vivo* gene transfer using nonviral vectors. This study was to examine whether our designed matrix, which consists of collagen, CaP, and a plasmid vector encoding for BMP2, enhances bone tissue regeneration in a rat bone defect model.

2.3.2 Materials and methods

Systemic administration of rhFGF4

Human FGF4 cDNAs vector were ligated to pET-29(+) vector (pET system, Novagen). After subcloning in JM109 and plasmid purification, the plasmids were transferred into BL21(DE3)pLysS, an E. coli strain used for protein expression. Protein expression was induced with isopropyl-b-Dthiogalactopyranoside (IPTG). The proteins were then purified using the STag Purification Kit (Novagen). The purified proteins were dialyzed against water using a minidialysis system (Bio-Tech International), and then freeze-dried.

Forty male ddY mice, 6 weeks old, were divided into eight groups and subcutaneously injected with rhFGF4s at doses of 0.03, 0.1, and 0.3 mg/kg every day for 2 weeks, which stimulated cellular proliferation of NIH3T3 cells, at doses of 0.03, 0.1, and 0.3 mg/kg. These rhFGF4s were dissolved in PBS containing 0.1% bovine serum albumin and injected. The five mice in the control group were injected with vehicle only.

After the 2 week injection course, the femurs were removed and contact microradiographs (CMRs) were taken of these ground sections using a soft X-ray radiographic apparatus (Sofron, SPO-M50).

For histomorphometric analysis, the sections were further ground down to 30 μm and stained with toluidine blue. Then, histomorphometric measurements were using an image analysis system (IBAS 2000, Carl Zeiss) to measure the histomorphometric parameters on the images of the areas. The measured parameters and the calculated parameters follow the previous report described in JBMR (A.M. Parfitt et al., 1987).

Local administration of rhFGF4

Thirty-two 10-week-old male Sprague-Dawley rats were divided into two equal groups; one group received local injection of rhFGF4 and the other received local injection of vehicle as control. An injection of 1 μg rhFGF4 (0.1 $\mu\text{g}/\mu\text{l}$) was given from the left tibial proximal intercondyle notch into the midshaft of the marrow cavity directly with a 21G needle.

The animals were killed under chloroform anesthesia on days 7 and 10. Tibiae were removed and photographed using soft X-rays. The bone mineral densities were measured with dual-energy X-ray absorptimetry. The trabecular and cortical bone marrow densities of the tibiae were measured with pQCT (Fig. 9A). After BMD measurements, the tibiae were fixed in 10% formalin, dehydrated, and embedded in methyl methacrylate resin (OsteoResin, Wako, Osaka, Japan). Then, longitudinal sections of 5- μm thickness were made via a microtome (Microtome 2050 Supercut, Reichert-Jung, Kandel Electronics, Inc., Orelan, PA) and stained by the Villanueva bone staining method. The x20 objectives of both light and fluorescence microscopes (Axiophot, Carl Zeiss, Oberkochen, Germany) were used to take optical images of the sections. Histomorphometric measurements were performed of the tibia using an image analysis system (IBAS 2000, Carl Zeiss).

BMP2 gene transfer at fracture site

cDNA of hBMP2 was inserted into pEGFP-N1 plasmid vector (Clontech, Mountain View, CA). hBMP2 encoding plasmid (bmp2) was precipitated in CaP solution (CalPhos Mammalian Transfection Kit, Clontech). An equal volume of 2% bovine type I atelocollagen solution (Atelocollagen Implant, Koken, Tokyo, Japan) was then added. Twelve micrograms of this mixture (50 μl) were lyophilized and designated "bmp2-CaP-collagen".

The bone segments across a 5-mm segmental tibial defect of male Wistar rats were fixed with stainless-steel screws. Implants were placed and held in the osteotomy. The specimens were fixed in 10% neutral formalin. Some specimens were embedded in methylmethacrylate resin and longitudinal sections that included the bone defects were then prepared. Undemineralized sections were stained with toluidine. For mechanical tests, the other samples were supported at the proximal and distal points on the jig, and force was applied to the middle of the bone defect perpendicularly at a displacement rate of 1 mm/min using a materials testing machine (Instron 1123, Canton, MA). Force and displacement data were stored in the computer.

The sites of the bone defects were collected and homogenized immediately for total RNA extraction (Isogen, Nippon Gene, Tokyo, Japan). And then RT-PCR was performed (SuperScript First-Strand Synthesis System for RT-PCR, Invitrogen, Carlsbad, CA; PureTaq Ready-To-Go PCR Beads, Amersham Biosciences, Piscataway, NJ). Initially, denaturing was carried out at 95°C for 5 min, followed by optimizing cycles: 95°C for 30 s for denaturing, optimized temperature for 30 s for annealing, and 72°C for 30 s for extension. Each RT-PCR product was electrophoresed in 2% agarose gel in TAE buffer and stained with ethidium bromide, followed by photography under ultraviolet light.

2.3.3 Results

Systemic administration of rhFGF4

Soft X-ray images revealed an increase in trabecular bone was evident dose-dependently in the CMRs in the rhFGF4-administered group (Fig. 8).

Fig. 8. Contact microradiographs (CMRs) of the longitudinal sections of femurs after administration with rhFGF4 (S. Kuroda et al., 1999).

Histomorphometric analysis revealed an increase in BV/TV and Tb.N, which represents an increase in trabecular bone. Furthermore, bone formation parameters (MS/BS and OS/BS) increased in a dosedependent manner, whereas a bone resorption parameter (ES/BS) was not affected (Table 1).

	rhFGF4 (mg/kg)			
	0 (Control)	0.03	0.1	0.3
BV/TV (%)	18.3 ± 2.5	23.4 ± 1.9	28.9 ± 1.5	31.1 ± 2.3
ES/BS (%)	10.5 ± 1.2	9.0 ± 1.6	9.8 ± 0.9	11.8 ± 1.1
MS/BS (%)	18.5 ± 2.7	20.0 ± 2.7	39.4 ± 2.5	43.1 ± 2.5
OS/BS (%)	17.5 ± 1.0	19.0 ± 2.6	30.4 ± 2.1	42.2 ± 1.9
Tb.Th (µm)	30.9 ± 1.3	28.8 ± 1.5	30.1 ± 2.3	33.7 ± 2.3
Tb.N (µm)	2.7 ± 0.2	3.0 ± 0.1	3.8 ± 0.2	3.8 ± 0.2

Table 1. The increase of trabecular bone after administration with rhFGF4 was measured and confirmed with histomorphometric parameters. Data are presented as the mean ± SE (n=5). a: Significantly different from controls, *p*<0.05. (S. Kuroda et al., 1999)

Local administration of rhFGF4

There were no visible or weight differences in the rats between the 2 groups at each time point, and neither the shapes nor sizes of the tibiae were affected by the local injection of rhFGF4 (data not shown). However, soft X-ray images demonstrated less radiolucence in the rhFGF4 group (Fig. 9A). DEXA analysis revealed increased BMD of the cancellous bone-rich zone of tibiae after the local injection of 1.0 mg of rhFGF4 and significance between the 2 groups at day 10 (Fig. 9B). Similarly, based on pQCT analysis (Fig. 9C), the trabecular BMD increased significantly in the rhFGF4 group from day 7 to day 10. Further, the higher BMD was maintained by the rhFGF4 injection over time. On the other hand, the cortical BMD exhibited no difference either between the groups or over time (data not shown).

Fig. 9. (A) Soft X-ray images of the tibiae. (B) BMD of the tibiae by DEXA. (C) Trabecular BMD of the mid shafts of the tibiae by pQCT. ^aSignificantly increased by time, ^bStatistically different between the groups, *p* < 0.05. (S. Kuroda et al., 2007)

Histomorphometric analysis elucidated increases in BV/TV, OS/BS, Ob.S/BS, MS/BS, ES/BS and Oc.S/BS in the rhFGF4 group at day 7 (Table 2), which represents high turnover of bone remodelling and derived increase of trabecular BMD. However, the ratios of parameters to BS were decreased at day 10; in particular, the ratios of OS, Ob.S, ES and Oc.S to BS were significantly decreased from day 7.

	Day 7		Day 10	
	Vehicle	rhFGF4	Vehicle	rhFGF4
BV/TV	15.3±6.7	23.9±3.8	15.0±5.6	30.6±7.8 ^a
OS/BS	16.5±3.1	29.3±3.5 ^a	13.4±1.8	17.5±1.6 ^b
MS/BS	18.6±3.0	31.7±2.5 ^a	12.6±0.7	23.6±7.2 ^a
ES/BS	16.6±4.0	23.5±1.4 ^a	10.4±1.8	16.2±1.1 ^a
Ob.S/BS	10.7±3.4	24.4±4.7 ^a	10.3±1.8	14.1±2.0 ^b
Oc.S/BS	10.6±2.9	22.3±2.8 ^a	11.2±2.4	14.1±3.3 ^b
Tb.Th	22.5±9.6	26.3±9.9	22.9±6.9	28.7±7.8
Tb.N	6.6±0.8	9.6±2.2	5.6±0.6	11.3±2.3 ^a

Table 2. Histomorphometry was analysed with following parameters. BV: bone volume; TV: tissue volume; BS: bone surface; OS: osteoid surface; MS: mineralized surface (single labeled by calcein); ES: eroded surface; Ob.S: osteoblast surface; Oc.S: osteoclast surface; Tb.Th: trabecular thickness; Tb.N: trabecular number. Data represents the mean of 4 samples from the both groups ± SD. ^a indicates values that are significantly different from control, *p* < 0.05. ^b indicates values that become significantly different in the same group, *p* < 0.05. (S. Kuroda et al., 2007)

BMP2 gene transfer at fracture site

Histological examinations indicated that the group treated with bmp2-CaP-collagen showed the most abundant osteogenesis (Fig. 10). The osteotomy site was connected with fibrous tissue only at 2 weeks. It was connected with callus and the gap was filled with newly formed cartilage and bone at 4 weeks after the operation. At 6 weeks, although there were still remnants of cartilage in the center, newly formed bone was remodeled to the cortical or cancellous bone and fused to the stump of the host bone, and the osteotomy sites became unclear. When treated with bmp2-collagen, the bone defect was bridged at 6 weeks but the bridged area was smaller and newly formed bone was less mature than that of the bmp2-CaP-collagen group. In the group treated with only collagen, a residue remained at 6 weeks, and although small callus formation from the host bone was observed, the defects were mainly filled with fibrous tissue.

Fig. 10. Histological images of the defects treated with different implants (M. Endo et al., 2006).

Longitudinal sections were prepared at 6 weeks after the operation. Sections were stained with toluidine blue (original magnification x 40). Mechanical strength to fracture at the osteotomy sites is presented in Fig. 11. The groups treated with bmp2 and collagen could be measured; however, the single implant of collagen did not induce the bone bridge. At 4 weeks, the bone treated with bmp2-CaP-collagen was stronger than that treated with bmp2-collagen. The mechanical strength of bone treated with bmp2-CaP-collagen was closely similar to that of the contralateral tibia at 6 weeks. In this group the fracture did not occur at the osteotomy site, but at the host bone.

Fig. 11. Mechanical strength of the tibiae treated with different implants (M. Endo et al., 2006).

Values are presented as mean ± standard deviation. The statistically significant difference is observed between G1 and G2 at 4 weeks ($p<0.05$). G1: bmp2-CaP-collagen; G2: bmp2-collagen; G3: collagen.

Both rat and human BMP2 gene expression were detected throughout the experimental period in Fig. 12: human BMP2 gene expression level did not alter so much with time, and, similarly, the expression level of rat BMP2 gene remained up to 8 weeks. The expression level of osteocalcin gene was elevated up to 8 weeks. On the other hand, VEGF genes were evenly expressed during the period. RANK and RANKL gene expression were enhanced initially, and the levels were maintained until 8 weeks.

Fig. 12. Gene expression at the defect site treated with bmp2-CaP-collagen (M. Endo et al., 2006). One to five μg of total RNA of each sample was reverse-transcribed to cDNA in 20 μl , 1 μl of which was used for PCR amplification in 50 μl . And then 8 μl out of 50 μl was run for a gel electrophoresis.

3. Discussion and conclusion

OVX

In OVX rats, both bone formation and resorption is accelerated; however, the unbalance, more bone resorption than bone formation, causes trabecular bone loss in long bones and vertebrates (Y. Otawara et al., 1983; T.J. Wronski et al., 1989a, 1989b; B.C. Toolan et al., 1992). In the present study, total femoral BMD of OVX rats decreased in DEXA analysis. This BMD decrease in OVX rats was due to the decrease in trabecular bone and cortical bone was not affected, which was revealed in pQCT analysis. These results are the same as previously reported by other investigators (P. Pastoureau et al., 1995; C.M. Bagi et al., 1996; S.A. Breen et al., 1996).

Firstly, BMD measurement of condylar region demonstrated BMD decrease in OVX rats, minus 14% from BMD of shame-operated one. Percent BMD decrease of this region was similar to the decrease of femoral BMD in OVX rats. Secondary, BMD of trabecular bone of molar region of mandible decreased in OVX rats, which was revealed in pQCT analysis. Notably, the extent of this BMD decrease (maximal 13% decrease) was less than the decrease of trabecular bone of the femur in OVX rats (30% decrease at the 17-mm-section). Thus, the

susceptibility of the bone of the molar region of the mandible to estrogen deficient condition was low compared to the bones of condylar region of the mandible and the femur.

In the present study, we found trabecular bone decrease of the molar region of the mandible in OVX-rats. Although the mechanism of the low susceptibility of the molar region of the mandible to estrogen deficient condition is not clear, it is likely that mechanical stress derived from functional occlusion is preventing the bone loss in this pathological condition. Elovic and his collaborators have clearly demonstrated that maxillary molar extraction together with ovariectomy causes more bone loss in the mandible than maxillary molar extraction alone (E. Klemetti et al., 1993a, 1993b, 1994; R.P. Elovic et al., 1994; E. Klemetti & P. Vainio, 1994; R.P. Elovic et al., 1995; L. Jahangiri et al., 1997), which supports this speculation.

GBR

Cell migration is likely influenced by the size of the inter-fibrous space in the molecular construction of the membranes (J. Behring et al., 2008). As such, cells were able to migrate inside the collagen membrane. The collagen was found to perform well as a new bone space maintainer excluding the adjacent soft tissue. Several cell-culture studies have explored the biocompatibility of GBR membranes by comparing their levels of inflammatory-related gene expression (A. Friedmann et al., 2008) and osteogenic markers (S.B. Idris et al.). However, only a few *in vivo* researches before this study had done so by developing and comparing gene expression profiles rather than performing radiological and histological analysis during osteogenesis in the bone defect (M. Nyan et al.). By allowing for observation of differences in cellular events between the experimental (with membrane) and control (no membrane) conditions, the development and comparison of gene expression profiles will permit molecular examination of the bone-healing process in the future study.

Systemic/local administration of rhFGF4

The most characteristic feature of the systemic effects of FGF4 is likely the stimulation of endosteal but not periosteal bone formation. This is elicited by proliferation of preosteoblastic cells in bone marrow, followed by recruitment of osteoblasts from preosteoblastic cells (T. Nakamura et al., 1995; S. Kuroda et al., 1999). Subsequently, the increase of cancellous bone becomes prominent (H. Mayahara et al., 1993; T. Nakamura et al., 1995). As suggested, FGF family members are of great importance for bone development and morphogenesis (B. Feldman et al., 1995; H. Ohuchi et al., 1995; R.A. Buckland et al., 1998). Further, the expression of FGF family members such as basic FGF is often upregulated during fracture repair and may contribute to the regeneration process (M.E. Bolander, 1992). Some reports have suggested that exogenous FGF family members accelerate bone fracture healing and wound healing when locally applied (H. Kawaguchi et al., 1994; T. Nakamura et al., 1998) and also recover bone mass that has been pathologically damaged because of ovariectomy or diabetes, for instance (H. Kawaguchi et al., 1994; C.R. Dunstan et al., 1999). Therefore, although FGF4 has not been detected in postnatal stages, it is assumed that exogenous FGF4 during tissue regeneration might have important aspects as well as basic FGF or other growth factors (R.K. Globus et al., 1989; M. Noda & J.J. Camilliere, 1989; M.E. Joyce et al., 1990; M.E. Bolander, 1992), and exogenous FGF4 as well as basic FGF may possibly enhance the local regeneration (M. Noda & R. Vogel, 1989).

The ratio of bone volume to tissue volume indicated that new bone formation seemed to have started before day 7 and this value became significantly higher on day 10 than that in the control group. The changes of these histomorphometric parameters might be attributed to an increase in the endosteum bone callous, which was supported by a prominent increase in the affected trabecular BMD but not the cortical BMD. Thus, rhFGF4 serves as an anabolic molecule in bone.

These studies were designed to analyze changes of bone mineral density or content with DEXA under osteoporosis, external growth factor intake and GBR in a skeletal defect, respectively. DEXA is widely used for obtaining an averaged mineral density of each part of a sample by directional scanning in as narrow a width as approximately 1 mm. These averages can be summed for the total bone area to observe the total mineral content and density. The densities are expressed in a unit of mg/cm² because the bone, originally three-dimensional, is recognized as a flat picture during the scanning process. While pQCT and micro computed tomography (μ CT) are provided for three-dimensional analyses of bone density and/or digital reconstructing of bone for several indices of morphometry, DEXA allows much easier and faster settings of samples and calculating of densities in regions of interest (ROI). Therefore, the key point and the characteristics of DEXA are giving an initial idea to get to know time-dependent changes of a sample or difference among samples. Overall, DEXA, pQCT and μ CT can provide convenient and prompt tools, which can perform accurate comparisons.

Gene transfer for BMP2

There are three phases in the bone regeneration process: 1) the early inflammatory phase; 2) the repair phase; and 3) the remodeling phase (V.I. Sikavitsas et al., 2001). In the early inflammatory phase, the hemorrhage and the subsequent hematoma are followed by infiltration of inflammatory cells and fibroblasts to the repair area. These events lead to vascularization and the formation of granulation tissue. The second phase is the repair phase, which is characterized by a callus. This phase begins with vascular ingrowth, osteoid secretion and the presence of collagenous fibers. A temporary callus consisting of cartilage is produced. In the remodeling phase, osteoblasts are active and the cartilage tissue is replaced with immature cancellous bone. Some cancellous bone is then converted to mature dense bone. Although histological examination was performed at the limited time points in the present study, it is likely that these sequential events occurred in the present bone defect. This repair process was clearly modified by the different combinations of implanted materials.

Constant expression of VEGF indicates importance of neovascularization during the healing process. Since RANK, receptor activator for NF κ B on the cellular membrane of osteoclast progenitors and osteoclasts, and its ligand RANKL, which is secreted from osteoblasts, are involved in osteoclast maturation and activation, maintained RANK expression suggests onward activation of osteoclasts leading to the bone remodeling including the callus. On the other hand, osteocalcin is a bone-specific matrix protein which is produced by mature osteoblasts. Thus, the increase of osteocalcin gene expression at 6 weeks implicates reaching the maturation of the defect site. Although rat BMP2 gene as well as human BMP2 gene diminished gradually, the gene expression remained detected over the period, suggesting enhanced osteoblast incorporation into the fractured site.

The mechanism of how the GAM system stimulates tissue regeneration is speculated as the following (J. Fang et al., 1996; J. Bonadio et al., 1999, 2000). CaP, in which plasmid vector is incorporated, has been used for *in vitro* gene transfer (C.F. Graham, 1973; A. Loyter et al., 1982a, 1982b; S. Kato et al., 1986; E. Orrantia & P.L. Chang, 1990; M. Werner et al., 1990; A.V. Zelenin et al., 1991; J.K. Burkholder et al., 1993; J.C. Sanford et al., 1993; T.A. Thompson et al., 1993). Since CaP stabilizes nucleic acids (A. Loyter et al., 1982a, 1982b), we speculated that CaP would be also useful for *in vivo* gene transfer (Y.W. Yang & J.C. Yang, 1997; S.Y. Watanabe et al., 1999; P. Batard et al., 2001). In the regeneration process, the cells surrounding GAM migrate into the matrix. Fundamentally, it is likely that the cells migrating into GAM are mainly fibroblastic cells and some of these cells can be regarded as targets for the plasmid. They engulf the plasmid vector, subsequently starting to produce an encoded protein. Thus, GAM acts as a bio-reactor for producing an encoded protein, which was human BMP2 in the present study. After transplanting our modified GAM, human BMP2 gene expression was observed at almost the same level throughout the experimental period. It is clear that human BMP2 produced by transfected cells enhanced bone regeneration in the present study. The duration of the gene expression in this gene transfer system depends on the site of the application and the size of the GAM, which presumably influences the period of the matrix degradation and the duration of gene expression. Although we did not observe cessation of human BMP2 gene expression, the expression of human BMP2 gene declined until time suggesting temporality of the expression of the transfect gene.

The results of the radiographic and histological analyses demonstrated that this critical size bone defect was bridged when it was treated with bmp2 and collagen. Notably, the regeneration of the defects treated with bmp2-CaP-collagen was more prominent than that of the defects treated with bmp2-collagen. These results were also confirmed in the biomechanical test.

Ideas to reduce and avoid the emergence of compromised bone status such as osteoporosis, fractures and critical skeletal defects, and to increase bone mineral density and bone volume must be a theme for minimizing the burden of fractures through interventions that help to achieve optimal peak bone mass, reduce excessive skeletal resorption, enhance bone formation.

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Osteoporosis is a public health issue worldwide. During the last few years, progress has been made concerning the knowledge of the pathophysiological mechanism of the disease. Sophisticated technologies have added important information in bone mineral density measurements and, additionally, geometrical and mechanical properties of bone. New bone indices have been developed from biochemical and hormonal measurements in order to investigate bone metabolism. Although it is clear that drugs are an essential element of the therapy, beyond medication there are other interventions in the management of the disease. Prevention of osteoporosis starts in young ages and continues during aging in order to prevent fractures associated with impaired quality of life, physical decline, mortality, and high cost for the health system. A number of different specialties are holding the scientific knowledge in osteoporosis. For this reason, we have collected papers from scientific departments all over the world for this book. The book includes up-to-date information about basics of bones, epidemiological data, diagnosis and assessment of osteoporosis, secondary osteoporosis, pediatric issues, prevention and treatment strategies, and research papers from osteoporotic fields.

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