bone tunnel, while eight samples from the experimental group failed due to rupture of the tendon near the tendon-bone junction, and two failed due to pulling out of the tendon from the tibia.

DISCUSSION

In the present study, we demonstrated that it is possible to induce ectopic bone formation in tendon consistently and then successfully generate a tendon-bone junction similar to normal enthesis at the tendon-bone interface. Further, the regenerated enthesis produced by transfer of the tendon containing the rhBMP-2-induced bone mass into host bone was shown to be functionally competent by mechanical load testing. This technique has the potential for use in regeneration of strong physiological fixation of tendons or ligaments to bone, although two series of surgical procedures are required to achieve this outcome.

It has been reported that rhBMP has the biological capacity to elicit bone formation even ectopically in subcutaneous tissue or muscle.[29,30] Intratendinous bone induction by BMP has not been reported before, and our findings clearly indicate that tendon consists of cells that are responsive to BMP and are capable of differentiating along the chondro-osseous pathway.[34–37] The process of intratendinous new bone formation followed that of endochondral ossification. The appearance of cartilage preceded bone formation, and most of the cartilage was resorbed and replaced by bone, except at the interface between the original tendon and the BMP-induced bone mass. However, one feature specific to the intratendinous new bone was the retention of tendinous collagen fibers connecting the tendon and the induced new bone, an essential morphological feature of the normal enthesis. The presence of Type I and Type II collagen fibers in the cartilage of the tendon indicates the presence of fibrocartilage, also a hallmark of the enthesis.[38–42] The structure of the tendon-bone junction was maintained after transfer to normal bone (of the tibia), and the intratendinous bone mass became firmly integrated with the host tibial bone. The strength of this attachment was confirmed by mechanical testing. Tendon rupture by pulling occurred predominantly in the region of the tendon outside the tibia, while the control tendon without BMP-induced bone pulled out of the tunnel without rupture of the tendon. The engineered enthesis thus exhibited functional and morphological features similar to those of normal enthesis, though the pullout strength of this enthesis may be inferior to that of normal enthesis as well as BTB reconstructions.[16–18]

Previous studies[21,22] reported administration of cytokines (BMP-2, TGF-β, etc.) to promote anchoring of the bone–tendon interface. Although bone formation around the tendon was demonstrated, connective tissue between bone and tendon was reduced compared with that in sham models.

Using the new technique described in this study, we have begun trials to reconstruct the ACL in animal models, in which two bone masses are generated by injecting rhBMP-2 into the flexor digitorum communis tendon to prepare bone-tendon-bone autografts. In these models, the length of the tendon between the two bone masses is adjusted to that of a normal ACL. The tendons will be strongly attached to the subchondral bone of the distal femur and proximal tibia by bony union. The results of studies following this preliminary one of reconstruction of the ACL will be described in subsequent reports.

A limitation of this study is that two surgeries are required, one to ossify the graft tendon, and another to harvest and reconstruct the knee, perhaps making clinicians reluctant to use this technique. However, hopefully our approach will avoid postoperative widening of the bone tunnels,[43,44] which probably occurs due to incomplete bonding of the transferred tendon to the bone tunnel walls,[45,46] and it might yield improved long-term outcomes for knees with ACL reconstruction.

In conclusion, we have presented a new technique using rhBMP-2 to successfully regenerate the tendon-bone junction. This approach has the potential for clinical use during reconstructive surgeries to restore the functional attachment of ligaments and/or tendons to bone.

ACKNOWLEDGMENTS

We thank the Genetic Institute and Astellas Pharma Inc. for kindly providing rhBMP. This work was supported in part by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (Project Grant 16109009), and Takeda Science Foundation and Hip Joint Foundation of Japan.

REFERENCES

1. Benjamin M, Kumai T, Milz S, et al. 2002. The skeletal attachment of tendons-tendon "entheses". Comp Biochem Physiol A Mol Integr Physiol 133:931–945.
2. Woo SL, Orlando CA, Gomez MA, et al. 1986. Tensile properties of the medial collateral ligament as a function of age. J Orthop Res 4:133–141.

 DOI 10.1002/jor

3. Noyes FR, Torvik PJ, Hyde WB, et al. 1974. Biomechanics of ligament failure. II. An analysis of immobilization, exercise, and reconditioning effects in primates. J Bone Joint Surg Am 56:1406–1418.
4. Furikado K, Fujioka H, Kurosaka M, et al. 2002. Comparison of mechanical and histological properties between the immature and mature tendon attachment. Int Orthop 26:318–321.
5. Nebelung W, Becker R, Urbach D, et al. 2003. Histological findings of tendon-bone healing following anterior cruciate ligament reconstruction with hamstring grafts. Arch Orthop Trauma Surg 123:158–163.
6. Robert H, Es-Sayeh J, Heymann D, et al. 2003. Hamstring insertion site healing after anterior cruciate ligament reconstruction in patients with symptomatic hardware or repeat rupture: a histologic study in 12 patients. Arthroscopy 19:948–954.
7. Weiler A, Hoffmann RF, Bail HJ, et al. 2002. Tendon healing in a bone tunnel. Part II: Histologic analysis after biodegradable interference fit fixation in a model of anterior cruciate ligament reconstruction in sheep. Arthroscopy 18:124–135.
8. Yoshiya S, Nagano M, Kurosaka M, et al. 2000. Graft healing in the bone tunnel in anterior cruciate ligament reconstruction. Clin Orthop Relat Res 376:278–286.
9. Goradia VK, Rochat MC, Grana WA, et al. 2000. Tendon-to-bone healing of a semitendinosus tendon autograft used for ACL reconstruction in a sheep model. Am J Knee Surg 13:143–151.
10. Grana WA, Egle DM, Mahnken R, et al. 1994. An analysis of autograft fixation after anterior cruciate ligament reconstruction in a rabbit model. Am J Sports Med 22: 344–531.
11. Panni AS, Milano G, Lucania L, et al. 1997. Graft healing after anterior cruciate ligament reconstruction in rabbits. Clin Orthop Relat Res 343:203–212.
12. Blickenstaff KR, Grana WA, Egle D. 1997. Analysis of a semitendinosus autograft in a rabbit model. Am J Sports Med 25:554–559.
13. Uhthoff HK, Sano H, Trudel G, et al. 2000. Early reactions after reimplantation of the tendon of supraspinatus into bone. A study in rabbits. J Bone Joint Surg Br 82:1072–1076.
14. Boyer MI, Harwood F, Ditsios K, et al. 2003. Two-portal repair of canine flexor tendon insertion site injuries: histologic and immunohistochemical characterization of healing during the early postoperative period. J Hand Surg [Am] 28:469–474.
15. Beynnon BD, Johnson RJ, Fleming BC, et al. 2002. Anterior cruciate ligament replacement: comparison of bone-patellar tendon-bone grafts with two-strand hamstring grafts. A prospective, randomized study. J Bone Joint Surg Am 84:1503–1513.
16. Leung KS, Qin L, Fu LK, et al. 2002. A comparative study of bone to bone repair and bone to tendon healing in patella-patellar tendon complex in rabbits. Clin Biomech 17:594–602.
17. Tomita F, Yasuda K, Mikami S, et al. 2001. Comparisons of intraosseous graft healing between the doubled flexor tendon graft and the bone-patellar tendon-bone graft in anterior cruciate ligament reconstruction. Arthroscopy 17: 461–476.
18. Park MJ, Lee MC, Seong SC. 2001. A comparative study of the healing of tendon autograft and tendon-bone autograft using patellar tendon in rabbits. Int Orthop 25:35–39.
19. Eriksson K, Anderberg P, Hamberg P, et al. 2001. There are differences in early morbidity after ACL reconstruction when comparing patellar tendon and semitendinosus tendon graft. A prospective randomized study of 107 patients. Scand J Med Sci Sports 11:170–177.
20. Kohn D, Sánder-Beuermann A. 1994. Donor-site morbidity after harvest of a bone-tendon-bone patellar tendon autograft. Knee Surg Sports Traumatol Arthrosc 2:219–223.
21. Rodeo SA, Suzuki K, Deng XH, et al. 1999. Use of recombinant human bone morphogenetic protein-2 to enhance tendon healing in a bone tunnel. Am J Sports Med 27:476–488.
22. Yamazaki S, Yasuda K, Tomita F, et al. 2005 The effect of transforming growth factor-beta1 on intraosseous healing of flexor tendon autograft replacement of anterior cruciate ligament in dogs. Arthroscopy 21:1034–1041.
23. Kyung HS, Kim SY, Oh CW, et al. 2003. Tendon-to-bone tunnel healing in a rabbit model: the effect of periosteum augmentation at the tendon-to-bone interface. Knee Surg Sports Traumatol Arthrosc 11:9–15.
24. Ishikawa H, Koshino T, Takeuchi R, et al. 2001. Effects of collagen gel mixed with hydroxyapatite powder on interface between newly formed bone and grafted achilles tendon in rabbit femoral bone tunnel. Biomaterials 22: 1689–1694.
25. Mutsuzaki H, Sakane M, Nakajima H, et al. 2004. Calcium-phosphate-hybridized tendon directly promotes regeneration of tendon-bone insertion. J Biomed Mater Res A 70:319–327.
26. Nawata K, Minamizaki T, Yamashita Y, et al. 2002. Development of the attachment zones in the rat anterior cruciate ligament: changes in the distributions of proliferating cells and fibrillar collagens during postnatal growth. J Orthop Res 20:1339–1344.
27. Messner K. 1997. Postnatal development of the cruciate ligament insertions in the rat knee. morphological evaluation and immunohistochemical study of collagens types I and II. Acta Anat (Basel) 160:261–268.
28. Rufai A, Benjamin M, Ralphs JR. 1992. Development and ageing of phenotypically distinct fibrocartilages associated with the rat Achilles tendon. Anat Embryol (Berl) 186: 611–618.
29. Saitou N, Okada T, Horiuchi H, et al. 2001. A biodegradable polymer as a cytokine delivery system for inducing bone formation. Nature Biotechnol 19:332–335.
30. Takaoka K, Yoshikawa H, Hashimoto J, et al. 1993. Purification and characterization of a bone-inducing protein from a murine osteosarcoma (Dunn type). Clin Orthop Relat Res 292:329–336.
31. Namikawa T, Terai H, Suzuki E, et al. 2005. Experimental spinal fusion with recombinant human bone morphogenetic protein-2 delivered by a synthetic polymer and beta-tricalcium phosphate in a rabbit model. Spine 30:1717–1722.
32. Hoshino M, Egi T, Terai H, et al. 2006. Repair of long intercalated rib defects using porous beta-tricalcium phosphate cylinders containing recombinant human bone morphogenetic protein-2 in dogs. Biomaterials 27:4934–4940.
33. Kawakami H, Shino K, Hamada M, et al. 2004. Graft healing in a bone tunnel: bone-attached graft with screw fixation versus bone-free graft with extra-articular suture fixation. Knee Surg Sports Traumatol Arthrosc 12:384–390.

34. Rutherford RB, Moalli M, Franceschi RT, et al. 2002. Bone morphogenetic protein-transduced human fibroblasts convert to osteoblasts and form bone in vivo. Tissue Eng 8: 441–452.
35. Salingcarnboriboon R, Yoshitake H, Tsuji K, et al. 2003. Establishment of tendon-derived cell lines exhibiting pluripotent mesenchymal stem cell-like property. Exp Cell Res 287:289–300.
36. Yonemori K, Imamura T, Ishidou Y, et al. 1997. Bone morphogenetic protein receptors and activin receptors are highly expressed in ossified ligament tissues of patients with ossification of the posterior longitudinal ligament. Am J Pathol 150:1335–1347.
37. Song J, Mizuno J, Hashizume Y, et al. 2006. Immunohistochemistry of symptomatic hypertrophy of the posterior longitudinal ligament with special reference to ligamentous ossification. Spinal Cord 44:576–581.
38. Amiel D, Frank C, Harwood F, et al. 1984. Tendons and ligaments: a morphological and biochemical comparison. J Orthop Res 1:257–265.
39. Thomopoulos S, Hattersley G, Rosen V, et al. 2002. The localized expression of extracellular matrix components in healing tendon insertion sites: an in situ hybridization study. J Orthop Res 20:454–463.
40. Kumagai J, Sarkar K, Uhthoff HK, et al. 1994. Immunohistochemical distribution of type I, II and III collagens in the rabbit supraspinatus tendon insertion. J Anat 185: 279–284.
41. Waggett AD, Ralphs JR, Kwan AP, et al. 1998. Characterization of collagens and proteoglycans at the insertion of the human Achilles tendon. Matrix Biol 16:457–470.
42. Kumagai J, Sarkar K, Uhthoff HK. 1994. The collagen types in the attachment zone of rotator cuff tendons in the elderly: an immunohistochemical study. J Rheumatol 21: 2096–2100.
43. Nebelung W, Becker R, Merkel M, et al. 1998. Bone tunnel enlargement after anterior cruciate ligament reconstruction with semitendinosus tendon using Endobutton fixation on the femoral side. Arthroscopy 14:810–815.
44. Clatworthy MG, Annear P, Bulow JU, et al. 1999. Tunnel widening in anterior cruciate ligament reconstruction: a prospective evaluation of hamstring and patella tendon grafts. Knee Surg Sports Traumatol Arthrosc 7:138–145.
45. Rodeo SA, Arnoczky SP, Torzilli PA, et al. 1993. Tendon-healing in a bone tunnel. A biomechanical and histological study in the dog. J Bone Joint Surg Am 75:1795–1803.
46. Liu SH, Panossian V, al-Shaikh R, et al. 1997. Morphology and matrix composition during early tendon to bone healing. Clin Orthop Relat Res 339:253–260.

DOI 10.1002/jor

Rheumatology 2007;46:1652–1656
Advance Access publication 12 September 2007

doi:10.1093/rheumatology/kem220

Serum keratan sulfate is a promising marker of early articular cartilage breakdown

S. Wakitani[1], M. Nawata[2], A. Kawaguchi[1], T. Okabe[3], K. Takaoka[1], T. Tsuchiya[4], R. Nakaoka[4], H. Masuda[5] and K. Miyazaki[5]

Objectives. To find serum markers that may serve as indices for an early diagnosis of degeneration or damage of the articular cartilage.
Methods. Twenty-four healthy volunteers, 19 individuals with knee trauma (KT) and 31 with knee osteoarthritis (OA) were evaluated. KT patients were divided into a group ($n=5$) with an injury <2 months old (recent KT) and a group ($n=14$) with that >2 months old (old KT). Articular cartilage damage was assessed using either arthroscopy or direct observation. Serum concentrations of hyaluronic acid (HA), cartilage proteoglycan aggrecan turnover epitope (CS846) and cartilage oligomeric protein (COMP) were measured using enzyme-linked immunosorbent assay kits and those of keratan sulfate (KS) and chondroitin-6-sulfate (C6S) using high-performance liquid chromatography.
Results. Serum KS in the recent KT group (2095 ± 594 ng/ml) was significantly higher than that in the old KT group (1373 ± 418 ng/ml; $P=0.021$), and serum COMP in the recent KT group (1572 ± 182 ng/ml) showed a tendency that was higher than that in the old KT group (1350 ± 250 ng/ml; $P=0.079$).
Serum KS in OA patients with Kellgren and Lawrence (KL) grades 0 and I (1456 ± 334 ng/ml) showed a tendency that was higher than that in OA patients with KL grades II, III and IV (1248 ± 220 ng/ml; $P=0.084$).
Conclusions. The serum concentration of KS correlated with the damage of the articular cartilage and it was significantly increased even at an early stage after the injury.

KEY WORDS: Keratan sulfate, Glycosaminoglycan, Cartilage oligomeric protein, Cartilage injury, Osteoarthritis, Serum marker.

Introduction

The prevalence of patients with articular cartilage defects among patients with symptomatic knees requiring arthroscopy has been reported as 5–20% [1–3]; when left untreated, osteoarthritic changes are observed on X-rays taken after 10–20 yrs [4, 5]. Thus, articular cartilage injury is considered a cause of osteoarthritis (OA). Even if there is no articular cartilage injury, degeneration of the articular cartilage is considered to begin in humans at a young age, and articular cartilage changes, such as changes in colour and fibrillation, can occur. Injury or early-stage alterations of the articular cartilage in OA cannot be detected using X-ray examination. Magnetic resonance imaging (MRI) can detect articular cartilage defects and cartilaginous quality changes to some extent, but this technique is not sensitive enough to detect early OA changes and is expensive to be used as a routine examination. Serum markers, on the other hand, are suitable as screening tests, and only patients with high values of serum markers should be subjected to MRI or arthroscopy to detect articular cartilage degeneration. If it were possible to detect OA or articular cartilage damage at an early stage, patients could be educated to prevent the progression of OA. Moreover, it would be useful to monitor the natural course of articular cartilage damage or repair after, for instance, autologous chondrocytes implantation, whose effectiveness is still controversial because there is no method to effectively evaluate cartilage repair.

In 1985, Thonar *et al.* [6] measured serum keratan sulfate (KS) using an enzyme-linked immunosorbent assay (ELISA) by anti-KS antibody (1/20/5-D-4), and suggested its usefulness as a marker of OA. However, the correlation was weak and it did not correlate with X-ray grading [7]. Many researchers have tried to detect the metabolic products of articular cartilage components (proteoglycan, type II collagen and non-collagenous proteins) in joint fluid or blood and thereby a marker of OA [8–11]. As reported by Okumura *et al.* [12], early OA articular cartilage destruction begins with a loss of glycosaminoglycans (GAGs) from articular cartilage surfaces, followed by collagenolysis. Thus, the first event in OA or articular cartilage damage is the release of GAGs, which play an important role in maintaining articular cartilage function. Consequently, early markers of articular cartilage damage or OA change might be among GAG metabolic products. We selected KS, chondroitin 6 sulfate (C6S), cartilage proteoglycan aggrecan turnover epitope (CS846) and hyaluronan (HA) as candidate markers, and cartilage oligomeric protein (COMP), which is not a component of GAGs but has been reported to be a marker of OA [9]. These components have been reported to correlate with OA, to some extent, but not with cartilage damage caused by degradation and/or injury. These metabolic products can be measured in joint fluid, serum and urine, but we measured them in serum because it is easy to collect.

We measured KS using high-performance liquid chromatography (HPLC), which has been reported to be more accurate than ELISA [13]; C6S using HPLC, and CS846, HA and COMP using ELISA. We measured these markers in healthy volunteers and in patients with knee trauma (KT) or OA, who were subjected to knee surgery and whose articular cartilage was optically assessed (by arthroscopy or direct observation). We examined the correlation of these markers with the articular cartilage assessment to evaluate their usefulness as markers of early articular cartilage breakdown caused by degeneration and/or injury but that showed no change by X-ray examination.

Patients and methods

This study was approved by the institutional Review Board of Marunouchi Hospital and was conducted in accordance with the Helsinki Declaration of 1975, revised in 1983. Written informed consent was obtained from the healthy volunteers and patients prior to their participation in the study.

[1]Department of Orthopaedic Surgery, Osaka City University Graduate School of Medicine, [2]Department of Orthopaedic Surgery, Marunouchi Hospital, [3]Department of Orthopaedic Surgery, Shinshu University School of Medicine, [4]Division of Medical Devices, National Institute of Health Sciences and [5]Department of Pharmaceuticals information, Seikagaku Corporation.

Submitted 11 April 2007; revised version accepted 19 July 2007.

Correspondence to: Shigeyuki Wakitani, MD, Department of Orthopaedic Surgery, Osaka City University Graduate School of Medicine, 1-4-3 Asahi, Abeno-ku, Osaka 545-8585, Japan. E-mail: wakitani@med.osaka-cu.ac.jp

© 2007 The Author(s)
This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/2.0/uk/) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Blood collection from healthy volunteers

Ten men and 14 women (23–52 yrs old) volunteered to participate in the study. The volunteers were healthy with no gross obesity, inferior limb malalignment, history of knee injury or knee disorders. Sera were collected and stored at −80°C.

Patients with KT or knee OA

Nineteen KT patients (11 men and 8 women; 20–54 yrs old) and 31 patients with knee OA (11 men and 20 women; 40–80 yrs old) who were diagnosed to undergo knee surgery participated in the study. X-rays of knee and lumbar spine were available for all the patients. Sera samples were collected before surgery and stored at −80°C. The condition of the knee articular cartilage was observed at the time of surgery either arthroscopically or by direct observation. Among the 19 KT patients, two had meniscal injuries, 12 had ligament injuries and five had both meniscal and ligament injuries. KT patients were divided into a group of 5 patients with injuries <2 months old (recent KT) and a group of 14 patients with injuries >2 months old (old KT). Among the 31 OA patients, eight underwent total knee replacement, one underwent a high tibial osteotomy and 22 underwent arthroscopic debridement.

Assessment of articular cartilage surfaces by X-ray and visual inspection

X-ray images were assessed using the Kellgren and Lawrence (KL) grading scale [14]. All the KT patients were KL grade 0. Seven of the OA patients were KL grade 0, seven were KL grade I, four were KL grade II, six were KL grade III and seven were KL grade IV. Articular cartilage damage was assessed using the Societé Francaise d' Arthroscopie (SFA) scaling system [15]. In brief, the degree of articular cartilage damage was estimated from 0 to IV according to the SFA grading scale. The width of the damaged area was evaluated as a percentage of the damaged area in the medial and medial femoro-tibial and patello-femoral areas, separately. The SFA score was then calculated using a coefficient. The SFA score represents not only the degree of articular cartilage surface damage, but also the width of the damaged area.

Determination of the serum markers

Keratan sulfate was determined by HPLC after digestion with keratanase II (Seikagaku Corporation, Tokyo, Japan) according to the method of Tomatsu *et al.* [13] Each serum sample (0.2 ml) was treated with a protease (actinase E: Kaken Pharmaceutical Co. Ltd., Tokyo) and the negatively charged substance containing KS was fractionated by Q sepharose and digested by keratanase II. The KS-derived β-galactosyl-(1-4)-6-*O*-sulfo-*N*-acetylglucosamine (m-ks) and β-6-*O*-sulfo-galactosyl-(1-4)-6-*O*-sulfo-*N*-acetylglucosamine (d-ks) were contained in the solution that was digested by the enzyme and were measured using HPLC. Standard KS derived from bovine cornea (Seikagaku) was used to measure KS under identical conditions; and the quantity of KS in each serum sample was calculated as the sum of m-ks and d-ks. To determine C6S concentration, 0.2 ml of each serum sample was first treated with chondroitinase ABC (Seikagaku). The quantity of unsaturated disaccharide contained in the digested fluid was determined and C6S was detected by HPLC [16]. For the determination of CS846, COMP and HA, the Aggrecan Chondroitin Sulfate 846 Epitope ELISA Kit (IBEX Technologies, Inc., Montreal, Quebec, Canada), Human COMP ELISA Kit (Kamiya Biomedical Company, Seattle, WA, USA) and Hyaluronan Assay Kit (Seikagaku Corporation) were used respectively.

Statistical analysis

To determine the statistical significance of inter-group differences, Steel's multiple comparison test for patient group *vs* control and Wilcoxon rank-sum test for inter-patient group were conducted, and the *P*-level was set at <0.05.

Results

Arthroscopic findings in KT patients and serum concentrations of KS, C6S, CS846, HA and COMP

All KT patients had articular cartilage damage. Their cartilaginous damage scores (SFA) for the recent KT group and the old KT group were 1.2 ± 0.7 and 3.8 ± 3.9, respectively. On X-ray examination, no changes were noted in the knee or intervertebral joints (Fig. 1).

The serum concentrations of KS, C6S, CS846, HA and COMP in KT patients are shown in Table 1. KS, C6S, CS846 and COMP were significantly higher in the recent KT group ($P=0.001$, $P=0.047$, $P=0.022$ and $P=0.001$, respectively), and KS and COMP higher in the old KT group (both $P<0.001$) than in controls.

X-ray and arthroscopic examination of OA patients and serum concentrations of KS, C6S, CS846, HA and COMP

The SFA scores of OA patients distributed by their KL grade are presented in Table 2. The SFA score increased in relation with

Fig. 1. Representative radiographs of the knee and lumbar spine and photograph of the articular cartilage in the knee of a 36-yr-old man with traumatic arthropathy. The X-ray grade, SFA score and serum KS concentration were normal, 0.7 and 2537 ng/ml, respectively. The serum KS concentration was high, although damage of the cartilage was minimal.

increased KL grade. Even in patients with KL grade 0 OA, degeneration or damage of the articular cartilage surface was observed by direct optical methods. In such patients, no X-ray findings were detected in the knee nor in the intervertebral discs (Fig. 2).

Serum concentrations of KS were significantly higher in most OA stages (KL grade 0: $P = 0.004$, I: $P < 0.001$, III: $P = 0.004$ and IV: $P = 0.008$) and serum COMP were significantly higher in all OA stages (KL grade 0: $P = 0.004$, I: $P = 0.002$, II: $P = 0.008$, III: $P = 0.002$ and IV: $P < 0.001$) than in controls. C6S and HA in patients with KL grade IV were significantly higher than in controls (both $P < 0.001$).

Table 1. Serum concentration of markers of cartilage degeneration or damage in patients with knee trauma

	Healthy subjects	KT patients	
		Recent trauma[a]	Old trauma[b]
n	24	5	14
SFA		1.2 ± 0.7	3.8 ± 3.9
KS (ng/ml)	910 ± 145	2095 ± 594*	1373 ± 418*
C6S (ng/ml)	97 ± 28	122 ± 10*	104 ± 22
CS846 (ng/ml)	137 ± 24	214 ± 77*	142 ± 46
COMP (ng/ml)	1030 ± 150	1572 ± 182*	1350 ± 250*
HA (ng/ml)	41 ± 15	44 ± 19	39 ± 12

The values are the mean ± S.D.
[a]Patients evaluated within 2 months after the injury.
[b]Patients evaluated >2 months after the injury.
*$P < 0.05$ *vs* Healthy subjects (Steel's multiple comparison test).
SFA, Societé Francaise d' Arthroscopie score; KS, keratan sulfate; C6S, Chondroitin-6-sulfate; CS846, cartilage proteoglycan aggrecan turnover epitope; COMP, cartilage oligomeric protein and HA, hyaluronic acid.

Comparison of the serum markers between patient groups

Since the serum concentrations of KS and COMP were higher in most stages of KT and OA than in controls, those differences between stages were compared in Fig. 3. The serum KS in the recent KT group (2095 ± 594 ng/ml) was significantly higher than that in the old KT group (1373 ± 418 ng/ml; $P = 0.021$) and those in OA patients with KL grades 0 and I (1456 ± 334 ng/ml) showed a tendency that was higher than that in patients with KL grades II, III and IV (1248 ± 220 ng/ml; $P = 0.084$). The serum concentrations of COMP in the recent KT group (1572 ± 182 ng/ml) showed a tendency that was higher than that in the old KT group (1350 ± 250 ng/ml; $P = 0.079$), but those in OA patients showed no difference between the patient group with KL grades 0 and I (1639 ± 434 ng/ml) and the patient group with KL grades II, III and IV (1731 ± 355 ng/ml).

Discussion

This study showed that the serum concentration of KS was high in patients with early-stage damage of the articular cartilage undetectable by X-ray imaging. Serum KS may be suitable as a screening test for articular cartilage damage and to monitor the natural course of articular damage or repair.

In the KT patients with recent injuries, KS was significantly higher than in those with old injuries, suggesting that serum

Table 2. Serum concentration of markers of cartilage degeneration or damage in OA patients

X-ray grade	Healthy subjects	OA patients 0	I	II	III	IV
n	24	7	7	4	6	7
SFA		2.4 ± 2.1	5.1 ± 3.5	28.8 ± 47.5	>100	>200
KS (ng/ml)	910 ± 145	1501 ± 360*	1411 ± 326*	1253 ± 241	1352 ± 242*	1155 ± 176*
C6S (ng/ml)	97 ± 28	116 ± 18	115 ± 15	102 ± 22	131 ± 22	157 ± 53*
CS846 (ng/ml)	137 ± 24	147 ± 67	151 ± 108	140 ± 70	112 ± 16	211 ± 184
COMP (ng/ml)	1030 ± 150	1710 ± 550*	1570 ± 310*	1580 ± 200*	1630 ± 470*	1907 ± 268*
HA (ng/ml)	41 ± 15	80 ± 65	72 ± 21*	76 ± 68	116 ± 76	258 ± 230*

The patients were grouped by their X-ray grade.
The values are the mean ± S.D.
*$P < 0.05$ *vs* healthy subjects (Steel's multiple comparison test).
SFA, Societé Francaise d' Arthroscopie score; KS, keratan sulfate; C6S, chondroitin-6-sulfate; CS846, cartilage proteoglycan aggrecan turnover epitope; COMP, cartilage oligomeric protein and HA, hyaluronic acid.

Fig. 2. Representative radiographs of the knee and lumbar spine and photograph of the articular cartilage in the knee of a 66-yr-old woman with KL grade 0 OA. The SFA score and serum KS concentration were 1.7 and 2145 ng/ml, respectively. The serum KS concentration was high, although the patient had early-stage OA.

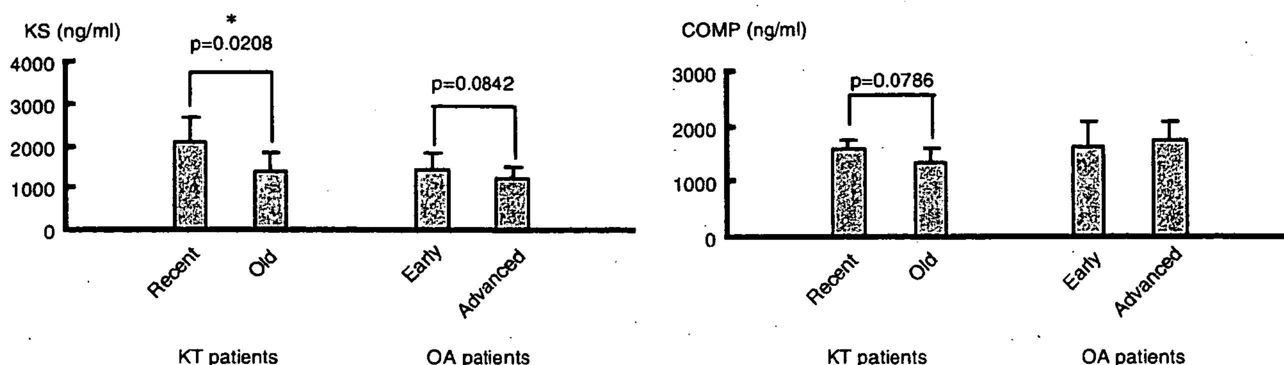


FIG. 3. Comparison of the serum markers between stage groups in KT or OA patients. KT patients were divided into a group with an injury <2 months old (recent) and a group with an injury >2 months old (old). OA patients were divided into a group with KL grades 0 and I (early) and a group with KL grades II, III and IV (advanced). Data are the mean ± S.D. *$P < 0.05$ (Wilcoxon rank-sum test).

KS might indicate the release of cartilaginous GAG in the early stage after injury in spite of moderate cartilaginous damage.

In OA patients, the serum concentrations of KS, C6S, HA and COMP were significantly higher than in healthy controls as reported previously [6, 17–19]. Among these parameters, KS was high in patients with KL grades 0 and I, indicating that KS might serve as a marker of early-stage OA. The KS concentrations in OA patients tended to decrease as the KL grade increased from 0 to IV, which reflects disappearance of the joint space. It may mean that in OA, a greater quantity of the cartilage matrix is released when the joint space has not yet narrowed. On the contrary, COMP was high in KL grade IV. This can be explained by the fact that COMP is a non-collagen protein that exists in the synovial membrane, meniscus and tendon, as well as in the cartilage and its increase is most likely related to the inflammation of various intra-articular tissues. The changes in C6S and HA were marked in KL grade IV, indicating that these are not markers of early-stage cartilage destruction.

KS is a component of proteoglycans found in the articular cartilage, intervertebral discs and corneas. Because corneas are relatively small tissues, serum KS mainly originates from articular cartilage and intervertebral discs. Thus, the serum concentration of KS is not only a marker of knee articular cartilage, but also of other joints and intervertebral discs. Therefore, before concluding that the elevated serum concentration of KS originated from damage to the knee joint articular cartilage, the possibility of spondyloarthropathy and OA in other joints must be examined. We verified that there were no X-ray changes in the lumbar spine nor symptoms caused by lumbar spinal abnormalities in KT patients (Fig. 1), although spondyloarthrotic changes existed in OA patients because most of these patients were of advanced age (Fig. 2). We verified that no OA symptoms were observed in joints other than the knee in these patients. We are planning to investigate serum KS in patients with spondyloarthropathy or intervertebral disk herniation in the future.

Serum KS is considered to reflect the normal metabolism of cartilage, and KS increases in case of mechanical injuries within a few months after injury. Budsberg *et al.* [20] found that serum KS increased 1–3 months after resection of the anterior cruciate ligaments of dog knees. In our report, KT patients who were evaluated within 2 months after the injury exhibited an acute release of KS. Although the SFA score of KT patients was very small, indicating that damage was confined, serum KS was high (Table 1). After the rapid release of KS ends, release from the injured surfaces continues at a relatively high rate. This phase is considered to continue for a few years to a couple of decades as in KT evaluated >2 months after the injury and in early-stage OA patients (KL grades 0 and I). The persistence of this condition leads to OA in a few decades. This phase corresponds to advanced OA (KL grades II, III, IV).

This report is the first study to show that serum KS increases early after an injury causing small articular damage and in patients with early-stage OA undetectable by X-ray imaging. As only a small volume of blood is required for the measurement of serum KS, this parameter may serve as a screening test to detect articular cartilage injury and it is expected to contribute greatly to the decision on a therapeutic strategy for the management of OA or cartilage injury.

Rheumatology key messagess

- Serum keratan sulfate correlates with damage of the articular cartilage.
- It may serve as a screening and monitoring test of the natural course of articular cartilage damage or repair.

Disclosure statement: The authors have declared no conflicts of interest.

References

1 Aroen A, Loken S, Heir S *et al.* Articular cartilage lesions in 993 consecutive knee arthroscopies. Am J Sports Med 2004;32:211–5.
2 Curl WW, Krome J, Gordon ES, Rushing J, Smith BP, Poehling GG. Cartilage injuries: a review of 31,516 knee arthroscopies. Arthroscopy 1997;13:456–60.
3 Hjelle K, Solheim E, Strand T, Muri R, Brittberg M. Articular cartilage defects in 1,000 knee arthroscopies. Arthroscopy 2002;18:730–4.
4 Messner K, Maletius W. The long-term prognosis for severe damage to weight-bearing cartilage in the knee: a 14-year clinical and radiographic follow-up in 28 young athletes. Acta Orthop Scand 1996;67:165–8.
5 Shelbourne KD, Jari S, Gray T. Outcome of untreated traumatic articular cartilage defects of the knee: a natural history study. J Bone Joint Surg Am 2003;85–A (Suppl 2):8–16.
6 Thonar EJ, Lenz ME, Klintworth GK *et al.* Quantification of keratan sulfate in blood as a marker of cartilage catabolism. Arthritis Rheum 1985;28:1367–76.
7 Campion GV, McCrae F, Schnitzer TJ, Lenz ME, Dieppe PA, Thonar EJ. Levels of keratan sulfate in the serum and synovial fluid of patients with osteoarthritis of the knee. Arthritis Rheum 1991;34:1254–9.
8 Belcher C, Yaqub R, Fawthrop F, Bayliss M, Doherty M. Synovial fluid chondroitin and keratan sulphate epitopes, glycosaminoglycans, and hyaluronan in arthritic and normal knees. Ann Rheum Dis 1997;56:299–307.
9 Mundermann A, Dyrby CO, Andriacchi TP, King KB. Serum concentration of cartilage oligomeric matrix protein (COMP) is sensitive to physiological cyclic loading in healthy adults. Osteoarthr Cartilage 2005;13:34–8.
10 Bruyere O, Collette J, Kothari M *et al.* Osteoarthritis, magnetic resonance imaging, and biochemical markers: a one year prospective study. Ann Rheum Dis 2006;65:1050–4.
11 Mazzuca SA, Poole AR, Brandt KD *et al.* Associations between joint space narrowing and molecular markers of collagen and proteoglycan turnover in patients with knee osteoarthritis. J Rhematol 2006;33:1147–51.
12 Okumura M, Tagami M, Fujinaga T. Measurement of serum and synovial fluid keratan sulphate and antibody to collagen type II in equine osteoarthritis. J Vet Med A 1998;45:513–6.
13 Tomatsu S, Okamura K, Maeda H *et al.* Keratan sulphate levels in mucopolysaccharidoses and mucolipidoses. J Inherit Metab Dis 2005;28:187–202.

14 Kellgren JH, Lawrence JS. Radiological assessment of osteo-arthrosis. Ann Rheum Dis 1957;16:494–502.
15 Ayral X, Altman RD. Arthroscopic evaluation of knee articular cartilage. In: Brandt KD, Doherty M, Lohmander LS, eds. Osteoarthritis. Oxford: Oxford University Press, 1998;494–505.
16 Shinmei M, Miyauchi S, Machida A, Miyazaki K. Quantitation of chondroitin 4-sulfate and chondroitin 6-sulfate in pathologic joint fluid. Arthritis Rheum 1992;35:1304–8.
17 Uesaka S, Nakayama Y, Shirai Y, Yoshihara K. Serum and synovial fluid levels of chondroitin sulfate in patients with osteoarthritis of the knee joint. J Nippon Med Sch 2001;68:165–70.
18 Salisbury C, Sharif M. Relations between synovial fluid and serum concentrations of osteocalcin and other markers of joint tissue turnover in the knee joint compared with peripheral blood. Ann Rheum Dis 1997;56:558–61.
19 Gemero P, Pipermo M, Gineyts E, Christgau S, Delmas PD, Vignon E. Cross sectional evaluation of biochemical markers of bone, cartilage, and synovial tissue metabolism in patients with knee osteoarthritis: relations with disease activity and joint damage. Ann Rheum Dis 2001;60:619–26.
20 Budsberg SC, Lenz ME, Thonar EJ. Serum and synovial fluid concentrations of keratan sulfate and hyaluronan in dogs with induced stifle joint osteoarthritis following cranial cruciate ligament transection. Am J Vet Res 2006;67:429–32.

J Bone Miner Metab (2007) 25:130–137
DOI 10.1007/s00774-006-0738-4

© Springer 2007

ORIGINAL ARTICLE

**Takuo Fujita · Hajime Orimo · Tetsuo Inoue
Kiyoshi Kaneda · Minoru Sakurai · Rikushi Morita
Kichizo Yamamoto · Yoichi Sugioka · Akio Inoue
Kunio Takaoka · Itsuo Yamamoto · Yuichi Hoshino
Hiroshi Kawaguchi**

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

Received: July 24, 2006 / Accepted: October 31, 2006

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 400mg 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, 200mg etidronate was used instead of 400mg (Group B, Low Dose Etidronate Group). In 138 patients, 1μg 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

T. Fujita (✉)
Calcium Research Institute, Katsuragi Hospital, 250 Makamicho, Kishiwada, Osaka 596-0842, Japan
Tel. +81-724-28-5141; Fax +81-724-27-1501
e-mail: fujita@katsuragi-hosp.or.jp

H. Orimo
University of Health Science, Tokyo, Japan

T. Inoue
Aoyama General Hospital, Toyohashi, Japan

K. Kaneda
Bibai Rosai Hospital, Bibai, Japan

M. Sakurai
Tohoku Central Hospital, Sendai, Japan

R. Morita
Shiga University of Medical Science Hospital, Otsu, Japan

K. Yamamoto
Hakuai Hospital, Yonago, Japan

Y. Sugioka
Kyushu Rosai Hospital, Fukuoka, Japan

A. Inoue
Dept. of Orthopedic Surgery, Yanagawa Rehabilitation Hospital, Yanagawa, Japan

K. Takaoka
Dept. of Orthopedic Surgery, Medical School, Osaka City University Graduate School of Medicine, Osaka, Japan

I. Yamamoto
Yamamoto Clinic, Shiga, Japan

Y. Hoshino
Dept. of Orthopedic Surgery, Jichi Medical School, Tochigi, Japan

H. Kawaguchi
Dept. of Orthopedic-Spinal Surgery, Graduate School of Medicine and Faculty of Medicine, University of Tokyo, Tokyo, Japan

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.

Table 1. Scoring method for the diagnosis of involutional osteoporosis

Item	Score
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

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.

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 200mg	137	2.527	4.814	<0.0001
	EHDP 400mg	137	3.639	5.861	<0.0001
	Alfacalcidol 1μg	140	−0.336	4.286	0.3295
Original data	EHDP 200mg	104	1.918	4.327	<0.0001
	EHDP 400mg	110	2.921	5.445	<0.0001
	Alfacalcidol 1μg	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 200mg	0.4441 (0.1921–1.026)	0.4031 (0.1598–1.0176)	0.5534 (0.1960–1.5025)
	EHDP 400mg	0.3466 <u>(0.1420–0.8464)</u>	0.3097 <u>(0.1172–0.8181)</u>	0.4959 (0.1679–1.4645)

Significant range is underlined

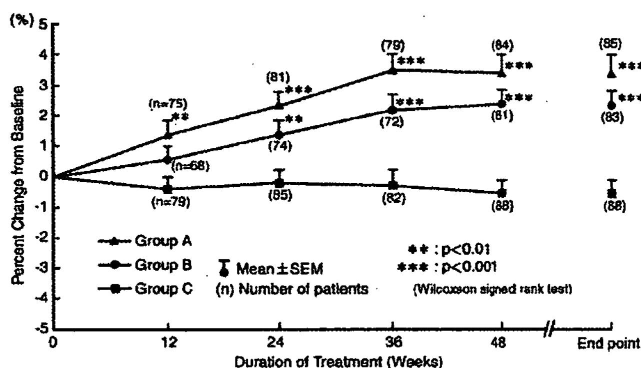


Fig. 1. Percent (%) changes of the lumbar spine bone mineral density (BMD) L2–L4 from baseline during the trial period (*vertical axis*) and duration of treatment in weeks (*horizontal axis*). Values for Group A at 12 weeks and subsequently those for Group B at 24 weeks and subsequently were both significantly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

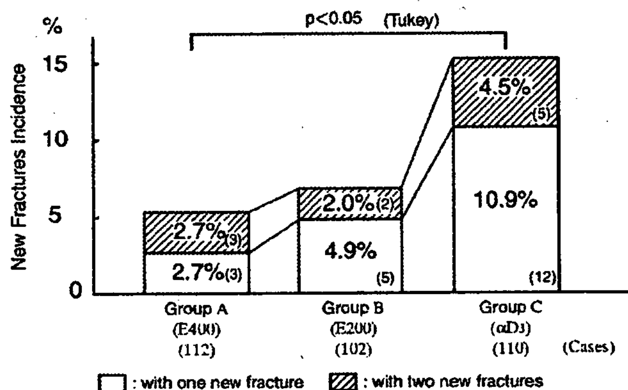


Fig. 2. Incident vertebral fracture during the test period. In Group C, one fracture occurred in 10.9% and two fractures in 4.5%; in Group A, the corresponding values were 2.7% and 2.7%, respectively, significantly lower than the former by the Tukey test; and in Group B, the corresponding values were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

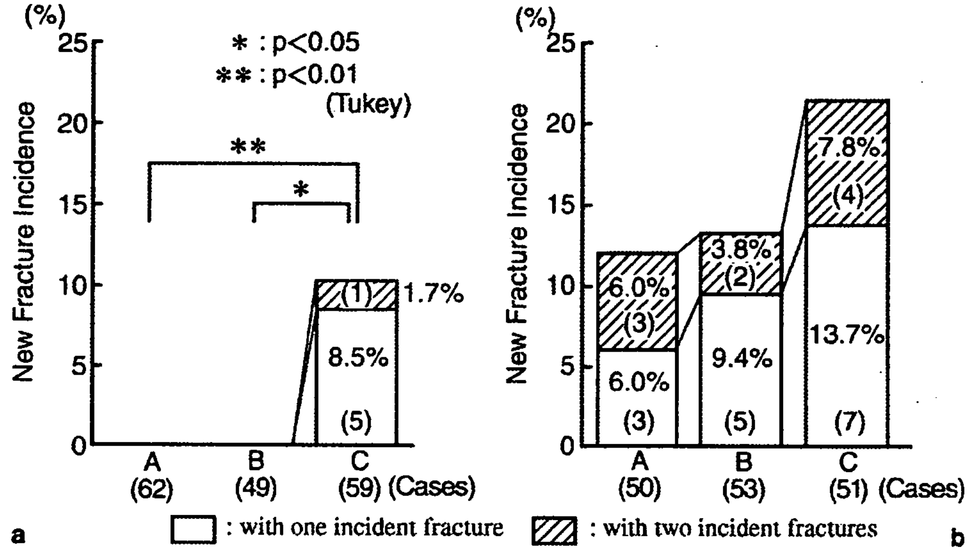
Group C ($P < 0.001$). The results of intention-to-treat analysis on lumbar spine BMD are summarized in Table 6. Significant increase was noted in both groups given etidronate. Figure 2 illustrates new fracture in patients with one new vertebral fracture and those with multiple new vertebral fractures. 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 400mg 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



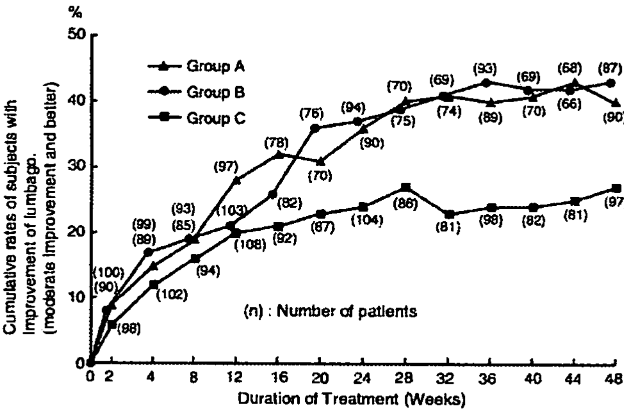


Fig. 4. Cumulative rates of definite improvement of backache: cumulative percentages of subjects with improvement of lumbago (*vertical axis*) and duration of treatment in weeks (*horizontal axis*). Group A and B gave higher values than Group C at 24 weeks

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-

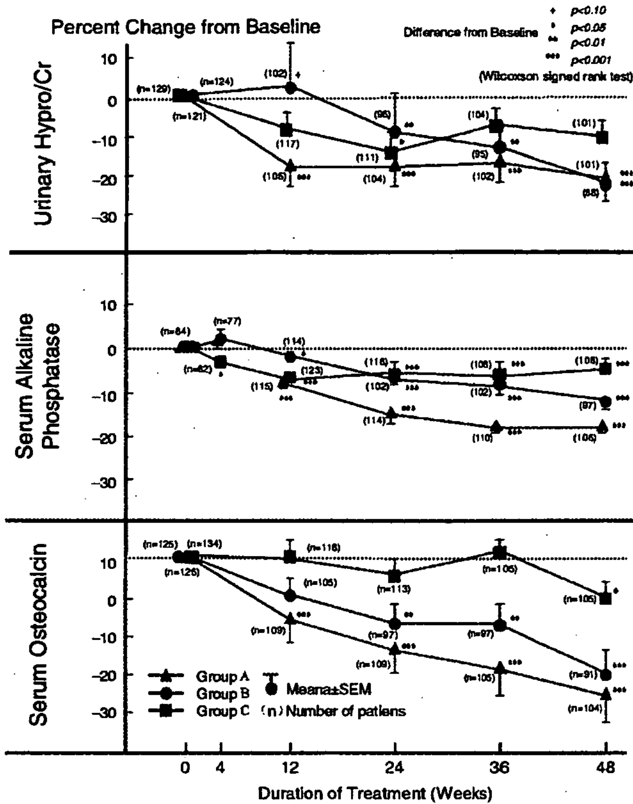


Fig. 5. *Top*: Urinary hydroxyproline/Cr (*vertical axis*) relative to duration of treatment in weeks (*horizontal axis*). A significant decrease of urinary hydroxyproline/creatinine ratio from the baseline value was noted after 12 weeks of treatment in Group A and after 24 weeks of treatment in Group 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 400mg 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, 500–550mg/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 400mg etidronate than 200mg 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.

Acknowledgments The loss of two original coauthors is deeply regretted. Dr. Masaaki Fukase of Kobe University passed away in March 1995, immediately after the Kobe earthquake. Dr. Hirotoshi Morii, Professor Emeritus, Osaka City University, passed away on April 6, 2006. This double-blind clinical test was supported by Dainippon Sumitomo Pharma, Osaka, Japan, with Dr. Y. Shimakoshi as the coordinator. The cooperation of Chugai Pharmaceutical Co., Ltd. and Procter & Gamble Pharmaceuticals is also appreciated. The following 36 centers participated the study: Hokkaido University (Orthopedic Surgery), Bibai Rosai Hospital (Orthopedic Surgery), Shin-Sapporo Orthopedic Hospital (Orthopedic Surgery), Eniwa Hospital (Orthopedic Surgery), Tohoku University (Orthopedic Surgery), The University of Tokyo (Geriatrics, Orthopedic Surgery, and Orthopedic Surgery of the University of Tokyo Hospital Branch), Kyorin University (Orthopedic Surgery), Tokyo Metropolitan Rehabilitation Hospital (Orthopedic Surgery), Yamanashi Medical College (Orthopedic Surgery), Hamamatsu University (Orthopedic Surgery), Aobadai Fukuchi Orthopedic Surgical Hospital, Toyama Medical and Pharmaceutical University (Orthopedic Surgery), Shakaihoken Takaoka Hospital (Orthopedic Surgery), Aichi Medical University (Clinical Laboratories), Hachiya Orthopedic Surgical Hospital, Shiga University of Medical Science (Radiology and Nuclear Medicine), Takashima Grand Hospital, Kyoto Municipal Hospital (Internal Medicine), Osaka City University (Second Department of Internal Medicine), Hoshigaoka Kouseinenkin Hospital (Orthopedic Surgery), Aijinkai Takatsuki Hospital (Internal Medicine), Komatsu Hospital (Orthopedic Surgery), Kobe University (Third Division, Department of Medicine), Hyogo College of Medicine (Orthopedic Surgery), Okayama University (Orthopedic Surgery), Kawasaki Medical School (Nuclear Medicine), Okayama City Hospital (Orthopedic Surgery), Tottori University (Orthopedic Surgery), San-in Rosai Hospital (Orthopedic Surgery), Kagawa Medical College (Orthopedic Surgery), Spine Injury Center (Orthopedic Surgery), Kurume University (Orthopedic Surgery), Nagasaki University (First Department of Internal Medicine and Orthopedic Surgery), and University of the Ryukyus (Orthopedic Surgery).

References

1. Sato M, Graser W (1990) Effect of bisphosphonates on isolated rat osteoclasts as examined by reflected light microscopy. J Bone Miner Res 5:31–40
2. Carano A, Teitelbaum SL, Konsek JD, Schlesinger PH, Blair HC (1990) Bisphosphonates directly inhibit the bone resorption activity of isolated avian osteoclasts in vitro. J Clin Invest 85:456–461

3. Hughes DE, MacDonald RR, Russell RGG, Gowen M (1989) Inhibition of osteoclasts-like cell formation by bisphosphonate in long term culture of human bone marrow. J Clin Invest 83:1930–1935
4. Heaney RP, Saville PD (1976) Etidronate disodium in postmenopausal osteoporosis. Clin Pharmacol Ther 20:593–604
5. Gennari C (1980) Disodium etidronate treatment of osteoporosis. In: Caniggia A (ed) Proceedings of the First International Symposium on Diphosphonate in Therapy, Pisa, Italy. Via Mazzini, Pisa, pp 133–146
6. Watts NB, Harris ST, Genant HK, Wasnich RD, Miller PD, Jackson RD, Licata AA, Ross P, Woodson GC III, Yanover MJ (1990) Intermittent cyclical etidronate treatment of postmenopausal osteoporosis. N Engl J Med 323:73–79
7. Cranney A, Guyatt G, Krolicki N, Welch V, Griffith L, Adachi JD, Shea B, Tugwell P, Wells G; Osteoporosis Research Advisory Group (ORAG) (2001) A meta-analysis of etidronate for the treatment of postmenopausal osteoporosis. Osteoporos Int 12:140–151
8. Ott SM, Woodson GC, Huffer WE, Miller PD, Watts NB (1994) Bone histomorphometric changes after cyclic therapy with phosphate and etidronate disodium in women with postmenopausal osteoporosis. J Clin Endocrinol Metab 78:968–972.
9. Hasling C, Charles P, Jensen FT, Mosekilde L (1994) A comparison of the effects of oestrogen/progestogen, high-dose oral calcium, intermittent cyclic etidronate and an ADFR regime on calcium kinetics and bone mass in postmenopausal women with spinal osteoporosis. Osteoporos Int 4:191–203
10. Miller PD, Watts NB, Licata AA, Harris ST, Genant HK, Wasnich RD, Jackson PD, Hoseyni MS, Schoenfeld SL, Valent DJ, Chesnut GH III (1997) Cyclical etidronate in the treatment of postmenopausal osteoporosis: efficacy and safety after seven years of treatment. Am J Med 103:468–476
11. Fujita T (1992) Vitamin D in the treatment of osteoporosis. Proc Soc Exp Biol Med 199:394–399
12. Orimo H, Shiraki M, Hayashi Y, Nakamura T (1987) Reduced occurrence of vertebral fracture in senile osteoporosis treated with 1-alpha(OH) vitamin D_3. Bone Miner 3:47–52
13. Papadimitropoulos E, Wells G, Shea B, Gillespie W, Weaver B, et al; Osteoporosis Methodology Group and The Osteoporosis Research Advisory Group (2002) Meta-analyses of therapies for postmenopausal osteoporosis. VIII: Meta-analysis of the efficacy of vitamin D treatment in preventing osteoporosis in postmenopausal women. Endocr Rev 23:560–569
14. De Leenheer AP, Bauwens RM (1985) Comparison of a cytosol radioreceptor assay with a radioimmunoassay for 1,25-dihydroxyvitamin D in serum or plasma. Clin Chim Acta 152: 143–154
15. Haddad JG, Chyu KJ (1971) Competitive protein-binding radioassay for 25-hydroxycholecalciferol. J Clin Endocrinol Metab 33:992–995
16. Slatopolsky E, Martin K, Morrissey J, Hruska K (1982) Current concepts of the metabolism and radioimmunoassay of parathyroid hormone. J Lab Clin Med 99:309–316
17. Sone T, Tomomitsu T, Fukunaga M (1995) Standardization of diagnosis with various dual X-ray absorptiometry systems (in Japanese). Osteoporosis Jpn 3:223–225
18. Jowsey J, Riggs BL, Kelly PJ, Hoffman DL, Bordier P (1971) The treatment of osteoporosis with disodium ethene-1,1-diphosphonate. J Lab Clin Med 78:574–584
19. Volkema R, Vismans F-J, Papapoulos SE, Pauwels EK, Bijvoet OLM (1989) Maintained improvement in calcium balance and bone mineral content in patients with osteoporosis treated with the bisphosphonates APD. Bone Miner 5:183–192
20. Storm T, Thamsborg C, Steiniche T, Genant HK, Soerensen OH (1990) Effect of intermittent cyclical etidronate therapy on bone mass and fracture rate in women with postmenopausal osteoporosis. N Engl J Med 322:1265–1271

CLINICAL ORTHOPAEDICS AND RELATED RESEARCH
Number 461, pp. 162–169
© 2007 Lippincott Williams & Wilkins

Reconstruction of Bone Defects Using rhBMP-2–coated Devitalized Bone

Susumu Taguchi, MD; Takashi Namikawa, MD; Makoto Ieguchi, MD; and Kunio Takaoka, MD, PhD

Massive bone defects often are caused by radical resection of bone tumors. Reconstruction of the defect by reimplantation of the resected bone segment after it has been devitalized is advantageous because of its ability to match the size of the defect. In addition, this technique carries a low risk for local recurrence of the tumor, avoids immunologic reaction, and is low in cost. However, limited osteogenic potential of the devitalized bone often leads to delayed union, gradual resorption, and mechanical weakness of the reimplanted segment. We applied rhBMP-2 in a biodegradable polymer delivery system to the devitalized bone. Middiaphyseal bone defects were created by resection in rat femurs. The resected segments were autoclaved at 135°C for 15 minutes, coated with a rhBMP-2-retaining paste on the outer surface, and then reimplanted into the defects. In a brief time, newly formed bone was seen on the surface of the devitalized bone. After 12 weeks, a solid bone mass encasing the dead bone segments was consistently formed and abundant new bone formation was visible in the segments as they were remodeled. The amount of new bone formed could be regulated by the amount of the rhBMP-2-retaining paste applied to the bone segments. This method presents a new approach for the reconstruction of bone defects.

Limb salvage surgery for treatment of malignant bone tumors has become an established modality with a low incidence of local tumor recurrence.[12,32] This successful treatment modality has been realized in combination with the advent of preoperative and/or postoperative adjuvant chemotherapy. Modern imaging technology has been a key factor because it permits delineation of the extent of the tumor dictating the margins needed for successful wide resection. Skeletal reconstruction after wide resection is essential to restore the supporting function of the involved limbs and trunk. Some treatment modalities include vascularized[24,31] or nonvascularized autogenous bone grafting, allogeneic bone grafting, and tumor prostheses. Disadvantages of these methods include limited bone sources; poor matching of bone grafts to the size of the defect; compromised mechanical strength of donor bone; risk for disease transfer with allogeneic bone; high cost of maintaining a bone banking system for allografting; and high incidence of aseptic loosening, prosthesis breakage, and infection resulting from prosthetic replacement.[15,16,20,35,36] Reimplantation of tumor-bearing bone segments devitalized after resection by means of autoclaving,[6] pasteurization,[14,28,29] or radiation[2,5] has been used to reconstruct diaphyseal long bone defects. This approach is most appropriate when the devitalized bone segment retains substantial mechanical strength. The advantages of this method are proportional sizing to accommodate the bone defect, low antigenicity with little immunologic reaction from the host, and low cost.[11] However, previous reports describe some disadvantages,[7,33] including the limited osteogenic potential of the devitalized bone graft, resulting in impaired union of the graft to the host bed and gradual resorption of the grafted bone with replacement by fibrous tissue leading to compromised mechanical strength or fracture of the graft.[1,9]

We hypothesized a reimplantation method that uses rhBMP-2 in a biodegradable polymer delivery system applied to the devitalized bone would improve on these disadvantages. We assessed the usefulness of this reconstruction method by answering three research questions: (1) Would application of rhBMP-2 to the reimplanted bone segments result in improved incorporation of the grafts with better preservation of length compared to implantation of devitalized bone grafts alone?; (2) Would the union

Received: May 29, 2006
Revised: November 24, 2006; January 28, 2007
Accepted: March 9, 2007
From the Department of Orthopedic Surgery, Graduate School of Medicine, Osaka City University, Osaka City, Japan.
Each author certifies that he or she has no commercial associations (eg, consultancies, stock ownership, equity interest, patent/licensing arrangements, etc) that might pose a conflict of interest in connection with the submitted article.
Each author certifies that his or her institution has approved the animal protocol for this investigation and that all investigations were conducted in conformity with ethical principles of research.
Correspondence to: Susumu Taguchi, MD, 1-4-3 Asahi-machi Abeno-ku Osaka City, Osaka Pref. Japan. Phone: 81-6-6645-3851; Fax: 81-6-6646-6260; E-mail: taguchi@med.osaka-cu.ac.jp.
DOI: 10.1097/BLO.0b013e318059ae44

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

of the augmented grafts to the host bed be evidenced by increased callus formation around the grafts and by evidence of early bone turnover of the devitalized bone leading to the appearance of new bone in the augmented grafts?; and (3) Would the augmented femoral, intercalated, devitalized bone segments acquire more sufficient mechanical bone strength than the unaugmented segments?

MATERIALS AND METHODS

Fifty inbred Sprague-Dawley male rats (7 weeks old, approximately 300 g body weight; Japan SLC Co, Shizuoka, Japan) were randomly divided into three groups (30 for the experimental group, 20 for two control groups) (Table 1).

Each animal was anesthetized by an intramuscular injection of ketamine (30 mg/kg body weight) and xylazine (10 mg/kg body weight). After removal of hair with an electric hair cutter and sterilization of the right thigh skin with 10% povidone iodine and 80% ethanol, the middiaphysis of the femur was exposed through a lateral longitudinal skin incision. An 8-mm-long bone segment with the periosteum attached was removed with a cutting saw. After removing the soft tissue from the bone segment, the bone segment then was autoclaved at 135°C for 15 minutes and reimplanted into the femoral defect. In the experimental group, the cortical surface of the reimplanted bone segment was coated with rhBMP-2-retaining paste (60 mg, 30 mg, or 15 mg in weight) prepared as described below. The cortical surfaces of the replanted segments from animals from the control groups were coated with either 60 mg paste without rhBMP-2 or no paste (CP and C groups, respectively). After reimplantation, all segments were fixed using an 18-gauge injection needle inserted as an intramedullary pin. The wound was closed with 4-0 nylon sutures.

The rhBMP-2-retaining paste consisted of rhBMP-2 (Genetics Institute, Boston, MA); polylactic acid/polyethylene glycol block copolymer (PLA-PEG), a synthetic biodegradable polymer with a molecular weight of 9200 (Taki Chemicals Co Ltd, Kakogawa, Japan); and beta-tricalcium phosphate (β-TCP) powder with a particle size less than 100 μm in diameter (Olympus Optics Co, Tokyo, Japan). The physicochemical characteristics and efficacy of the block copolymer as carrier material for rhBMP-2 has been reported.[21,23] To generate the rhBMP-2-retaining paste, we warmed 300 mg PLA-PEG polymer to 50°C, added 100 μg rhBMP-2 and 300 mg β-TCP powder, mixed well, and cooled to yield a doughlike paste. Another batch that lacked rhBMP-2 was made and used as a control. Both batches were stored in a deep freezer at −70°C until use.

At surgery, the animals from the experimental group were subdivided into three groups (10 per respective group) and 60 mg, 30 mg, or 15 mg of the rhBMP-2-retaining paste was put on the cortical surface of the autoclaved bone segments. These groups were designated BP60, BP30, and BP15, respectively. During the postoperative period, all rats were fed normal diets. All animals were housed and fed in cages without any limitation of motion and with free access to food and water.

The effect of the application of the rhBMP-2-retaining paste on the incorporation of the reimplanted autograft segments into the femoral defect was evaluated by radiographic, histologic, and biomechanical tests. Plain radiographs of the hind limbs were taken under anesthesia to check for new bone formation in and around the femoral defect sites. The appearance of callus was assessed radiographically and mean radiographic scores were calculated for each group according to the modified scoring system proposed by Yuehuei[37] (Table 2). Loss of length of the right femurs resulting from collapse of the devitalized bone segments was measured with calipers and was expressed as a percentage of the length of the contralateral, intact femur. The femurs obtained at each respective interval (2, 4, 8, and 12 weeks after surgery) from each group were processed for routine histologic examination to identify newly formed cartilage, bone, and marrow tissue. Before histologic processing, the intramedullary fixation pins were removed from the femurs. The specific areas of interest for the radiographic and histologic examinations were the junctions of the devitalized bone with the vital cut ends and the circumference of the reimplanted devitalized bone segments. The samples were fixed in neutral-buffered 10% formalin solution, decalcified in 5% formic acid at 4°C, dehydrated in a gradient of ethanol solutions, embedded in paraffin, sectioned at the midsagittal plane at 5-μm thickness, and stained with hematoxylin and eosin. Tartrate-resistant acid phosphatase (TRAP), a marker enzyme specific to osteoclasts, was used to identify these cells in contact with the devitalized bone segments. Briefly, deparaffinized sections were placed in the TRAP staining solution consisting of acetate buffer (pH 5.0) containing 50 mmol/L sodium tartrate, 25 mg/mL naphthol AS-MX phosphate (Sigma Chemical Co, St Louis, MO), and 0.5 mg/mL fast red violet salt (Sigma). The specimens were incubated with the solution at 37°C for 20 minutes. After the solution was removed by washing, the specimens were counterstained with hematoxylin. All of the prepared histologic sections were observed under a light

TABLE 1. Implant Assignment

Group	rhBMP-2 (μg)	PLA-PEG (mg)	β-TCP (mg)	Concentration of rhBMP-2 (% weight)	Number of Animals
BP60	10	30	30	0.0166	10
BP30	5	15	15	0.0166	10
BP15	2.5	7.5	7.5	0.0166	10
CP60	0	30	30	0	10

PLA-PEG = polylactic acid/polyethylene glycol; β-TCP = beta-tricalcium phosphate

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

TABLE 2. Radiographic Scoring System

Category	Score
New bone formation in the defect	
Full (across the defect)	3
Moderate (> 50%)	2
Mild (< 50%)	1
None	0
Proximal osteotomy union	
Union	3
Moderate bridge (> 50%)	2
Mild bridge (< 50%)	1
Nonunion	0
Distal osteotomy union	
Union	3
Moderate bridge (> 50%)	2
Mild bridge (< 50%)	1
Nonunion	0
Graft-host bone junction	
Cortex to cortex (both side)	3
Cortex to cortex (one side)	2
Cortex to trabecula	1
No connection	0
Remodeling	
Full remodeling cortex	2
Intramedullary canal	1
No remodeling	0
Maximum total score	14

microscope. At 12 weeks postoperatively, five animals from each group were sacrificed and the femurs of both sides were harvested. After being radiographed, the harvested femurs were subjected to mechanical testing. The mechanical strength of the femurs with autoclaved bone segments was tested in two steps, ie, manual bending and three-point bending strength tests using a mechanical apparatus (EZ Graph; Shimazu Co, Kyoto, Japan) equipped with a computer for data acquisition. When the reimplanted site of the femur was unstable and flexed easily with mild manual bending, repair of the defect was judged to have failed. When the defect site was solid, the femur was judged to be consolidated and repaired. For quantitative evaluation of the consolidation, the samples were subjected to the three-point bending machine test to evaluate maximum bending strength of the reimplanted femur. Contralateral femurs also were tested in the same manner to obtain normal standard values. The femurs were placed horizontally on two rounded supporting bars located at a distance of 20 mm. The bone was loaded at the midpoint of the diaphysis at the osteogenesis area, including the transplantation site in the anteroposterior plane, by lowering the third bar at a rate of 1 mm per minute until fracture occurred and the load displacement curve was recorded. The ultimate load, and the force causing fracture, was determined from the load displacement curve.

Intergroup comparisons of the radiographic scores, differences in femoral length, and results of the biomechanical testing of the femurs were performed using the Kruskal-Wallis test. Significance was defined as p values less than 0.01. When appropriate, differences between experimental groups were evaluated with the post hoc Bonferroni/Dunn test. All sections and radiographic images were viewed by two individuals (TN, KT) who were blinded to the results.

RESULTS

When assessed by gross appearance at 12 weeks after surgery, the reimplanted femurs harvested from the BP15 group were found to be completely repaired as opposed to none of those in the control groups CP60 and C (Fig 1). The average lengths of the reimplanted femurs expressed as a percentage of the contralateral intact femur after 12 weeks were 92.3% in the C group and 97.8% in the CP60 group. No reduction in the femoral length was seen in the

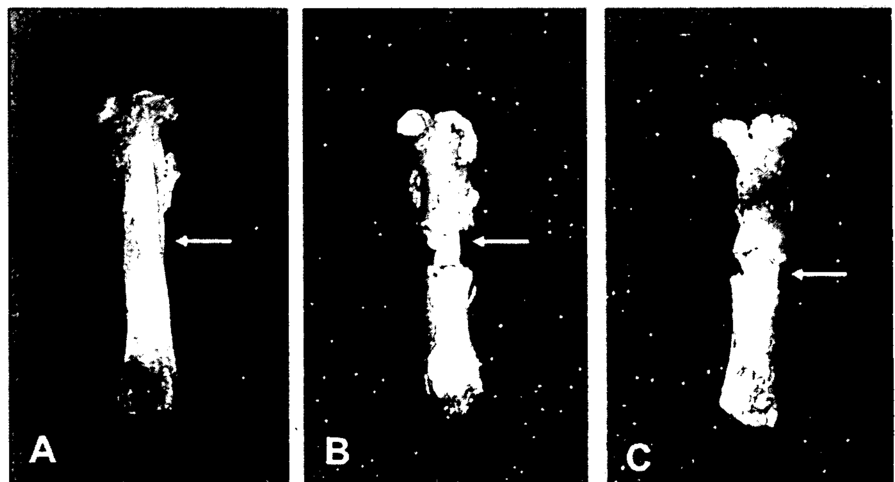


Fig 1A–C. The gross appearance of femurs harvested 12 weeks after reimplantation of 8-mm-long autoclaved segments into intercalated defects is shown. (A) The devitalized bone segment was coated with 15 mg rhBMP-2-retaining paste (containing 2.5 μg rhBMP-2) at surgery. (B) The segment was coated with 30 mg paste without rhBMP-2. (C) The segment was not coated with the paste. The reimplanted bone segment (arrow) is completely repaired in (A) but not in (B) and (C).

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

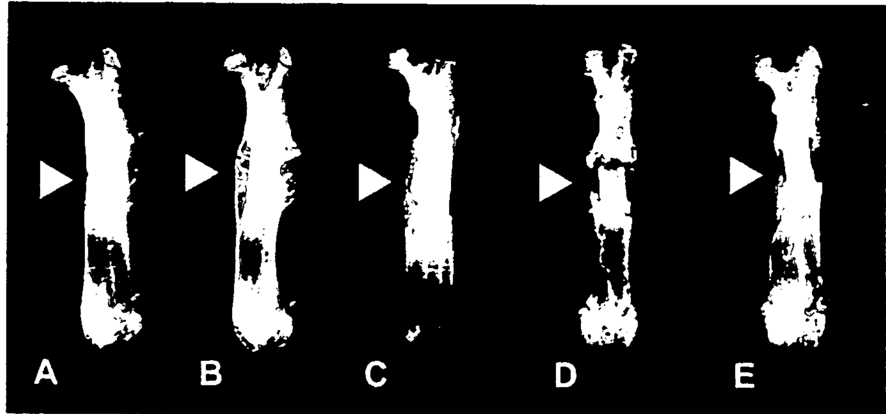


Fig 2A–E. Radiographs of femurs reimplanted with autoclaved bone segments with or without rhBMP-2-retaining paste and harvested 4 weeks after surgery are shown. (A) In the BP60 group, autoclaved bone was coated with 60 mg rhBMP-2-retaining paste (containing 10 μg rhBMP-2). (B) In the BP30 group, autoclaved bone was coated with 30 mg rhBMP-2-retaining paste (containing 5 μg rhBMP-2). (C) In the BP15 group, autoclaved bone was coated with 15 mg rhBMP-2-retaining paste (containing 2.5 μg rhBMP-2). (D) In the CP60 group, autoclaved bone was coated with 60 mg paste without rhBMP-2. (E) In the C group, autoclaved bone was left uncoated. Arrowheads indicate the original defect sites reimplanted with autoclaved bone segments. The callus is more evident bridging the cut ends of femur defects in the BP groups. In control animals (the CP60 and C groups), callus formation was less obvious than in the BP groups.

BP60 (100.7%), BP30 (100.7%), and BP15 (100.5%) groups. The control femurs (CP60 and C) were shortened ($p < 0.01$) compared with those in the BP groups.

At 4 weeks after reimplantation, encapsulating callus formation was observed in all BP groups. Shell-shaped calcified shadows bridging the cut ends of the femur and encasing the reimplanted devitalized bone were seen. In the controls (CP60 and C), small areas of callus were seen, but these did not bridge the cut ends (Fig 2). At 12 weeks, the callus containing the reimplanted devitalized bone in the BP60 and BP30 group animals was remodeled to form cortical bone, with the unresorbed, reimplanted bone visible inside the new cortical bone. In the BP15 group animals, the bone defects were repaired with normal anatomic appearance. The femurs from the controls showed no obvious new bone or callus formation around the devitalized bone segments (Fig 3). The radiographic scores were higher ($p < 0.01$) in all BP groups compared with those of the controls at 4, 6, and 12 weeks after reimplantation (Fig 4).

Histologic sections prepared from all BP groups revealed qualitatively similar reactions over the experimental period. At 4 weeks, the newly formed bone was partially remodeled to form cortical bone connecting the cut ends of the femur, and new living bone and bone marrow were present in the marrow space. In the controls, no new bone was observed and the dead bone segments were not united to the cut ends of the femur (Fig 5). At 12 weeks, the diameter of the repaired bone in the BP groups remained larger than that of normal femurs, and part of the cortical bone of the reimplanted segment appeared to be incorporated to the host bone (Fig 6).

Fig 3A–E. Radiographs of femurs reimplanted with autoclaved bone segments with or without rhBMP-2-retaining paste and harvested 12 weeks after surgery are shown. (A) BP60, (B) BP30, (C) BP15, (D) CP60, and (E) C groups are described in Figure 2. Arrowheads indicate the original defect sites reimplanted with autoclaved bone segments. Newly formed bone mass size was proportional to the amount of paste implant material in the BP groups. The femurs from the control animals (the CP60 and C groups) showed no obvious new bone or callus formation around the devitalized bone segments.

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.