表 4　膝 OA の候補遺伝子として報告されたもの

| 膝 OA の候補遺伝子として報告されたもの | 膝 OA および類縁疾患との関連 |
|---|---|
| COMP (cartilage oligomeric matrix protein) | 偽性軟骨無形成症 |
| COL11A1 (human type-XI procollagen gene) | Stickler 症候群 |
| COL2A1 (human type-II procollagen gene) | 軟骨形成不全，脊椎骨端異形成症など多数 |
| VDR (vitamin D receptor gene) | 骨粗鬆症，骨棘形成 |
| Aggrecan | 手指 OA |
| COL9A1 (human type-IX procollagen gene) | 股関節 OA |
| COL9A3 (human type-IX procollagen gene) | 股関節 OA |
| IGF1 (insulin-like growth factor 1) | 手指 OA，脊椎 OA |
| CRTL1 (cartilage matrix protein gene 1) | 手指 OA，股関節 OA |
| ER (estrogen receptor) | 骨粗鬆症 |
| PAPSS2 (3'-phosphoadenosine 5'-phosphosulfate synthase) | 脊椎骨端異形成症 |
| ASPN (Asporin) | 膝 OA |
| AnK | CPPD 沈着 |
| CALM1 (calmodulin1) | 股関節 OA |
| FRZB (serected frizzeled-related protein-3) | 股関節 OA (女性) |
| IL-1 | 股関節 OA |
| MATN3 (matrilin 3) | 手指 OA |
| IL-4L | 股関節 OA |
| ADAM12 (metalloprotease) | 股関節 OA |

CPPD：ピロリン酸カルシウム結晶

### 9. 下肢アライメント，スラスト運動

Sharma ら[33]は膝 OA 患者を調べ，膝内反・外反アライメントが内側・外側型膝 OA を有意に進行させると報告し，われわれも松代膝検診において膝内反アライメントが膝 OA 発症の危険因子であることを明らかにしている[26]．また，立脚歩行初期にみられる膝の急激な内反運動であるスラスト運動は膝 OA の有力な危険因子と考えられており，その関連性が松代膝検診や Chang ら[6]によって示されている．

### 10. 骨粗鬆症

膝 OA に関する過去の疫学調査では，変形性関節症と骨粗鬆症は逆の作用を持つという仮説に基づいて研究が行われた．その結果，Framingham study[12]や Chingford study[13]では高骨密度と膝 OA の関連性が示され，わが国でも須藤ら[37]が同様の結果を報告している．しかし，近年の研究では高骨密度は膝 OA 発症に影響するが膝 OA の進展には低骨密度が関連するという報告もあり，現時点では骨粗鬆症と膝 OA の関連性は明らかであるが，その作用機序については今後の研究が待たれている．

### 11. 性ホルモン

Framingham study[41]や Chingford study[36]では，エストロゲン補充療法 (ERT) は膝 OA に予防的に作用する結果が示されたが有意ではなかった．近年，ERT とアレンドロネートの併用が膝 OA の軟骨下骨変性に予防的に作用することが示されており[16]，今後疫学研究においても大規模な前向き調査が必要と考えられる．

### 12. 微量栄養素

Sowers ら[35]は，抗酸化物質としてのビタミン A，C，E およびベータカロチンは膝 OA の発症には影響しないものの進行および疼痛の軽減に有効であると述べており，McAlindon ら[23]は血中 25-hydroxy vitamin D の低下が膝 OA の進行を助長すると報告している．

### 13. 遺伝子(表4)

膝OAの遺伝形式は多因子遺伝であり，原因遺伝子よりも感受性遺伝子として研究される場合が多い．膝OAの遺伝性については，Kellgrenら[19]が全身性関節症(GOA)の報告以後，軟骨形成不全症やStickler症候群の原因遺伝子として同定されたCOMPやCOL2A1を足がかりにして多くの遺伝子多形が発見された．さらに，これらの遺伝子多形の相関解析が行われているが，現在まで明らかな膝OAの候補遺伝子として特定されたものはない[18,28]．本疾患の複雑な病態を考えると今後大規模な集団での解析が必要と考えられる．

## おわりに

膝OAはcommon diseaseであり，その発症と進行には多くの因子が関与している．これらのメカニズムおよびその自然経過を明らかにするためには，大規模集団に対する長期間の疫学的縦断研究は極めて重要である．

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# Impaired bone fracture healing in matrix metalloproteinase-13 deficient mice

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**Abstract**

Vascular and cellular invasion into the cartilage is a critical step in the fracture healing. Matrix metalloproteinase-13 (MMP-13) is a member of the zinc-dependent endopeptidase family and plays an important role in remodeling of extracellular matrix. Therefore we investigated the possible involvement of MMP-13 in a murine model of stabilized bone fracture healing. Repair of the fracture in MMP-13 deficient (MMP-13$^{-/-}$) mice was significantly delayed and characterized by a retarded cartilage resorption in the fracture callus. Immunohistochemistry indicated severe defects in vascular penetration and chondroclast recruitment to the fracture callus in MMP-13$^{-/-}$ mice. Consistent with the observations, the chondrocyte pellets cultured from the MMP13$^{-/-}$ mice exhibited diminished angiogenic activities when the pellets were co-cultured with endothelial cells. These results suggest that MMP-13 is crucial to the process of angiogenesis during healing of fracture, especially in the cartilage resorption process.


*Keywords:* MMP-13; Fracture healing; Extracellular matrix; Angiogenesis; Chondroclast

Bone fracture triggers a steady cascade of bone regeneration. Under optimal conditions, fractured bone heals without scar formation and fully recovers its morphological and biomechanical properties. This reparative process consists of a variety of molecular and cellular events, which recapitulate several aspects of skeletal development [1]. Although various growth factors and cytokines that participate in fracture healing have been identified [2], the precise mechanism behind these processes has not been fully elucidated.

Matrix metalloproteinases (MMPs) constitute a family of zinc-dependent proteinases which have essential roles in degradation of extracellular matrix (ECM) components such as collagens and proteoglycans. They are involved in normal development and tissue dysfunction under various pathophysiological conditions including wound healing, arthritis, and tumor development [3]. Among the members of secreted-type MMPs, MMP-9, and MMP-13 are thought to be important to normal skeletal development in mice [4–6]. MMP-13 is primarily expressed in osteoblasts and hypertrophic chondrocytes, while MMP-9 is mainly expressed in osteoclasts [7]. In contrast to the difference in their expression patterns, MMP-9 deficient (MMP-9$^{-/-}$) mice and MMP-13 deficient (MMP-13$^{-/-}$) mice exhibited similar skeletal phenotypes characterized by the elongation of hypertrophic cartilage zone in the growth plates [4–6]. The skeletal defects in MMP-9$^{-/-}$ mice are explained by

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the impaired angiogenesis as vascular invasion of calcified cartilage is a crucial step for endochondral bone formation [8]. Because of the critical involvement of MMP-13 in endochondral bone development, we conducted the following experiments to analyze the role of MMP-13 in fracture healing.

In the current study, we generated a stabilized bone fracture at the middle of the tibia in MMP-13$^{-/-}$ mice and the healing process was then compared with those of WT mice. Cartilage formation and the angiogenic activity of chondrocytes derived from MMP-13$^{-/-}$ and WT mice were also analyzed using an *in vitro* chondrocyte culture system. The lack of MMP-13 leads to a severe delay in fracture healing, which is characterized by prolonged absorption of the fracture callus. In addition, chondrocytes derived from MMP-13$^{-/-}$ mice exhibit diminished angiogenic activity. These observations deepen our understanding of fracture healing and further underscore the importance of MMP-13 during this pathological condition.

## Materials and methods

*Animals.* MMP-13$^{-/-}$ and WT male littermates in a C57BL/6J and 129/Sv hybrid background were generated from the intercross between heterozygous MMP-13$^{+/-}$ mice. The generation of MMP-13$^{-/-}$ mice is described elsewhere [9]. All experiments were performed according to the protocol approved by the Laboratory Animal Care and Use Committee of Keio University School of Medicine.

*Fracture model.* Bone fractures were generated essentially as previously described [10]. Thirty 8-week-old mice were used in each group. Briefly, under general anesthesia with xylazine (0.2 mg /10 g body weight, Bayer) and ketamine (0.5 mg/10 g body weight, Sankyo), an anterior knee incision was made, and a transverse osteotomy was performed at the middle of the tibia with a bone saw (Volvere GX, NSK Nakanishi). Fractured bones were repositioned and stabilized by inserting the inner pin of a 23-gauge spinal needle intramedullary. The mice were euthanized by cervical dislocation at designated time points and their tibiae were excised.

*Radiological analysis.* Bone radiographs were taken with a soft X-ray instrument (CMB-2, SOFTEX). Microarchitecture of the fracture callus was evaluated by using a micro-CT system (Scan Xmate-A100S40, Comscantecno). Calcified area and bone mineral content (BMC) of the entire tibiae were measured by a single energy X-ray absorptiometry utilizing a bone mineral analyzer for small animals (PIXImus, LUNAR), and gain of the calcified area and % gain of the BMC were calculated.

*Histological analysis.* The harvested tibiae were fixed in 4% paraformaldehyde, decalcified in 0.5 M EDTA (pH 7.4), embedded in paraffin, and then cut into 4-μm sections. Alcian blue and van Gieson stainings were performed according to the standard procedure. Ratio of cartilage area and bone area to total callus area was measured by a planimetric method using NIH Image.

Histochemical detection of tartrate resistant acid phosphatase (TRAP) and immunohistochemistry of type II collagen, type X collagen, MMP-9, CD31, and cathepsin K were performed as previously described [11,12].

*Cell cultures.* Rib chondrocytes were isolated from neonatal MMP-13$^{-/-}$ and WT littermates, and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and antibiotics. Human umbilical vein endothelial cells (HUVEC, Kurabo) were cultured in HuMedia-EG2 medium (Kurabo). All the cells were maintained at 37 °C in a humidified $CO_2$ incubator. Any chemicals if not mentioned otherwise were purchased from Sigma.

*Cell differentiation assay.* To induce chondrogenic differentiation, chondrocyte 3D culture was performed as previously described [13]. Briefly, chondrocytes were suspended in atelopeptide collagen solution (0.5% atelopeptide collagen (Kawaken Fine Chemicals)/5 mM NaOH/26 mM $NaHCO_3$/20 mM Hepes/one volume of 10 times concentrated α-minimal essential medium (αMEM)) at a density of $1 \times 10^7$ cells/ml. Each 20 μl of the mixture was placed into the bottom of 15 ml conical tubes (Falcon) and incubated for 1 h at 37 °C to form a gel. DMEM with 200 ng/ml recombinant human bone morphogenetic protein-2 (kindly provided by Astellas Pharma Inc.), 5 μg/ml insulin and antibiotics (chondrogenic medium) was gently poured onto the gel at a volume of 1 ml. The paraffin sections of the pellets were made after fixation with 4% paraformaldehyde and stained with Alcian blue.

*Angiogenesis assay.* For the *in vitro* angiogenesis assay, the chondrocyte pellets that had been cultured in chondrogenic medium for 3 weeks were co-cultured with HUVEC. HUVEC were suspended in a collagen gel solution (Cellmatrix type IA (Nitta Gelatin)/5 mM NaOH/26 mM $NaHCO_3$/20 mM Hepes/one volume of 10 times concentrated αMEM) at a density of $1 \times 10^6$ cells/ml, and 400 μl of the mixture was poured into each well of 24-well plates. The chondrocyte pellets were then dropped at the center of each well and incubated for 30 min at 37 °C to form a gel. These cells were cultured together in HuMedia-EG2 medium and the angiogenic reactions were monitored for 2 weeks.

*Statistical analysis.* Means of groups were compared by analysis of variance, and significance of differences was determined by post hoc testing using Bonferroni's method.

## Results

### *MMP-13$^{-/-}$ mice exhibit delayed fracture healing*

To investigate the effect of MMP-13 on fracture healing, we generated a stabilized tibial fracture model in mice and assessed the healing process by radiological evaluation (Fig. 1A). In plain radiographs of WT mice, the calcified callus appeared at post-fracture week (PFW) 1, progressed to form a bony bridge by PFW3, and then gradually decreased in size by PFW10. In contrast, in the MMP-13$^{-/-}$ mice, a radiolucent zone was apparent in the fracture callus even at PFW3, suggesting that with the loss of MMP-13 the bony bridging was delayed. In axial CT images, the WT mouse callus contained a small noncalcified area at PFW2, which was subsequently replaced by calcification at PFW3. However, in MMP-13$^{-/-}$ mice, the callus consisted mainly of noncalcified tissue at PFW2, and although calcification progressed, there remained an area of noncalcified tissue at PFW3.

To quantify the extent of callus formation, the calcified area and BMC in the fractured and control tibiae were measured by a bone densitometer. As shown in Fig. 1B, both parameters increased for 3 weeks during the modeling phase and then decreased during the remodeling phase in WT mice. In contrast, MMP-13$^{-/-}$ mice showed a significant reduction in these parameters at PFW2-3 during the modeling phase. There were no differences in the calcified area and BMC between the two genotypes during the remodeling phase (PFW4-10). These results indicate that MMP-13 deficiency causes a delay in fracture healing through impaired bone modeling due to retarded calcification of the fracture callus.

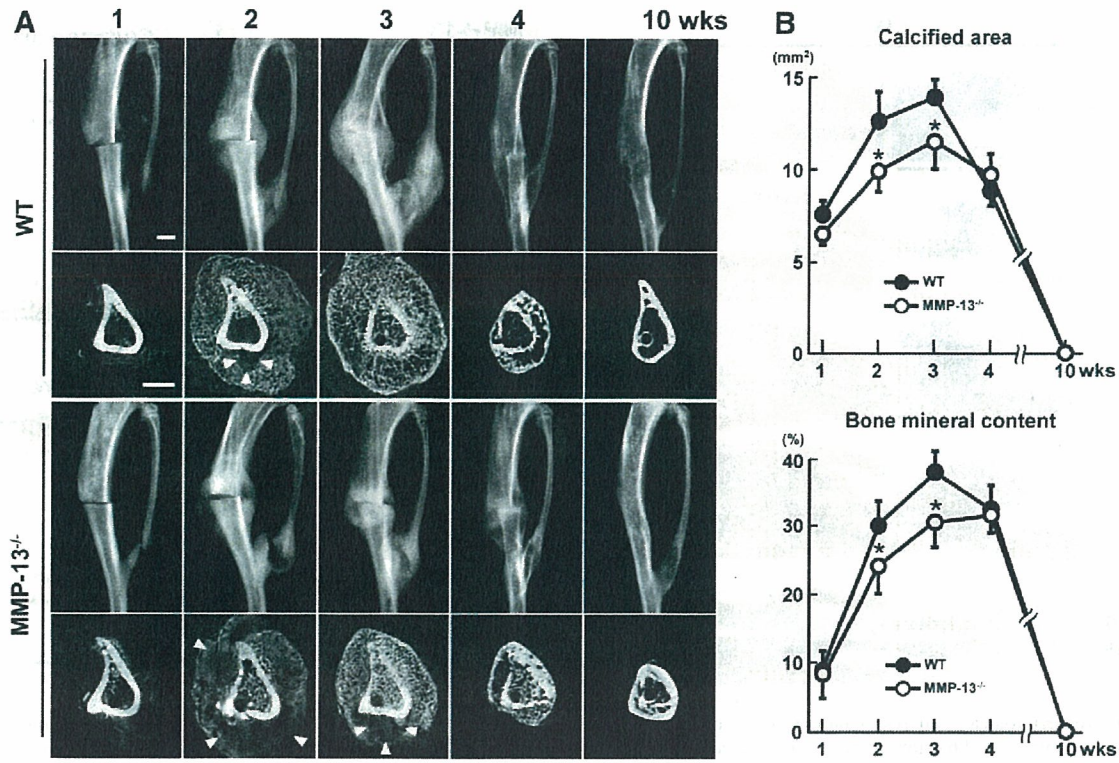


Fig. 1. Radiological analyses of bone fracture healing in WT and MMP-13$^{-/-}$ mice. (A) Plain radiographs (upper row) and CT images (lower row) of the representative fractured tibiae in WT and MMP-13$^{-/-}$ mice at PFWs 1–10. The arrowheads indicate non-calcified areas. Scale bar, 1 mm. (B) Time course changes of the calcified area and bone mineral content of the callus at the fracture site measured by single energy X-ray absorptiometry. Data are expressed as the means (symbols) ± SD (error bars) of 6 mice per genotype. $^{*}p < 0.05$ versus WT.

*MMP-13 deficiency impairs the replacement of cartilage with bone in the fracture callus*

Remodeling of bone requires the resorption of cartilage, which provides initial stabilization to the fractured bone [2]. To evaluate the turnover of cartilage to bone in the fracture callus, the ratio of cartilage area and bone area to total callus area (CA/TA and BA/TA, respectively) were measured utilizing a planimetric method in histological sections (Fig. 2). The cartilage area was stained blue with Alcian blue staining, and the bone area was stained red with van Gieson. In WT mice, CA/TA reached its peak value at PFW1 and then decreased by PFW4, whereas BA/CA increased to reach a plateau by PFW3. Compared to WT mice, MMP-13$^{-/-}$ mice exhibited higher CA/TA values and lower BA/TA values at PFW2-3, indicating that loss of MMP-13 interferes with cartilage-to-bone replacement.

*MMP-13 deficiency impairs vascular and chondroclast invasion of cartilage*

To further investigate the mechanisms underlying the impaired bone healing in MMP-13$^{-/-}$ mice, we performed immunohistochemical analysis of the fracture callus at PFW2 (Fig. 3). Type X collagen, a marker for hypertrophic chondrocytes, was more prevalent in the MMP-13$^{-/-}$ cartilage than in the WT cartilage, while type II collagen was equivalent between the genotypes (Fig. 3A–D). MMP-9 was expressed in hypertrophic chondrocytes and chondroclasts, and appeared to be up-regulated in the MMP-13$^{-/-}$ mice (Fig. 3E and F). In WT mice, the CD31-immunostained capillaries penetrated into the cartilage extending their cytoplasmic processes (Fig. 3G), and cathepsin K/TRAP-positive chondroclasts were directly attached to the cartilage matrix (Fig. 3I and K). In contrast, MMP-13$^{-/-}$ mice showed minimal cartilaginous breakage associated with capillary invasion (Fig. 3H), and the chondroclasts were not attached to the cartilage matrix (Fig. 3J and L). These findings suggest that the impaired cartilage resorption in the MMP-13$^{-/-}$ mouse callus is associated with the inability of capillaries and chondroclasts to invade the cartilage.

*MMP-13 is required for the angiogenic activation of cartilage*

In order to clarify the cellular mechanism underlying these abnormalities, we examined *in vitro* the differentiation of rib chondrocytes isolated from WT and MMP-13$^{-/-}$ littermates (Fig. 4). Calvarial osteoblasts cultured from MMP-13$^{-/-}$ mice exhibited no difference in proliferation and differentiation compared with those from WT mice,

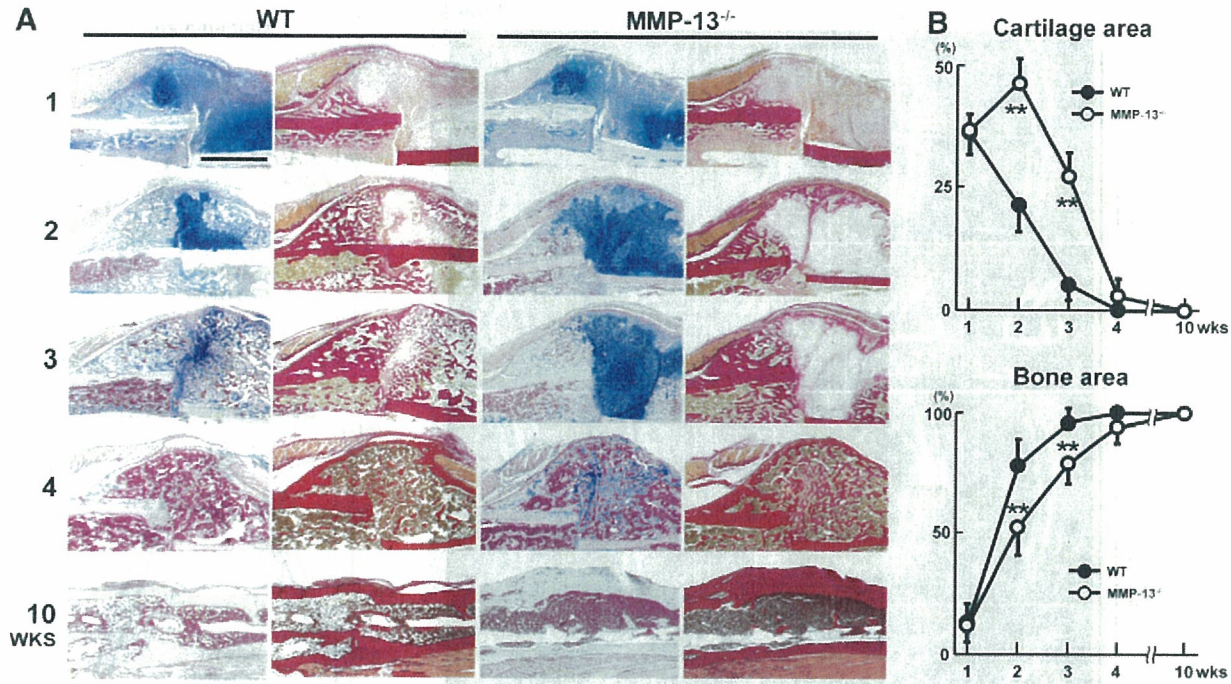


Fig. 2. Histomorphological analyses of bone fracture healing in WT and MMP-13$^{-/-}$ mice. (A) Alcian blue staining (right column) and van Gieson staining (left column) of the fractured tibia sections from WT and MMP-13$^{-/-}$ mice. Scale bar, 1 mm. (B) Ratios of cartilage area and bone area to total callus area of the fractured site, which were measured by a planimetric method. Data are expressed as the means (symbols) ± SD (error bars) of 6 mice per genotype. $^{**}p < 0.01$ versus WT.

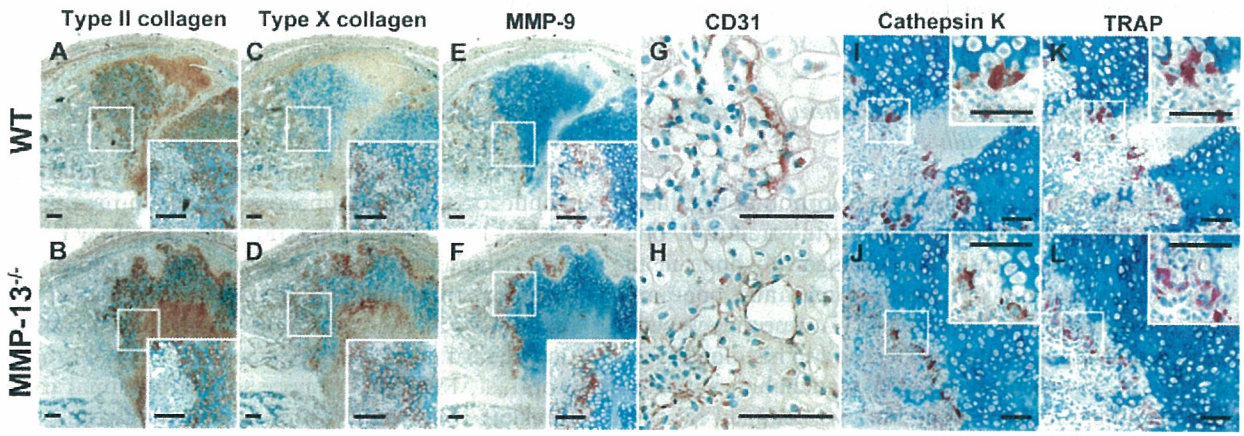


Fig. 3. Immunohistochemical and histochemical analyses of bone fracture healing in WT and MMP-13$^{-/-}$ mice. (A–J) Immunohistochemistry of type II collagen (A,B), type X collagen (C,D), MMP-9 (E,F), CD31 (G,H) and cathepsin K (I,J) in the fracture callus of WT and MMP-13$^{-/-}$ mice at PFW2. (K,L) Histochemistry for TRAP in the fracture callus of WT and MMP-13$^{-/-}$ mice at PFW2. All insets are higher magnification images of each figure. Note that the expression of type X collagen and MMP-9 in hypertrophic chondrocytes and/or chondroclasts appear to be up-regulated in MMP-13$^{-/-}$ mice compared to WT mice (C–F). Also note that and cathepsin K/TRAP-positive chondroclasts are unattached to cartilage matrix in MMP-13$^{-/-}$ mice (J,L). Scale bar, 80 μm.

and the cell proliferation rate of MMP-13$^{-/-}$ chondrocytes was also normal (data not shown).

To evaluate the cartilage formation, chondrocytes were cultured in 3D collagen gel pellets. During the first 2 days the WT pellets shrunk and the cartilage matrix formation by the chondrocytes was observed at the pellet periphery on day 7. By 21 days cartilage maturation proceeded toward the center forming a mature cartilage pellet. In the MMP-13$^{-/-}$ pellets, the gel contraction rate on day 2 and matrix synthesis on day 7 were reduced compared to WT pellets, although the matured pellets exhibited no histological differences between genotypes on day 21.

To further investigate the angiogenic property of the cartilage pellets, we developed a co-culture system of the chondrocyte pellets with HUVEC. After 1 week of co-culture, extensive vascular sprouting was observed around the

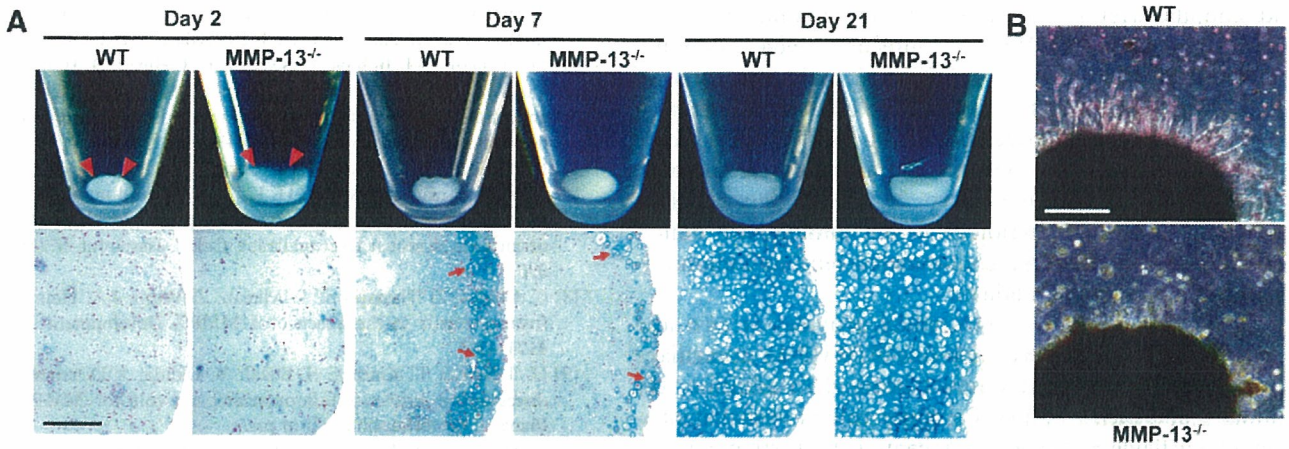


Fig. 4. Cultured chondrocytes from WT and MMP-13$^{-/-}$ mice. (A) Gross appearance (upper row) and Alcian blue stainings (lower row) of chondrocyte pellets on days 2, 7, and 21. Chondrocytes from WT and MMP-13$^{-/-}$ mice were cultured in 3D collagen gel pellets. Note that MMP-13$^{-/-}$ pellet is reduced in gel contraction rate on day 2 (arrowheads) and Alcian blue-positive matrix synthesis on day 7 (arrows). Scale bar, 500 μm. (B) *In vitro* angiogenesis assay. Chondrocyte pellets were co-cultured with HUVEC in collagen gel. Note that WT pellets are surrounded by numerous vascular sproutings, which are barely seen with the MMP-13$^{-/-}$ pellets. Scale bar, 250 μm.

WT pellets, although the MMP-13$^{-/-}$ pellets exhibited limited sprouting (Fig. 4B). These results suggest that MMP-13 is involved in the angiogenic activation of chondrocytes via matrix remodeling.

## Discussion

MMP-13 is the major proteinase that cleaves interstitial fibrillar collagens (type I, II, and III collagens) [14]. Deficiency of MMP-13 causes a transient elongation of the hypertrophic zone in the growth plate during the early stages of growth and development, indicating a role for this enzyme in endochondral bone development [5,6]. Since the bone fracture healing process is thought to recapitulate skeletal development [1], and since MMP-13 is highly expressed in the fracture callus [7], we hypothesized that MMP-13 plays an important role in fracture healing. Utilizing a mouse fracture model and a chondrocyte pellet culture, the present studies demonstrate that the lack of MMP-13 leads to a significant delay in the fracture healing process, and that MMP-13 plays a critical role in the maturation of chondrocytes and the induction of angiogenesis.

During the bone fracture healing process, fracture callus consisting of cartilage tissue is transiently formed and subsequently resorbed and replaced with osseous tissue; hence the cartilage resorption process is an important step for fracture healing. In general, the vascular invasion is a critical rate-limiting determinant for bone formation [15], and at the chondroosseous junction there are three key players in this process: hypertrophic chondrocytes, capillaries, and chondroclasts [16]. Among them, hypertrophic chondrocytes are the major cell type producing MMP-13 in the fracture callus and, in MMP-13$^{-/-}$ mice, the cartilage resorption was delayed due to the impairment of the invasion of capillaries and chondroclasts into the cartilage. These data re-emphasize the role of vascular and chondroclast invasion during the fracture callus resorption and indicate that MMP-13 production by chondrocytes is a prerequisite for this process.

Since primary chondrocytes from the rib cartilage were not capable of effectively producing ECM in a monolayer culture, we utilized a 3D pellet culture model to mimic the *in vivo* environment. Cells/collagen gel composites are known to undergo shrinkage during culture [17], and early gel contraction and subsequent matrix synthesis in the MMP-13$^{-/-}$ chondrocyte pellets were both suppressed compared to those observed in WT controls, suggesting that cell–ECM and/or cell–cell interactions caused by gel contraction are important for chondrocyte development and that MMP-13 is involved in these processes. These findings are similar to those seen in mice deficient in α1 integrin, a key molecule for cell–ECM interaction, which exhibited reduced cartilage ECM synthesis during fracture healing [18]. Moreover, interactions of chondrocytes with their ECM are required for the expression of angiogenic activities of chondrocytes [19]. When chondrocyte pellets and endothelial cells from the MMP-13$^{-/-}$ mice were co-cultured, tubular formation was considerably reduced in the pellets from MMP-13$^{-/-}$ mice, indicating that MMP-13 is critical for the angiogenic activities of chondrocytes. These data strongly support the notion that MMP-13 contributes to the fracture healing process by regulating both chondrocyte development and vascular induction.

MMP-9 deficiency has been shown to cause delayed fracture healing [8] in a manner similar to that seen in the MMP-13$^{-/-}$ mice. MMP-9$^{-/-}$ mice exhibit hindered cartilage resorption mainly due to suppressed angiogenesis, which can be rescued by local injection of recombinant vascular endothelial growth factor. Similar to the MMP-13$^{-/-}$ mice, elongation of the hypertrophic cartilage zone is also observed in the MMP-9$^{-/-}$ mice [4–6], although the expression pattern of these enzymes are different; in bone tissue MMP-9 is expressed mainly in osteoclasts and chondroclasts while MMP-13 is expressed mainly in osteoblasts

and chondrocytes [7]. In line with the findings of the growth plates of MMP-13$^{-/-}$ mice in which MMP-9 mRNA expression is up-regulated [5], the intensity of MMP-9 expression in the fracture callus was higher in MMP-13$^{-/-}$ mice compared to that in WT mice. These findings indicate a possible compensatory/redundant mechanism between these two MMPs during cartilage resorption in both physiological and pathological conditions, where chondrocytes, endothelial cells, and chondroclasts are intricately regulated.

In conclusion, the current study provides *in vivo* and *in vitro* evidence for the critical role of MMP-13 in cartilage resorption during bone fracture healing. In addition, the finding that MMP-13 plays a major role in vascular invasion of cartilage provides an incentive to further study the mechanism underlying the pro-angiogenic function of MMP-13. Moreover, it will be interesting to examine the compensatory role of MMPs, such as MMP-9 and MMP-13, in fracture healing. Taken together, this study provides new insight into the role of MMP-13 in fracture healing, which in turn has implications for bone regenerative medicine.

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ORIGINAL ARTICLE

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# Clinical effect of bisphosphonate and vitamin D on osteoporosis: reappraisal of a multicenter double-blind clinical trial comparing etidronate and alfacalcidol

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**Abstract** As inhibitors of bone resorption, bisphosphonates and vitamin D derivatives have been extensively used for the treatment of osteoporosis in various parts of the world, but the clinical effects of these two groups of agents have rarely been compared in detail. A multicenter, prospective, double-blind controlled study was started comparing the effects of etidronate and alfacalcidol (1-alpha-hydroxycholecalciferol) in 414 patients with established osteoporosis from 36 centers. Among these patients, 135 were given 400 mg etidronate daily at bedtime for 2 weeks followed by 10 weeks off treatment, and this cycle was repeated four times along with a placebo indistinguishable from the alfacalcidol capsule daily throughout the 48 weeks of study (Group A, High Dose Etidronate Group). In 133 patients, 200 mg etidronate was used instead of 400 mg (Group B, Low Dose Etidronate Group). In 138 patients, 1 μg alfacalcidol was given daily throughout the 48-week study period along with a placebo indistinguishable from the etidronate tablet in four separate periods of 2 weeks (Group C, Control Group). Dual-energy X-ray absorptiometry of the lumbar spine (L2–L4) was performed before the beginning of the study and every 12 weeks thereafter. Changes in spinal deformity were also assessed based on the lateral thoracic and lumbar spine X-ray films taken before and after the study. The lumbar spine bone mineral density (BMD) changes were +3.4% ± 0.6% (mean ± SEM) in Group A, +2.4% ± 0.5% in Group B, and −0.5% ± 0.4% in Group C, the former two being significantly higher than the last. New occurrence of spinal compression fracture was also significantly reduced in Group A compared to Group C. In patients without previous fracture at entry, incident fracture was 10.2% in Group C, but 0% in Groups A and B. In patients with prevalent fracture at entry, corresponding figures were 21.5% (Group C), 12.0% (Group A), and 13.2% (Group B), respectively. Alfacalcidol maintained lumbar spine BMD, preventing a decrease for 48 weeks, and etidronate significantly increased it further, demonstrating its usefulness in the treatment of established osteoporosis.

**Key words** etidronate · alfacalcidol · osteoporosis · DXA (dual energy X-ray absorptiometry) · fracture

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## Introduction

Etidronate is the first bisphosphonate developed for the treatment of osteoporosis, based on its potent inhibitory effect on osteoclastic resorption [1–3]. Although several studies including prospective, placebo-controlled double

blind trials indicated positive effects of etidronate in preventing bone loss in osteoporosis [4–10], its effect has rarely been compared with those of other drugs. In addition to the well-known estrogen, calcitonin and vitamin D derivatives, especially alfacalcidol (1-alpha-hydroxycholecalciferol) have been extensively used for the treatment of osteoporosis in Japan [11,12], possibly because of the profound calcium deficiency caused by Japanese dietary habits. Vitamin D and its derivatives decrease vertebral fracture, and may also decrease nonvertebral fractures, according to a meta-analysis [13]. To evaluate the clinical effect of etidronate, alfacalcidol was therefore employed as the active control drug in a multicentered, prospective, double-blind program involving 414 patients with established osteoporosis at 36 centers in Japan.

## Patients and methods

### Test subjects

This study was started on 414 patients with established primary osteoporosis with scores higher than 4 according to the scoring method for the diagnosis of involutional osteoporosis established by the Osteoporosis Research Group sponsored by the Health and Welfare Ministry (Chairperson: Dr. Hajime Orimo) (Table 1). Patients with primary or secondary hyperparathyroidism, and secondary osteoporosis caused by renal failure, vitamin D deficiency, rheumatoid arthritis, bone metastases of malignant tumors, multiple myeloma, trauma or corticosteroid use, and osteomalacia, were excluded from the study. This study was conducted from July 1990 to June 1992.

### Study design

Over a period of 8 weeks before the beginning of the study (washout period), specific treatments for osteoporosis including estrogens, calcitonins, vitamin D derivatives, and ipriflavone were withheld. Because no bisphosphonate had been therapeutically administered before this study and subsequent government authorization, no subjects had taken any bisphosphosphonate before their participation. A prospective, randomized, double-blind-controlled comparative study among the three groups was constructed as follows. Informed oral consent was obtained from each proposed test subject as to voluntary participation to this study.

The 414 test subjects were given either 400 mg etidronate (Group A), 200 mg etidronate (Group B), or 1 μg alfacalcidol (Group C) according to the preset randomized order. The key of the randomized samples was kept by the controller until the finalization of the data, when it was opened and the results were analyzed. To assure the blindness, a placebo with indistinguishable appearance was provided whenever the true drug was not given. The 48-week test period was divided into four equal parts consisting of 12 weeks each. Each morning, Groups A and B were given one alfacalcidol placebo. At bedtime, only during the first 2 weeks of each 12-week period, Group A was given two 200 mg etidronate tablets, Group B one etidronate placebo tablet and one etidronate 200 mg tablet, and Group C two etidronate placebo tablets. No calcium supplements were given in any of the three groups.

**Table 1.** Scoring method for the diagnosis of involutional osteoporosis

| | |
|---|---|
| 1. Decrease of bone mass: score 3 | |
| Bone mineral density (AP spine by DXA) less than 2 SD of young adult mean and/or X-ray evidence of vertical trabecular loss | |
| 2. Fracture | |
| One vertebra | 1 |
| More than two vertebrae | 2 |
| Proximal femur | 3 |
| Radius | 1 |
| 3. Premenopausal female | −1 |
| 4. Backache | 1 |
| 5. Serum Ca, P, and alkaline phosphatase | |
| Normal | 1 |
| One abnormal value | 0 |
| More than two abnormal values | −1 |

Each item is given scores specified, and evaluation is based on the total of the scores. In case the total is above 5, diagnosis of osteoporosis is definite. Total score 4 indicates that osteoporosis is likely. Total score 3 indicates that osteoporosis is suspected. In case the total is 2 on less, osteoporosis is unlikely

AP, antiroposterior; DXA, dual-energy X-ray absorptionetry

### Measurement of efficacy

Each test subject was seen by the physician in charge of the project at each center every 2 weeks and asked for symptoms, especially backache, as well as those possibly related to side effects. X-ray pictures of the lumbar and thoracic spine were taken at anteroposterior and lateral projections at the beginning and end of the study. Vertebral fracture was defined by a decrease of the anterior height (wedge deformity) or middle height (biconcave deformity) to less than 80% of the intact posterior height. In case the posterior height was also decreased (flat deformity or crush fracture), the decrease of the anterior, middle, or posterior height to less than 80% of the posterior height of the adjacent intact vertebra was used as the criterion for the deformity. Incident fractures were analyzed by the logistic method in the whole series, and cases with and without fracture at the start of the test, to calculate the odds ratio.

For bone measurements, dual energy X-ray absorptiometry (DXA) of L2–L4 was performed at the beginning of the study and every 12 weeks thereafter using a QDR-1000 (Hologic), XR-26 (Norland), or DPX (Lunar). The results were expressed as percent (%) of the initial value, and the same method of measurement with the same apparatus was used for each patient throughout the study period. Subjects with compressive or osteophytic deformities, interfering with accurate bone mineral density (BMD) measurement in two adjoining vertebrae of L2–L4 were excluded from the series.

Biochemical measurements were carried out as follows. Vitamin D metabolites (25-hydroxyvitamin D and 1,25-

dihydroxyvitamin D) were measured at the beginning of the study [14,15]. Serum midportion-parathyroid hormone (PTH) was measured by using a double-antibody immunoradiometric method [16] and osteocalcin by an immunoradiometric assay at the beginning of the study and every 12 weeks thereafter, along with routine blood count, biochemical tests including serum Ca, P, and alkaline phosphatase, and urinalysis.

Statistical analysis was conducted by Student's $t$ test, Tukey test, and Wilcoxson signed rank test. Lumbar BMD data were subjected to intention-to-treat (ITT) analysis without exclusion cases, filling defective sites with change-free values. The occurrence of new fractures was analyzed by odds ratio estimates in a logistic procedure.

## Results

Of the 414 test subjects who entered the study, data on 406 subjects, 135 from Group A, 133 from Group B, and 138 from Group C were analyzed after exclusion of 8 subjects: 1 for not conforming to the admission criteria of the study because of an associated disease, 2 for being subjected to other forms of therapy for osteoporosis interfering with the evaluation of the results, 4 for failing to show up after the initial visit and 1 for failing to withhold the treatment during the 8 weeks of the washout period. Administration of the test drug had to be discontinued before the 36th week, precluding the planned final measurement at the 48th week in 26, 23, and 26 subjects in groups A, B, and C, respectively. The reasons for the discontinuation and dropout are summarized in Table 2. DXA measurement proved to be unacceptable because of scoliosis, spondylosis deformans, ligamentous calcification, or localized hyperosteosis in 23, 26, and 23 subjects in Groups A, B, and C, respectively. In total, 49 subjects were excluded from each group, making the total number of subjects with analyzable DXA data 83 in Group A, 85 in Group B, and 88 in Group C, a total of 256 subjects. After exclusion of subjects in whom differentiation of the osteoporotic and nonosteoporotic causes of pain was difficult, data from 340 subjects were analyzed for pain assessment.

The prestudy background of the test subjects in these three groups are shown in Tables 3, 4, and 5. Each group consisted mainly of females with a small number of males. Mean age, time after menopause, body weight, number of spinal fractures, and spinal BMD were indistinguishable among the three groups, confirming the homogeneity of the patients. No evidence of vitamin D deficiency or marked secondary hyperparathyroidism was found in any of these patients. The criteria for the diagnosis of osteoporosis adopted in the present study were intended to exclude non-osteoporotic decrease of BMD such as osteomalacia and primary hyperparathyroidism. Compared to the current criteria by the World Health Organization (WHO) or the Japanese Society of Bone and Mineral Research, it may tend to overemphasize the frequency of confounding non-osteoporotic conditions. In view of the mean lumbar BMD of 0.714 ± 0.124 (mean ± SD), median of 0.712, and 25%–75% range of 0.621–0.801 g/cm$^2$ in Group A, 0.717 ± 0.131, 0.703, and 0.626–0.790, respectively, in Group B, and 0.716 ± 0.133, 0.731, and 0.608–0.803, respectively, in Group C as the QDR-1000 equivalent, most of the subjects appear to conform the current criteria for osteoporosis, i.e., less than –2.5 SD (WHO) or –30% from the young adult mean (0.708 g/cm$^2$). BMD values were converted to QDR-100 equivalent by calculation using the equations shown in Sone et al. [17].

As shown in Fig. 1, spinal BMD significantly increased from the baseline level after 12 weeks in Group A and after 24 weeks in Group B, and was maintained at approximately the same level in Group C. The mean percent change of spinal BMD at the end of the trial was +3.4% in Group A, +2.4% in Group B, and –0.5% in Group C. The values for Groups A and B were both significantly higher that for

**Table 2.** Summary of the reasons for discontinuation or dropout

| Reasons | A | B | C | Total |
|---|---|---|---|---|
| Discontinuation | | | | |
| Complications or their aggravation | 1 (1) | 2 (2) | 2 (1) | 5 (1) |
| Drug-related adverse events | 4 (3) | 1 (1) | 2 (1) | 7 (2) |
| Refusal by patient | 3 (2) | 0 (0) | 0 (0) | 3 (1) |
| Irregular timing for consultation | 0 (0) | 1 (1) | 0 (0) | 1 (0) |
| Irregular timing for drug ingestion | 1 (1) | 0 (0) | 0 (0) | 1 (0) |
| Others | 0 (0) | 4 (3) | 2 (1) | 6 (1) |
| Dropout | | | | |
| Improvement of subjective symptoms | 4 (3) | 7 (5) | 3 (2) | 14 (3) |
| Absence of effect | 1 (1) | 0 (0) | 0 (0) | 1 (0) |
| Complication and their aggravation | 0 (0) | 1 (1) | 1 (1) | 2 (0) |
| Drug-related adverse events | 0 (0) | 0 (0) | 1 (1) | 1 (0) |
| Burdens of housework or business | 3 (2) | 3 (2) | 3 (2) | 9 (2) |
| Poor cooperation by patients | 4 (3) | 7 (5) | 9 (7) | 20 (5) |
| Change of physician or clinic | 1 (1) | 2 (2) | 0 (0) | 3 (1) |
| Others | 1 (1) | 3 (2) | 4 (3) | 8 (2) |
| Totals | 23 (17) | 31 (23) | 27 (20) | 81 (20) |

Numbers of subjects are followed by numbers in parentheses indicating percentage of the total number of the subjects in the group

**Table 3.** General prestudy background data in 406 subjects

| Group | A | B | C | Total | Results of statistical analysis |
|---|---|---|---|---|---|
| Total number of patients | 135 | 133 | 138 | 406 | |
| Sex | | | | | |
| Males | 10 (7) | 7 (5) | 6 (4) | 23 (6) | NS |
| Females | 125 (93) | 126 (95) | 132 (96) | 383 (94) | |
| Age (years) | | | | | |
| –59 | 30 (22) | 28 (21) | 26 (19) | 84 (21) | NS |
| 60–69 | 58 (43) | 45 (34) | 67 (49) | 170 (42) | |
| 70–79 | 35 (26) | 48 (36) | 39 (28) | 122 (30) | |
| 80– | 12 (9) | 12 (9) | 6 (4) | 30 (7) | |
| Mean ± SD | 67 ± 9 | 68 ± 9 | 66 ± 8 | | |
| Time after menopause (years) | | | | | |
| –9 | 22 (18) | 22 (17) | 21 (16) | 65 (17) | NS |
| 10–19 | 47 (38) | 33 (26) | 58 (44) | 138 (36) | |
| 20– | 54 (43) | 68 (54) | 52 (39) | 174 (45) | |
| Unknown | 2 (2) | 3 (2) | 1 (1) | 6 (2) | |
| Mean ± SD | 18 ± 8 or 9 | 20 ± 10 | 18 ± 9 | | |
| Body weight (kg) | | | | | |
| –39 | 12 (9) | 16 (12) | 17 (12) | 45 (11) | NS |
| 40–49 | 55 (41) | 54 (41) | 49 (36) | 158 (39) | |
| 50–59 | 48 (36) | 54 (41) | 54 (39) | 156 (38) | |
| 60– | 20 (15) | 9 (7) | 18 (13) | 47 (12) | |
| Mean ± SD | 50 ± 8 | 48 ± 8 | 49 ± 8 | | |

Subjects were divided into three groups after the initial exclusion of 8 subjects from the original 414 with percentages in parentheses according to the intention-to-treat principle
NS, no significant difference

**Table 4.** Bone-related pre-study background data in the subjects at start divided into three groups

| Group | A | B | C | Total | Results of statistical analysis |
|---|---|---|---|---|---|
| Number of subjects at start | 135 | 133 | 138 | 406 | |
| Number of spinal fractures | | | | | |
| 0 | 77 (57) | 63 (47) | 67 (49) | 207 (51) | NS |
| 1 | 26 (19) | 28 (21) | 30 (22) | 84 (21) | |
| 2 | 16 (12) | 12 (9) | 12 (9) | 40 (10) | |
| 3 | 3 (2) | 9 (7) | 9 (7) | 21 (5) | |
| 4 | 5 (4) | 7 (5) | 3 (2) | 15 (4) | |
| 5 | 3 (2) | 4 (3) | 4 (3) | 11 (3) | |
| 6 or more | 4 (3) | 8 (6) | 11 (8) | 23 (6) | |
| Unknown | 1 (1) | 2 (2) | 2 (1) | 5 (1) | |
| BMD L2–L4 (QDR equivalent) ($g/cm^2$) | | | | | |
| Mean ± SD | 0.717 ± 0.131 | 0.714 ± 0.127 | 0.716 ± 0.133 | | NS |

Percentages are shown in parentheses according to the intention-to-treat principle
BMD, bone mineral density

**Table 5.** Background laboratory data in the three groups

| Group | | A | B | C | Results of statistical analysis |
|---|---|---|---|---|---|
| Number of subjects at start | | 135 | 133 | 138 | |
| Serum Ca (mg/dl) | | 9.19 ± 0.49 | 9.24 ± 0.52 | 9.16 ± 0.53 | NS |
| Serum P (mg/dl) | | 3.51 ± 0.48 | 3.58 ± 0.54 | 3.52 ± 0.49 | NS |
| Serum Alkaline Phosphatase | (IU) | 183.7 ± 77.9 | 189.3 ± 95.6 | 183.4 ± 74.7 | NS |
| | (KA) | 7.43 ± 1.95 | 7.61 ± 2.35 | 8.01 ± 2.76 | |
| | (BL) | 4.11 ± 2.83 | 3.49 ± 1.94 | 2.92 ± 1.05 | |
| Urinary Ca/Cr | | 0.21 ± 0.14 | 0.23 ± 0.14 | 0.21 ± 0.14 | NS |
| Urinary P/Cr | | 0.66 ± 0.37 | 0.69 ± 0.32 | 0.67 ± 0.36 | NS |
| Urinary hydroxyproline/Cr | | 0.022 ± 0.014 | 0.024 ± 0.020 | 0.022 ± 0.011 | NS |
| Parathyroid hormone (PTH) (pg/ml) | | 455.1 ± 195.2 | 449.5 ± 204.8 | 451.2 ± 181.7 | NS |
| Serum osteocalcin (ng/ml) | | 8.24 ± 4.23 | 8.64 ± 4.88 | 8.47 ± 4.37 | NS |
| Serum 1,25(OH) vitamin D (ng/ml) | | 52.19 ± 29.01 | 52.67 ± 21.07 | 49.55 ± 17.45 | NS |
| Serum 25(OH) vitamin D (ng/ml) | | 21.42 ± 7.59 | 21.91 ± 7.39 | 21.82 ± 7.30 | NS |

Date are mean ± SD according to the intention-to-treat principle

**Table 6.** Rate of change of DXA Values (% change): intention-to-treat (ITT) analysis using constant figures to fill the defective sites

| Method | Treatment | Number of cases | Mean (%) | SD | *P* values on signed-rank test |
|---|---|---|---|---|---|
| ITT | EHDP 200 mg | 137 | 2.527 | 4.814 | <0.0001 |
| | EHDP 400 mg | 137 | 3.639 | 5.861 | <0.0001 |
| | Alfacalcidol 1 μg | 140 | −0.336 | 4.286 | 0.3295 |
| Original data | EHDP 200 mg | 104 | 1.918 | 4.327 | <0.0001 |
| | EHDP 400 mg | 110 | 2.921 | 5.445 | <0.0001 |
| | Alfacalcidol 1 μg | 103 | −0.255 | 3.728 | 0.3295 |

Final data consisted of last observation carried forward (LOCF)
EHDP, etidronate

**Table 7.** Logistic procedure on incident fractures (Fx)

| | | Whole series | Fx (−) at start | Fx (+) at start |
|---|---|---|---|---|
| Odds ratio (confidence interval) | EHDP 200 mg | 0.4441 (0.1921–1.026) | 0.4031 (0.1598–1.0176) | 0.5534 (0.1960–1.5025) |
| | EHDP 400 mg | 0.3466 (0.1420–0.8464) | 0.3097 (0.1172–0.8181) | 0.4959 (0.1679–1.4645) |

Significant range is underlined

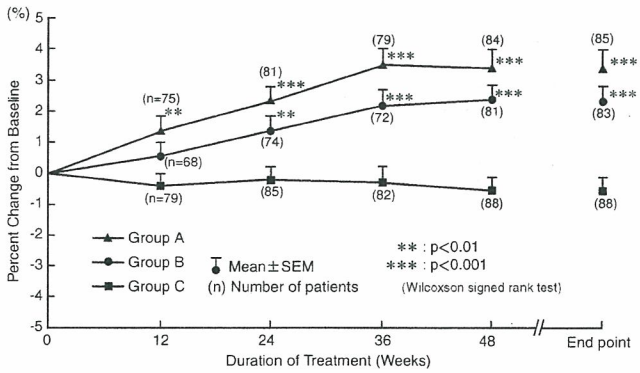


**Fig. 1.** Percent (%) changes of the lumbar spine bone mineral density (BMD) L2–L4 from baseline during the trial period (*vertical axis*) and duration of treatment in weeks (*horizontal axis*). Values for Group A at 12 weeks and subsequently those for Group B at 24 weeks and subsequently were both significantly higher from the baseline, whereas those for Group C did not change at any time, without a significant difference from baseline

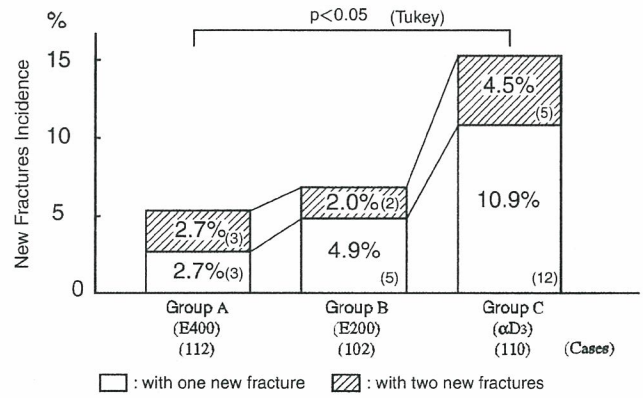


**Fig. 2.** Incident vertebral fracture during the test period. In Group C, one fracture occurred in 10.9% and two fractures in 4.5%; in Group A, the corresponding values were 2.7% and 2.7%, respectively, significantly lower than the former by the Tukey test; and in Group B, the corresponding values were 4.9% and 2.0%

Group C ($P < 0.001$). The results of intention-to-treat analysis on lumbar spine BMD are summarized in Table 6. Significant increase was noted in both groups given etidronate. Figure 2 illustrates new fracture in patients with one new vertebral fracture and those with multiple new vertebral fractures. In 10.9 and 4.5% of Group C, one and two new spinal fractures, respectively, occurred during the trial period, whereas such fractures occurred only in 2.7 and 2.7% of Group A and 4.9 and 2.0% of Group B.

Logistic analysis of incident fracture is summarized in Table 7. Significant reduction was noted only in the group given 400 mg etidoronate, in the whole series, and the group without prevalent fracture at the start of the study. The difference between Groups A and C was significant at $P < 0.05$ by the Tukey test. These patients were separated into those without fracture at entry and those with fracture at entry (Fig. 3). Among those without fracture at entry, one fracture occurred in 8.5% and two fractures in 1.7% of Group C, but none occurred in Groups A and B. The difference in fracture incidence between Groups A and C was significant at $P < 0.01$ and that between Groups B and C at $P < 0.05$. Among those with fracture at the beginning, 13.7% of Group C sustained one new fracture and 7.8% more than two, and the corresponding figures were 6.0 and 6.0% in Group A and 9.4 and 3.8% for Group B; however, the differences among the three groups were not significant.

In all the three groups, backache improved compared to the baseline level after 4 weeks. After 12 weeks, the cumulative rate of improvement reached 20%, and Groups A and B showed better results than in Group C (Fig. 4).

Changes of biochemical markers of bone turnover are shown in Fig. 5. Urinary hydroxyproline/Cr, a bone resorption marker, tended to fall in all three groups, especially in Group A (Fig. 5). Two bone formation markers showed

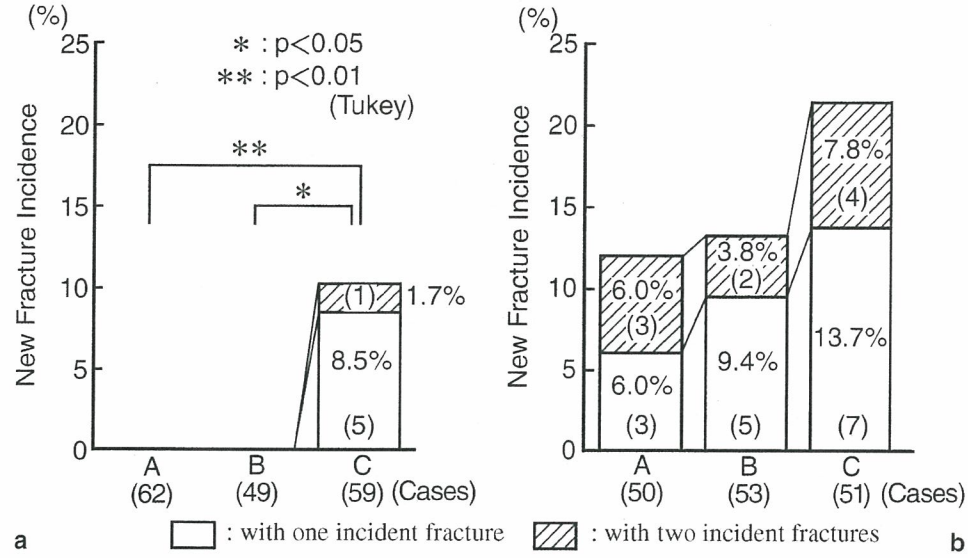


**Fig. 3.** Patients analyzed in Fig. 4 were divided into (**a**) those without prevalent fracture at entry and (**b**) those with prevalent fractures at entry. **a** One fracture occurred in 8.5% and two fractures in 1.7% in Group C, whereas no fractures occurred in Groups A and B. **b** One fracture occurred in 13.7% and two fractures in 7.8% in Group C; the corresponding figures were 6.0% and 6.0% in Group A and 9.4% and 3.8% in Group B, with less remarkable difference among the three groups. In subjects without prevalent fracture at entry, incident fracture occurred significantly less frequently in Group A ($P < 0.01$) and in Group B ($P < 0.05$) than in Group C by the Tukey test

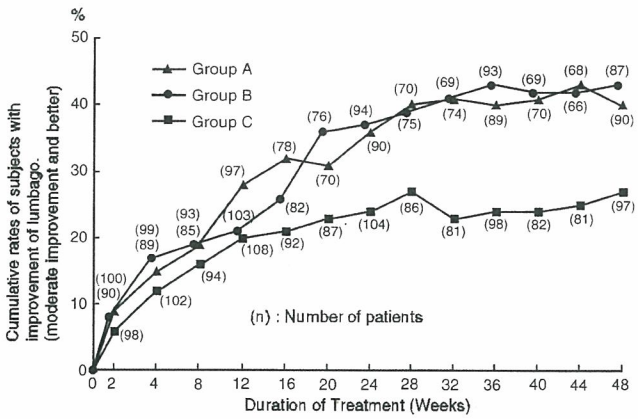


**Fig. 4.** Cumulative rates of definite improvement of backache: cumulative percentages of subjects with improvement of lumbago (*vertical axis*) and duration of treatment in weeks (*horizontal axis*). Group A and B gave higher values than Group C at 24 weeks

**Table 8.** Side effects shown according to the intention-to-treat principle

| Group | A | B | C |
|---|---|---|---|
| Number of patients | 135 | 133 | 138 |
| Patients with side effects | 14 | 8 | 10 |
| Episodes of side effects | 18 | 11 | 13 |
| Gastrointestinal episodes | 12 | 9 | 6 |
| Oral episodes | 4 | 1 | 0 |
| Dermal episodes | 1 | 1 | 1 |
| Electrolyte imbalance | 1 | 0 | 3 |
| Others | 0 | 0 | 3 |

different transitions; serum alkaline phosphatase fell from the preadministration level in all groups, especially markedly in Group A (Fig. 5), and serum osteocalcin fell progressively in Group A, followed by Group B, throughout the trial, but stayed almost unchanged in Group C (Fig. 5). Serum calcium showed a transient fall in Group A after 12 weeks but stayed within the normal range in all other groups. Serum P showed transient rises in all groups after 4 weeks, but returned to the normal range thereafter. Serum PTH showed a significant fall from the preadministration level after 12 weeks in Group C, but stayed within the normal range in Groups A and B, with a tendency for a slight rise toward the end of the trial. Urinary Ca/Cr ratio and P tended to rise in Group C, but stayed almost constant, except for mild transient falls, in Groups A and B.

Side effects are summarized in Table 8. Some side effects were seen in 14 of 135 patients in Group A, 8 of 133 in Group B, and 10 of 138 in Group C. Gastrointestinal side effects were seen most frequently, and others were rather infrequent, without remarkable difference among the three groups.

## Discussion

Etidronate, 200 or 400 mg daily for 2 weeks followed by a 10-week interval, repeated in four cycles, significantly increased spinal BMD measured by DXA over the basal level and also above the level maintained by daily administration of 1 μg alfacalcidol throughout the 48 weeks of the trial. New occurrence of spinal fracture deformity was also reduced by the administration of cyclic administration of 200 or 400 mg etidronate from the level obtained by the administration of alfacalcidol, especially in those without spinal fracture at the beginning of the trial.

The effect of etidronate to increase bone density has been reported in several prospective controlled studies [18–20], but the effect on spinal fracture incidence has been rather controversial. Alfacalcidol was also reported to increase BMD and to reduce the number of spinal fracture significantly better than inactive placebo. No inactive placebo was used in the present study because it was thought that 48 weeks was too long a period to maintain osteopo-

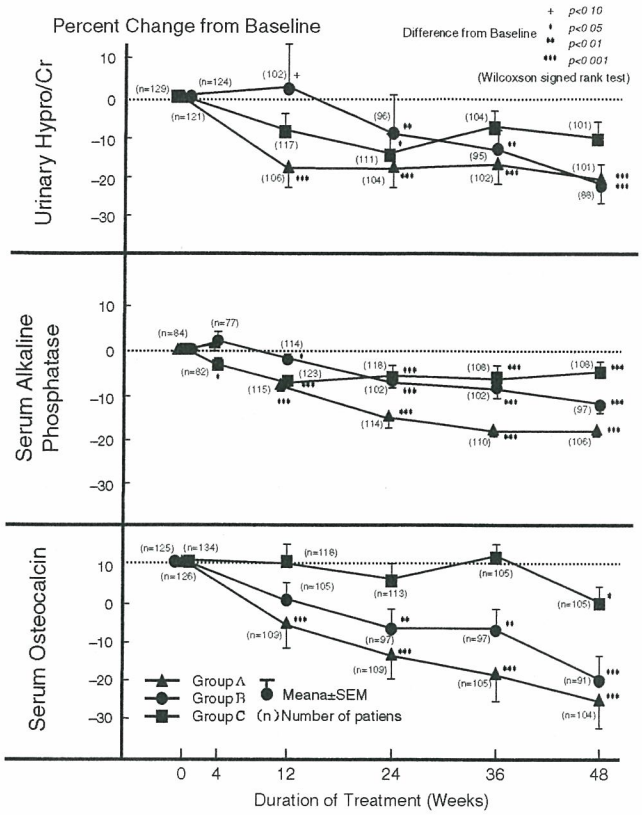


**Fig. 5.** *Top*: Urinary hydroxyproline/Cr (*vertical axis*) relative to duration of treatment in weeks (*horizontal axis*). A significant decrease of urinary hydroxyproline/creatinine ratio from the baseline value was noted after 12 weeks of treatment in Group A and after 24 weeks of treatment in Group C and Group B by Wilcoxon's signed rank test ($P < 0.0001$). *Middle*: On the *vertical axis* is shown percent serum alkaline phosphatase and on the *horizontal axis* duration of treatment in weeks. A significant decrease of alkaline phosphatase was already noted after 4 months of treatment in Group C ($P < 0.05$). After 12 months of treatment, a highly significant decrease was noted in Groups A and B ($P < 0.0001$), and a significant difference was also noted in Group C. Decreases persisted thereafter in all groups. *Bottom*: Serum osteocalcin (*vertical axis*) and duration of treatment (*horizontal axis*). A highly significant decrease of serum osteocalcin was already noted after 12 months of treatment in Group A, but not in Groups B and C; after 24 months, a significant decrease was noted in Groups A and B. In Group C, it was not until after 48 weeks that a slight but significant decrease appeared

rotic patients on an inactive placebo. Alfacalcidol was shown to maintain BMD, at least at the pretrial level, whereas age-related decrease of bone density was otherwise expected. Because cyclic etidronate therapy for 2 weeks on and 10 weeks off at 200 or 400 mg per day gave a significantly higher BMD and lower incidence of spinal fracture, etidronate appeared to be a useful drug to inhibit the progress of osteoporosis in this group of patients. The mean age of the patients who participated the study was 67, beyond the so-called immediate postmenopausal range and higher than that in most of the previous studies, which may add some significance to this study in enlarging the range of osteoporotic patients treated with this drug in addition to the genetic and nutritional differences. Calcium intake by Japanese, 500–550 mg/day, for instance, is generally lower than ordinary intake by Westerners. The cumulative rate of good responders to treatment as to backache suggested a more favorable response to 400 mg etidronate than 200 mg etidronate or alfacalcidol, especially after 6 months of treatment.

As a limitation of the present study, admission criteria for the study were rather complex because of the concern about nonosteoporotic bone disease. The number of subjects for DXA data analysis became unexpectedly smaller because of degenerative changes of the spine in greater age. The design of the study, involving prospective double-blind control, made a long-term continuation of more than 48 weeks difficult. The proposed duration of the present study, 48 weeks, was thought to be too short to provide sufficient data allowing accurate analysis of the frequency of incident fracture. Lumbar BMD was therefore chosen as the primary endpoint. Further studies of longer duration are desirable to provide more definite evidence of the effect of etidronate on incident fracture, especially because of the rather unexpectedly favorable findings in the present study.

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# Local Application of Recombinant Human Fibroblast Growth Factor-2 on Bone Repair: A Dose–Escalation Prospective Trial on Patients with Osteotomy

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**ABSTRACT:** Based on preclinical evidence in animal models, the present study examined the clinical efficacy and safety of recombinant human fibroblast growth factor-2 (rhFGF-2) to accelerate bone repair in a dose-escalation prospective trial. One of three dosages (200, 400 or 800 μg) of rhFGF-2 in a biodegradable gelatin hydrogel was injected during surgery into the osteotomy site of 59 knee osteoarthritis patients undergoing high tibial osteotomy, and 57 of them were monitored for 16 weeks. The rhFGF-2 dose dependently increased the percentage of patients with radiographic bone union, and decreased the average time needed for such union. The percentages of patients with an absence of pain and full-weight bearing were also greater in the higher dosage groups than in the low dosage group, especially in the clinically critical periods 6, 8, and 10 weeks. Neither blood chemistries nor clinical adverse events were associated with the rhFGF-2 dosages. We therefore conclude that the rhFGF-2 in gelatin hydrogel dose dependently accelerated radiographic bone union of a surgical osteotomy with a safety profile at least at the dosages used, suggesting the clinical efficacy of this agent for bone repair. © 2007 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 25:480–487, 2007

**Keywords:** fibroblast growth factor; fracture; bone; human

## INTRODUCTION

Development of synthetic materials with osteogenic properties is a major goal of the orthopedic research field. Because growth factors are expressed during various phases of bone repair, there has been considerable interest in their use as therapeutic agents to enhance the repair in accordance with the recent advent of their recombinant proteins.[1] Fibroblast growth factors (FGFs) are a family of 23 structurally related polypeptides that are characterized by their affinity for the glycosaminoglycan heparin-binding sites on cells, and are known to play a critical role in angiogenesis and mesenchymal cell mitogenesis.[2] The most abundant FGFs in normal adult tissue are fibroblast growth factor-1 (FGF-1 or acidic FGF) and FGF-2 (basic FGF), both of which have been identified during the early stages of bone repair, although FGF-2 is a more potent mitogen than FGF-1.[3] In skeletal tissues, FGF-2 is accumulated in bone matrix and acts as an autocrine/paracrine factor.[4] Several genetic diseases with abnormalities in bone and cartilage formation, such as achondroplasia and thanatophoric dysplasia type II, have been shown to be due to mutations of genes for FGFs or their receptors,[5] suggesting the importance of FGFs in bone and

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cartilage formation. We and others have reported the anabolic effect of local and systemic administrations of FGF-2 on bone formation using several animal models including nonhuman primates.[6–15] A single local application of FGF-2 facilitated the healing of bone fracture and segmental bone defect in normal and diabetic rats, rabbits, dogs, and monkeys;[6–12] stimulated bone formation in callotasis bone lengthening in rabbits;[13] and increased bone mass intraosseously in normal and ovariectomized rats and rabbits.[14] In addition, a daily systemic administration of FGF-2 facilitated endosteal bone formation.[15] Regarding the delivery system for clinical use, previous animal studies have revealed that FGF-2 exerted the most potent anabolic activity when a synthetic bioabsorbable hydrogel prepared through glutaraldehyde crosslinking of gelatin was used as the carrier.[11,16,17] Based on these preclinical studies, the present study for the first time evaluated the efficacy and safety of the use of recombinant human fibroblast growth factor-2 (rhFGF-2) in the biodegradable gelatin hydrogel for bone repair in a clinical dose–escalation trial.

## MATERIALS AND METHODS

### Materials

rhFGF-2 was provided by Kaken Pharmaceutical Co., Ltd. (Tokyo, Japan). Biodegradable gelatin hydrogel was prepared through the glutaraldehyde crosslinking of acidic gelatin that was purified from natural bovine bone, as reported previously.[16,17]

### Study Design

The present study was designed with a reasonable sample size as the first exploratory trial that examined the effects of rhFGF-2 administration in humans with safety and accuracy. Between November 2000 and March 2004, 59 patients (40–74 years old) with osteoarthritis in the medial compartment of the knee, who were planning to undergo closing wedge high tibial osteotomy, were enrolled for this dose–escalation trial at 16 medical centers and hospitals in Japan. Exclusion criteria included use of medications that were known to affect bone and cartilage metabolism (e.g., bisphosphonates, vitamin D derivatives, calcitonin, vitamin $K_2$, ipriflavone, estrogen, androgen, calcium supplement, corticosteroids), osteoporosis, diabetes, malignant tumor, and prior surgery on bone or joint. Pain medications were not restricted, and all patients used nonsteroidal anti-inflammatory drugs (NSAIDs) before or during the observation period, although the duration was not significantly different among the groups. After approval of the protocol by the Institutional Review Boards (IRB) at all participating institutions and after the patients had provided written informed consent, patients were assigned in order of enrollment to one of three investigational groups that received a total dosage of 200, 400, or 800 μg of rhFGF-2 in a biodegradable gelatin hydrogel carrier (the total reconstituted volume was 1 mL). The rhFGF-2 dosages were determined based on previous preclinical results of osteotomy experiments on rats, rabbits, dogs, and monkeys[7–9,12] through normalizing by the cross section area of the bones. Safety of a dosage was assessed and confirmed before proceeding to a higher trial dosage. For the surgery, through an anterior approach a closing wedge osteotomy was performed, aiming at 164–170° of postoperative femorotibial angle (FTA), with fixations by an external fixator, an internal plate or an internal staple, depending on the institution. Following wound irrigation and hemostasis, the rhFGF-2 hydrogel was injected into the osteotomy site just prior to the soft-tissue closure. A drain without suctioning was placed as far as possible from the osteomomy to avoid the potential consequences on the bioavailability of the agent.

### Radiographic Assessment

Standard X-ray pictures of the anteroposterior and lateral projections were taken immediately after the surgery and at least every 2 weeks thereafter up to 16 weeks postoperatively, even if radiographic bone union was achieved at earlier time points. A panel of two orthopedic surgeons and one musculoskeletal radiologist, blinded to patient data including treatment and time following the surgery, independently assessed the presence or absence of bone union. Radiographic bone union was defined as the existence of apparent bridging by bony beam across the osteotomy gap on anteroposterior and lateral projections in patients with external and internal staple fixations, and on anteroposterior projection in those with internal plate fixation.

### Clinical Assessment

Clinical assessment included the presence of pain at the osteotomy site and the ability for full weight-bearing on the operated leg. Although neither NSAIDs nor analgesics were restricted during the observation period, pain free was defined as no pain at the osteotomy site during daily activities without the pain medications. From 6 weeks after surgery, as much weight bearing as possible was allowed unless the patient felt pain while walking. In addition, clinical healing was defined as both the radiographic bone union and the absence of pain at the osteotomy site. These criteria were evaluated every 2 weeks up to 16 weeks, even if there was no clinical problem at earlier time points.

### Blood Chemistries

The serum calcium and phosphate levels were measured by orthocresolphthalein complexone and direct molybdic method, respectively (SRL Inc., Tokyo, Japan), preoperatively, and 2 and 4 weeks postoperatively. The calcitonin and osteocalcin levels were measured by

radioimmunoabsorbent assay (RIA; SRL Inc.). The serum level of FGF-2 was monitored by enzyme-linked immunoabsorbent assay (ELISA; Kaken Pharmaceutical Co., Ltd., Tokyo) before and 2, 4, 24, and 2 weeks after the surgery. All patients were also screened for antibodies to FGF-2 and gelatin preoperatively and 2 and 4 weeks postoperatively, by ELISA (Kaken Pharmaceutical Co., Ltd.) and fluorescence enzyme immunoabsorbent assay (FEIA; SRL Inc.), respectively.

### Safety Assessment

Safety was monitored according to the number and duration of adverse events. An adverse event was defined as any local or systemic sign, symptom, syndrome, illness, medical condition, or abnormal laboratory data that occurred or worsened after surgery, regardless of causality or treatment group. Infections were conservatively defined as any suspected or confirmed superficial or deep bone or soft-tissue infection, with or without bacteriological confirmation. Retrospectively, all adverse events were classified as serious or nonserious according to ICH Guidelines (International Conference of Harmonization Guideline Clinical Safety Data Management: Definitions and Standards for Expedited Reporting. Federal Register, March 1, 1995).

### Statistical Analyses

Baseline characteristics of patients were tested using one-way analysis of varience (ANOVA) or Fisher's exact test. Dose response effects among three dosages of rhFGF-2, and pair-wise comparisons were evaluated by generalized Wilcoxon test and Tukey-Kramer method, respectively. Differences in the number and duration of adverse events were evaluated by Fisher's exact test. Comparison of the serum concentrations was performed with one-way ANOVA and paired $t$-test. A $p$-value of $<0.05$ for analysis of safety variables was considered significant. Data analyses were performed using SAS version 8.0 (SAS Institute Inc., Cary, NC).

## RESULTS

### Demographics

Fifty-nine patients were enrolled from 16 institutions, and two patients (one for spontaneous retraction of the informed consent and the other for the onset of heart failure) were withdrawn, so that investigations were performed on 57 patients (97%). There were no statistically significant differences (all $p > 0.05$) for any of the baseline data among the groups treated with 200, 400, and 800 μg of rhFGF-2 (Table 1). The low, middle, and high dosage groups consisted of 20, 18, and 19 patients with no significant difference in gender. The average age, body height, weight, and body mass index at surgery were also comparable among the three groups. Although the aimed FTA and the fixation method varied among the institutions, three was no significant difference in the corrected angle or the hardware used among the groups (all $p > 0.05$).

### Radiographic Bone Union

The interrater reliability of the three independent reviewers has a weighted kappa coefficient of 0.60–0.68, suggesting an excellent reproducibility among them. All osteotomies were confirmed to show similar bone defects without radiographic

**Table 1.** Baseline Patient Demographics

| | rhFGF-2 | | | |
|---|---|---|---|---|
| | 200 μg | 400 μg | 800 μg | $p$-Value |
| Number of patients | 20 | 18 | 19 | |
| Males/females | 7/13 | 8/10 | 11/8 | 0.378 |
| Age (years) (range) | 62.2 ± 9.2 (41–74) | 57.4 ± 6.9 (44–72) | 59.7 ± 9.7 (40–71) | 0.252 |
| Height (cm) (range) | 156.7 ± 8.0 (147.0–178.6) | 157.1 ± 8.1 (145.8–170.0) | 159.3 ± 9.4 (142.3–175.0) | 0.603 |
| Weight (kg) (range) | 63.9 ± 9.6 (50.0–84.0) | 68.1 ± 10.7 (51.0–88.3) | 64.8 ± 9.8 (43.0–82.5) | 0.415 |
| Body mass index ($kg/m^2$) (range) | 26.0 ± 3.2 (21.3–34.5) | 27.6 ± 4.5 (22.4–36.9) | 25.5 ± 2.7 (19.1–28.5) | 0.167 |
| Corrected angle (degrees) | | | | 0.847 |
| <10 | 0 | 1 | 1 | |
| 10–14 | 10 | 9 | 7 | |
| 15–19 | 8 | 5 | 9 | |
| ≥20 | 2 | 3 | 2 | |
| Fixation methods | | | | 0.177 |
| External fixation | 12 | 5 | 7 | |
| Internal plate fixation | 6 | 12 | 11 | |
| Internal stabple fixation | 2 | 1 | 1 | |

Data are mean ± SD.


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