

Treatment of fracture non-union with tissue-engineered bone grafts

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Abstract

When pseudoarthrosis occurs due to poor union of a bone fracture, cancellous bone with high cellular activity is generally harvested from the ilium and then grafted to the affected area to facilitate bone union. However, graft harvest is associated with frequent complications and pain. If a tissue engineering approach was available, bone defects could be repaired without damaging normal tissues. Subjects were 13 graft patients with pseudoarthrosis (average age, 56.9 years; range, 17-82 years). Pseudoarthrosis affected the thoracolumbar spine, the femur, the clavicle, and the metatarsal bone. From the ilium (tibia in one patient), 10-20 mL of bone marrow fluid was collected, and then, it was cultured in the standard medium containing minimum essential medium (MEM). After 2 weeks in primary culture, cells were subsequently incubated with porous beta-tricalcium phosphate (TCP) in order to prepare tissue-engineered bone, according to the previously reported modified culturing technique. Tissue-engineered bone (TEB) was grafted around the non-union site of each affected bone. In all patients, X-ray and CT showed good bone formation at three months after surgery. The TEB graft underwent remodeling and bone union was confirmed. In 3 patients, bone biopsy was performed during removal of internal fixation and bone regeneration was confirmed histologically. This tissue-engineered artificial bone provides a graft with a high regenerative capacity that can be prepared by minimally invasive aspiration. Compared with iliac bone grafts, it is possible to markedly reduce postoperative pain and the loss of autogenous bone.

Introduction

Fracture non-union is permanent failure of healing following a broken bone (fracture). Non-union is a serious complication of a fracture and may occur when the fracture moves

too much, has a poor blood supply or gets infected. The normal process of bone healing is interrupted or stalled. A pseudo-joint (pseudoarthrosis) develops between the two fragments with cartilage formation and a joint cavity. The patient complains of persistent pain at the fracture site and may also notice abnormal movement or clicking at the level of the fracture. An x-ray plate of the fractured bone shows a persistent radiolucent line at the fracture. In general, if a nonunion is still evident at 6 months post injury it will remain unhealed without specific treatment, usually orthopedic surgery. Surgical treatment includes removal of all scar tissue from between the fracture fragments, immobilization of the fracture with metal plates, rods and or pins and autogenous bone graft. Autogenous bone grafting involves utilizing bone obtained from the same individual receiving the graft. Bone can be harvested from non-essential bones, such as from the iliac crest.

Autogenous bone grafts are ideal from the viewpoint of rejection and osteogenic potential, and also produce the best results clinically. When pseudoarthrosis occurs due to poor union of a fracture, cancellous bone with a high cellular activity is generally harvested from the ilium and grafted into the affected region to promote bone union.¹⁻³ However, autogenous bone cannot be used to reconstruct extensive bone defects, as there is naturally a limit to the amount that can be harvested. This method also has the drawback of causing damage to normal bone and soft tissues.

Harvesting cancellous bone from the ilium is associated with severe postoperative pain, and patients experience more pain at the donor site than at the graft site, resulting in poor patient satisfaction.⁴⁻¹⁶ Management of pain at the donor site is often difficult for both patients and their medical professionals. There have been reports of pelvic deformity and persistent pain lasting more than one year after surgery. If a tissue engineering approach could be used to produce autogenous bone by *ex vivo* culture, extensive bone defects could be repaired without damaging normal tissues.

The bone marrow cells are known to include stem cells that can differentiate in various directions.¹⁷ Culturing these marrow mesenchymal cells with differentiation factors and biomaterials can achieve regeneration of bone, cartilage, subcutaneous tissue, and so on.¹⁸⁻²⁰ Maniopoulos *et al.*²¹ reported the formation of calcified bone-like tissue when marrow cells were cultured with dexamethasone and beta-glycerophosphate, and stated that the mineralized matrix exhibited bone morphogenetic protein (BMP)-like activity. The mineralized matrix formed around these cells was morphologically similar to the actual bone matrix found in the body.²² It was also

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demonstrated by biochemical and gene expression studies that this culture method achieved differentiation of cells with a high level of osteoblastic activity.²³⁻²⁵ The cultured tissue was shown to be bone tissue that contained bone matrix and had a high level of osteoblastic activity, suggesting that this was a form of organ culture rather than cell culture. Combining this cultured bone tissue with synthetic bone material has allowed the production of tissue engineered bone (TEB) that contain proliferating bone marrow cells with an increased osteogenic potential.²⁶⁻²⁹

We have succeeded in developing TEB which shows a regenerative capacity comparable to that of cancellous bone.³⁰ In addition, we have improved the culture technique and have been able to successfully treat pseudoarthrosis using TEB grafts with a high bone regeneration capacity. We suggest that the TEB graft fabricated by autogenous marrow mesenchymal cell culture with ceramic as a scaffold makes it possible to treat pseudoarthrosis as is done using autogenous bone grafts, but with the minimally invasive procedure of bone marrow aspiration. Here, we report on the results obtained in 13 patients.

Materials and Methods

The present method of bone regeneration therapy using marrow mesenchymal cells was approved in 2000 by the university ethics review board. Our subjects were 13 patients with pseudoarthrosis and an average age of 56.9 years (range: 17-82 years). The thoracolumbar spine was involved in 10 patients, while nonunion affected the femur in 1 patient, the clavicle in 1 patient, and the metatarsal in 1 patient.

After the patients all gave informed consent, 10-20 mL of bone marrow fluid was collected from the iliac crest under local anesthesia (Figure 1 and Figure 2.1). To prevent bone marrow fluid coagulation, 1 ml heparin (1000 units, Fuji Pharma Co., Ltd., Tokyo, Japan) was placed in a 10-ml injector beforehand and was mixed with the bone marrow fluid. Bone marrow fluid was centrifuged (1000 rpm, 5 min), and after eliminating the supernatant and adding 5 mL physiological saline, it was again centrifuged. The supernatant was aspirated, and the heparin was removed prior to incubation. Bone marrow fluid was placed in a T75 flask (BD Falcon, Franklin Lakes, NJ, USA) with the standard medium of minimum essential medium (MEM; Sigma Co., St. Louis, MO, USA) containing an antibiotic (gentamicin; Schering Plough, Kenilworth, NJ, USA), 15% autogenous or fetal bovine serum (MP Biomedicals, Tokyo, Japan), and placed in a carbon dioxide incubator (temperature: 37°C; humidity: 100%; CO₂: 5%) (Figure 2.2). Culture medium was exchanged three times per week. After 2 weeks, the mesenchymal cells were treated with 0.1% trypsin (Nacalai Tesque, Kyoto, Japan), and the resulting cells were centrifuged at 1000 rpm for 5 minutes. The centrifuged cells were rinsed once using physiological saline, and a cell suspension of 10⁶ cells/mL was prepared. Then 1/10 of the cells thus obtained were cultured in T75 flasks containing standard medium, while the remaining cells were seeded onto porous beta-TCP (OSferion, G2, Olympus Co., Tokyo, Japan) in 6 well-plate (Falcon) and subcultured in the osteogenic medium of the standard medium containing 10 mM Na β-glycerophosphate (Merck&Co., Inc., Whitehouse Station, NJ, USA), 80 μg/mL vitamin C phosphate (L-Ascorbic Acid Phosphate Magnesium Salt n-Hydrate, C₆H₆O₉PMg_{2/3}.nH₂O, Wako Pure Chemical Industries, Ltd., Osaka, Japan), 10⁻⁸M dexamethasone (Sigma) and 10⁻⁷M estriol (Nacalai Tesque) for 3 weeks (Figure 2.3). Subcultures were done in a carbon dioxide incubator (temperature: 37°C; humidity: 100%; CO₂: 5%). Culture medium was exchanged three times per week. At one week before transplantation, mesenchymal cells from the culture in standard medium were reseeded to prepare cultured artificial bone. The cultured artificial bone, namely, tissue engineered bone (TEB) was rinsed twice with physiological saline, packed under aseptic conditions (Figure 2.4), and refrigerated until use in the operating room (Figure 2.5). Immediately before the finish of culture, the medium was tested for bacteria, fungi, mycoplasma in BML Inc. (Tokyo, Japan), and endotoxins to confirm that the bone grafts were not contaminated. Endotoxin test was performed using assay kit (Endospey ES-6 Set, Seikagaku Corp., Tokyo, Japan).

A portion of the TEB was also tested for alkaline phosphatase (ALP) in order to evaluate its osteogenic capacity. TEB were washed twice with phosphate buffer saline (PBS), then rinsed with water and stained with 0.5 mg of naphthol-AS-MX phosphate sodium salt (Sigma) and 0.5 mg of Fast red violet B salt (Sigma) /mL in AMP buffer (1.0 mM MgCl₂, 10 mM p-

nitrophenyl phosphate in 0.056M 2-amino-2-methylpropanol) for 10 min.

After staining TEB were rinsed with tap water.

The use of autogenous serum or fetal bovine serum for culture was decided before harvesting the marrow fluid by consultation with the patient at the time of obtaining informed consent.

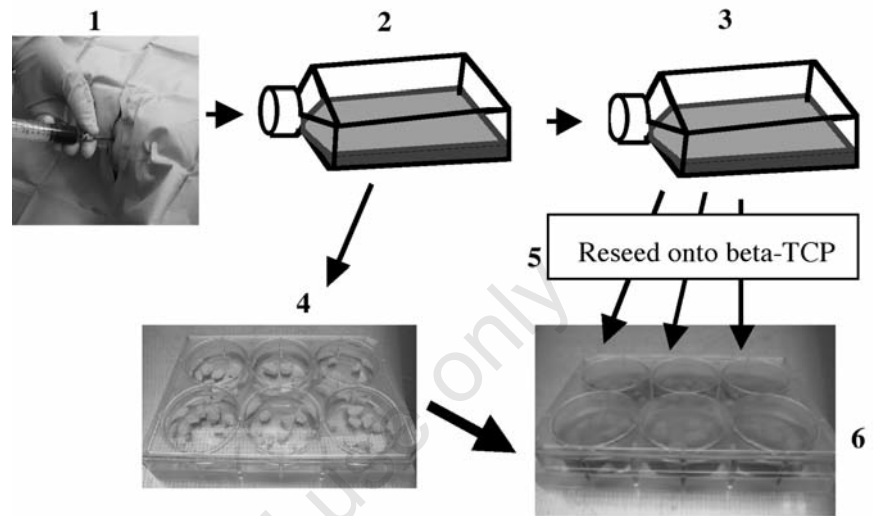


Figure 1. The preparation for tissue engineered bone grafts.

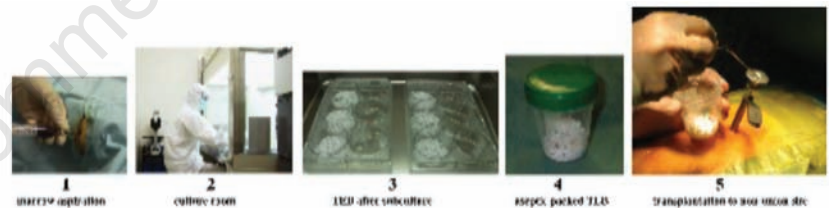


Figure 2. Preparation and transplantation of tissue engineered bone grafts. (case 5)



Figure 3. ALP stain of the tissue engineered bone (left) after culture in case 1. ALP stain showed significant osteoblastic activity of TEB (right)



Figure 4. The intraoperative photographs in case 1. A showed non union site. The TEB was grafted around the non-union site (B). After 1 year, the plate was removed and favorite bone union was observed (C).

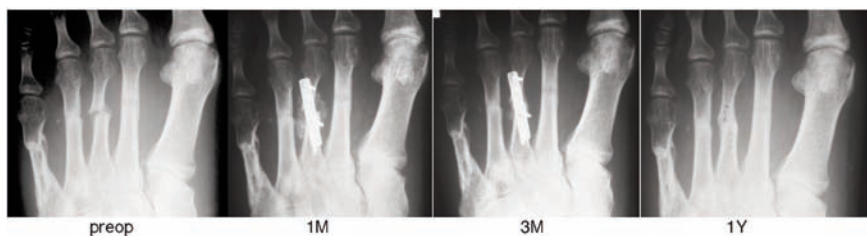


Figure 5. X ray of case 1 with metatarsal pseudoarthrosis. TEB grafts was observed around previous non union site at 1 month postoperation. Beta-TCP was absorbed and bone union was observed at 3 months. The plate and screws were removed at 1 year postoperation.

Grafting of tissue-engineered bone at sites of non-union

In all patients, TEB was grafted at the site of nonunion of long bone shaft or vertebrae. In 3 patients with nonunion of long bones, TEB was grafted around the affected site in each long bone, and internal fixation was performed as necessary. In 10 patients with thoracolumbar pseudoarthrosis, the bone graft was implanted into the pedicles (Figure 2.5). In 7 of these 10 patients, internal fixation with pedicle screws or hooks and rods was added.

Postoperatively, the progress of bone union was followed by radiography and CT scanning. In 3 patients, bone biopsy was performed during removal of internal fixation in order to confirm bone regeneration.

Results

Preoperative evaluation of tissue-engineered bone

ALP (which indicates osteoblastic activity) was measured in the TEB samples to evaluate

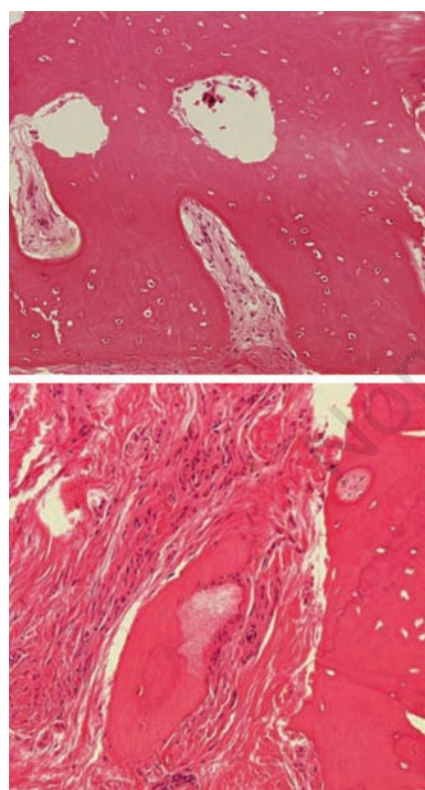


Figure 6. Histological data of decalcified HE stain 1 year after surgery. Biopsy specimens were obtained from the fracture site. Histological examination revealed mature bone tissue at the fracture site (top). Beta-TCP (arrow) surrounded by bone tissue was observed around previous non union site (down).

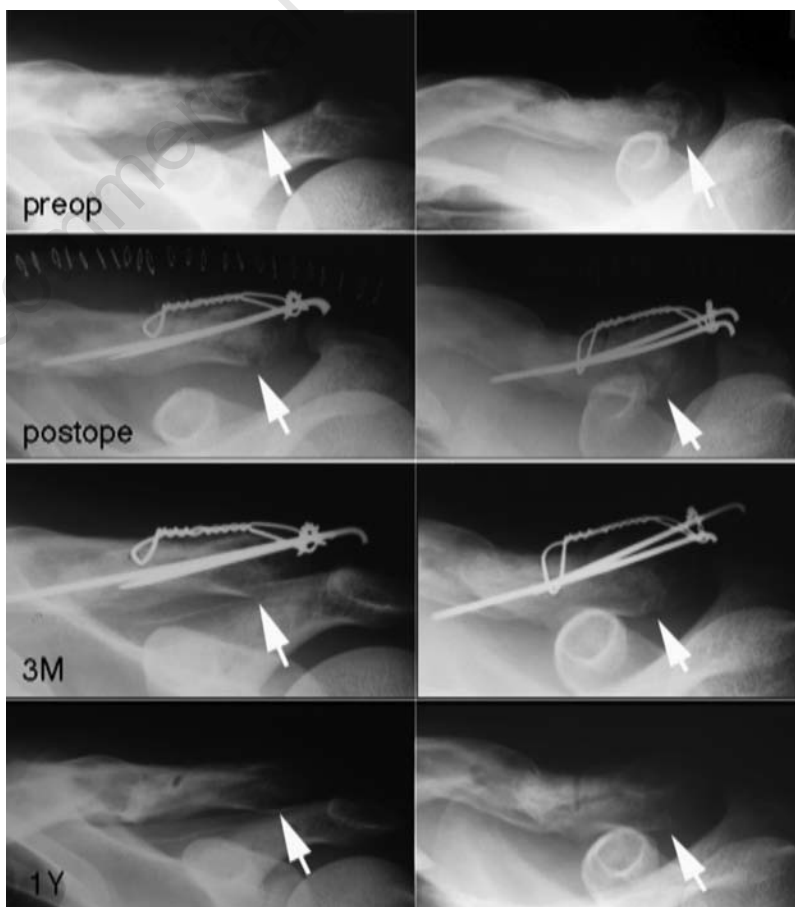


Figure 7. X ray of case 2 with clavicular pseudoarthrosis. TEB grafts was observed in previous non union site at postoperation. TEB was absorbed and replaced by bone at 3 months. The plate and screws were removed at 1 year postoperation.

osteogenic potential, and a high level of alkaline phosphatase activity was found in all patients regardless of their age. In Case 1, TEB samples were stained for alkaline phosphatase and reddish-brown areas were seen that confirmed high osteoblastic activity (Figure 3). Culture of the growth medium for bacteria, fungi, and mycoplasma, as well as testing for endotoxin, immediately before grafting was negative in all patients. No infection of the TEB occurred after grafting. Fetal bovine serum was used for culture in 10 patients and autologous serum was employed in 3 patients.

Clinical profile

In the 3 patients with long bone pseudoarthrosis (Cases 1-3), the cause was trauma and 6 months or more had passed since the injury. They had no underlying systemic diseases.

All 10 patients with spinal pseudoarthrosis (Cases 4-13) were symptomatic. Nine patients had suffered osteoporotic fractures, including 1 patient (Case 4) with osteoporosis secondary to steroid therapy for autoimmune disease and 3 patients (Cases 5, 6, and 12) with rheumatoid arthritis. The remaining 1 patient (Case 13) underwent open reduction of a traumatic fracture, but pseudoarthrosis occurred and the screws were broken. The pseudoarthrosis was located at T10 in 1 patient, T12 in 5 patients, L1 in 3 patients, L2 in 1 patient, and L5 in 1 patient. In 3 of the 10 patients (Cases 4-6), instrumentation was not performed. TEB was inserted percutaneously in 1 of these 3 patients (Case 6). Instrumentation was done in 7 patients (Cases 7-13), and 7 patients (Cases 4, 6, 8, 10-13) used pedicle plugs (Pentax Co., Tokyo, Japan) after grafting.

The mean postoperative follow-up period was 15.4 months (range: 6-40 months). All of the 13 patients were followed up for 6 months or more. Wound healing was good and there were no adverse events related to TEB grafting.

Postoperative course of patients with long bone pseudoarthrosis

In Case 1 (metatarsal pseudoarthrosis), radiography at 3 months after grafting revealed that the TEB grafted near the pseudoarthrosis had been almost completely absorbed and remodeled, suggesting good bone union (Figure 4AB and 5). After 1 year, the graft was absorbed and good bone union was achieved radiographically. The hardware was removed (Figure 4C and 5). At the time of surgery, good bone cortex formation was visible (Figure 4C). In addition, bone biopsy was performed from previous non-union site during operation of the removal of plate and screws. The histological data showed that the mature bone tissue was confirmed in previous non-union site (Figure 6-left), and β -TCP sur-

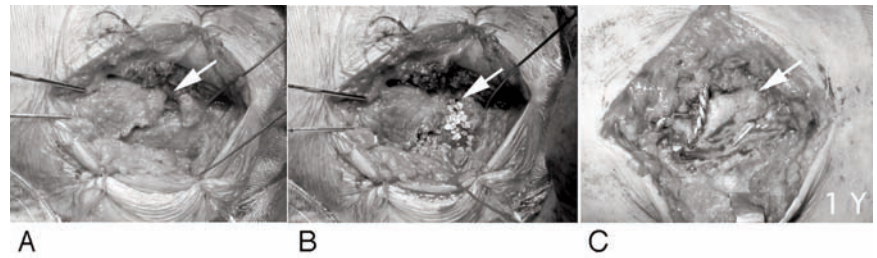


Figure 8. The intraoperative photographs in case 2. (A) The arrow shows non union site. (B) The TEB (arrow) was grafted around the non-union site. C. After 1 year, the plate was removed and favorite bone union was observed.

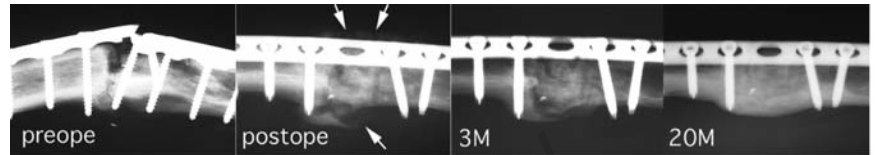


Figure 9. X ray of case 3 with femur pseudoarthrosis. Arrows indicate TEB grafts.

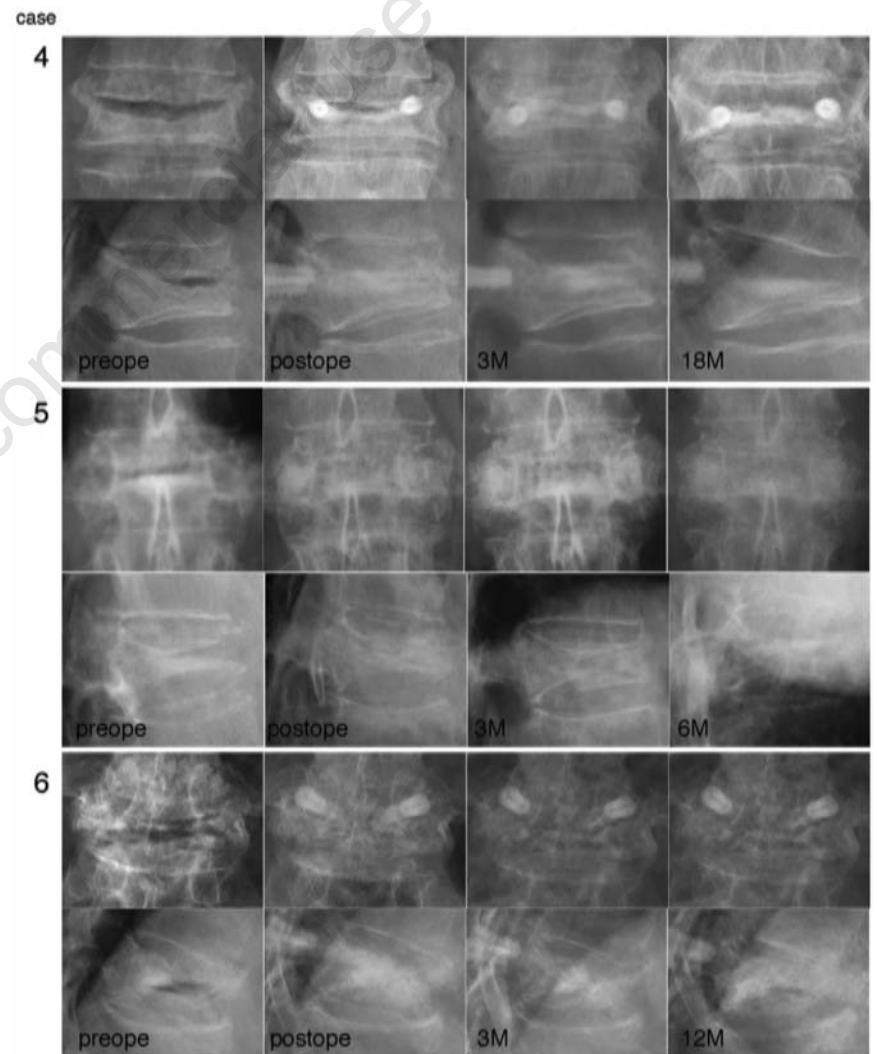


Figure 10. X ray of case 4-6 who underwent TEB grafting without instrumentation.

rounded by bone tissue was observed around previous non-union site (Figure 6-right). Near the fracture site, bone regeneration was seen around a calcified substance that was suggested to represent the artificial bone (Figure 6). No new fractures occurred during follow-up for 25 months.

In Case 2 (clavicular pseudoarthrosis), the TEB graft inserted into the defect was remodeled, absorbed, and replaced by bone at 3 months after grafting, resulting in good bone union. After 1 year, the artificial bone graft was absorbed and replaced by bone on radiographs (Figure 7). At the time of hardware removal after 1 year, bone formation was visible and good bone union was confirmed (Figure 8). No new fracture occurred during follow-up for 20 months.

In Case 3, because of bone harvesting from both iliac crests for multiple trauma, autogenous bone could not be obtained, so bone regeneration therapy using TEB was recommended to the patient. Bone marrow fluid was collected from the tibia. The TEB graft (arrow in Figure 9) was absorbed after 3 months and good bone union was achieved at 1 year (Figure 9). The patient refused to undergo removal of the hardware. No new fracture was observed during follow-up for 40 months.

Postoperative course of patients with vertebral pseudoarthrosis

Figure 10 shows radiographs obtained during follow-up in patients who underwent TEB grafting without instrumentation. In Case 6, the TEB graft was placed percutaneously. In all patients, the defects observed on radiography improved at 3 months after surgery, indicating bone union, and pain resolved or improved. Bone formation progressed for over 6 months. In Case 4, vertebral collapse, breakdown of a vertebra resulting in a decreased height of its body, showed progression, but union was eventually achieved. In Cases 5 and 6, vertebral collapse did not show progression and the vertebral body was reformed.

Figure 11A and B show patients who underwent grafting with instrumentation. Instrumentation was used in patients with a burst fracture or highly active patients. Favorable calcification was seen at 3 months after surgery. In Cases 8 and 13, radiolucent areas suggesting absorption of bone were observed around the TEB graft. In patients followed up for over 6 months after surgery, calcification at the site of the bone graft was decreased, showing that the artificial bone had been absorbed and replaced by host bone, indicating favorable bony union. X rays of Cases 8 and 13 showed excellent bone regeneration by TEB graft at the non-union site (Figure 11A and B). Three months after surgery, back pain had either resolved or showed improvement. Case 11 had pseudoarthrosis of two vertebrae

(T12 and L1), and both revealed good bone union at 3 to 6 months after surgery. One patient complained of lumbar pain around the fixed caudal part of the grafted vertebra at 6 months after surgery. Cases 5, 6, and 12 had rheumatoid arthritis, but a good therapeutic outcome was achieved even in the presence of this disease. In case 13, the bone biopsy was performed when the internal fixation device was removed at 2 years after surgery. The histological finding showed mature bone formation with marrow tissue.

Discussion

We have already reported that TEB can be prepared by combining cultured bone tissue and recently developed artificial bone materials with a high level of biocompatibility.²⁴⁻³³ Because TEB includes active osteogenic cells as well as mineralized matrices with BMP activity. Our previous biochemical study

showed that high ALP activity and significant osteocalcin content indicating osteogenic ability could be detected in rat TEB.^{25,26} SEM study of rat TEB demonstrated that mineralized collagenous matrices together with osteogenic cells was observed on surface of the pore areas of TEB,^{18,25,26} and that the differentiated osteogenic cells synthesized mineralized collagenous matrices and cement line on the artificial during culture.^{25,26} Therefore, TEB has a high osteogenic response in *in vivo* situations. When TEB was transplanted into *in vivo*, bone formation can begin immediately. High ALP activity and significant osteocalcin content could be detected at 1 week after transplantation.²⁶ Bone regeneration after transplantation of TEB was also demonstrated at the significant level of gene expression of ALP and osteocalcin,³⁰ and could be maintained for a long period.²⁹ Thus, bone regeneration by transplantation of TEB is considered to be a superior method.¹⁸ Furthermore, it was reported that TEB had superior bone regenerative potential compared with a bone marrow mesenchymal

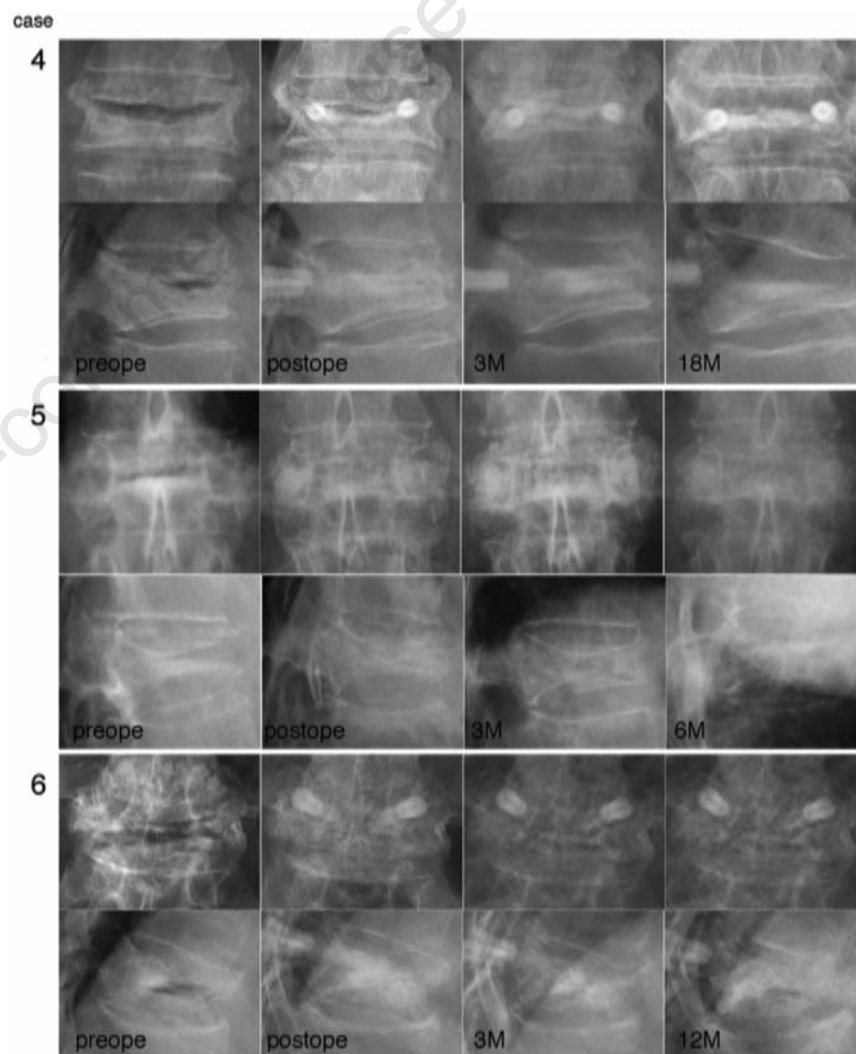


Figure 11-A. X ray of case 7-10 who underwent grafting with instrumentation.

cell/ceramic composites.³⁴

Human TEB prepared by culturing human bone marrow cells obtained through iliac marrow aspiration also has high osteogenic ability. Biochemical study showed that high ALP activity and significant osteocalcin content could be detected in human TEB.^{31,32} In SEM study of human TEB, mineralized collagenous matrices together with osteogenic cells was observed in the pore areas of TEB.^{31,32} When human TEB was transplanted into immunodeficient nude mice, human bone formation was observed. Using immunoassay, bone regeneration could be demonstrated by detection of human osteocalcin, a specific bone protein.³¹

Our previous immunohistochemical analysis²⁰ confirmed the presence of human CD34 (a marker for stem cells), vimentin (a marker for human fibrous tissue), and Factor VIII (a marker for vascular endothelial cells), thus suggesting that human marrow mesenchymal cells are capable of regenerating fibrous and vascular tissues. At the same time, the results clarified that tissue can be regenerated by grafting cultured marrow mesenchymal cells.

The ability of bone marrow cells to regenerate bone has been reported to decrease with aging.³⁵ It has also been reported that the ability of human bone marrow stromal cells to differentiate into osteoblasts decreases with aging.³⁶ However, the previous study of TEB developed by our culture technology did not show any correlation between the ability of bone marrow cells to regenerate bone and the age of the patients from whom the samples were obtained.³¹ Bone regeneration due to autogenous transplantation of TEB was also confirmed in beagle dogs.³³

However, when marrow fluid is collected from humans and cultured, there are 21 individual differences with regard to the number of cells and the level of mitotic activity, so rapid bone regeneration is not certain unlike animal studies. Therefore, based on the technique of Maniopoulos *et al.*,²¹ we have established a new culture technique. First, we found that adding estriol to the osteogenic medium enhanced bone regeneration *in vitro* by more than two-fold. Therefore, we included estriol as an osteogenic factor in the culture medium. Second, we found that a large quantity of osteogenic cells could be layered over artificial bone material, and we succeeded in preparing TEB with more than twice the osteogenic activity.³⁷

Using this method to create TEB, artificial material with a high regenerative capacity was prepared by aspiration, which is minimally invasive. Thus, when compared with the harvesting of iliac bone grafts, it is possible to markedly reduce postoperative pain. In addition, harvesting bone from the ilium is associated with considerable bleeding, while TEB grafts make it possible to avoid bleeding dur-

ing harvesting and surgery. Moreover, the duration of surgery is shorter. Therefore, the present technique represents a method for minimally invasive autogenous bone grafting without sacrificing autogenous bone. Harvesting cancellous bone from the ilium of children during the growth stage damages the epiphyseal plate, so pelvic deformity occurs and becomes worse with time. In women with little subcutaneous fat, the iliac defect is easily noticeable and the cosmetic outcome is poor.

In the present study, we used beta-TCP as the scaffold for fabricating TEB. Beta-TCP is a ceramic that is absorbed and eventually replaced by host bone.³⁸ Tissue compatibility

has been shown to be superior to other synthetic materials.³⁹ However beta-TCP does not have osteoinductive ability in subcutaneous sites.⁴⁰ In patients with long bone pseudoarthrosis, grafts of TEB with a beta-TCP scaffold were absorbed within a few months after grafting. After 1 year, the component of beta-TCP in TEB had been fully absorbed in the vicinity of the pseudoarthrosis and bone union was complete. It usually takes several years for β -TCP to be absorbed, but beta-TCP coated with cultured bone cells was absorbed and underwent remodeling into bone within a few months. The presence of cultured cells accelerated the absorption and remodeling of beta-

case

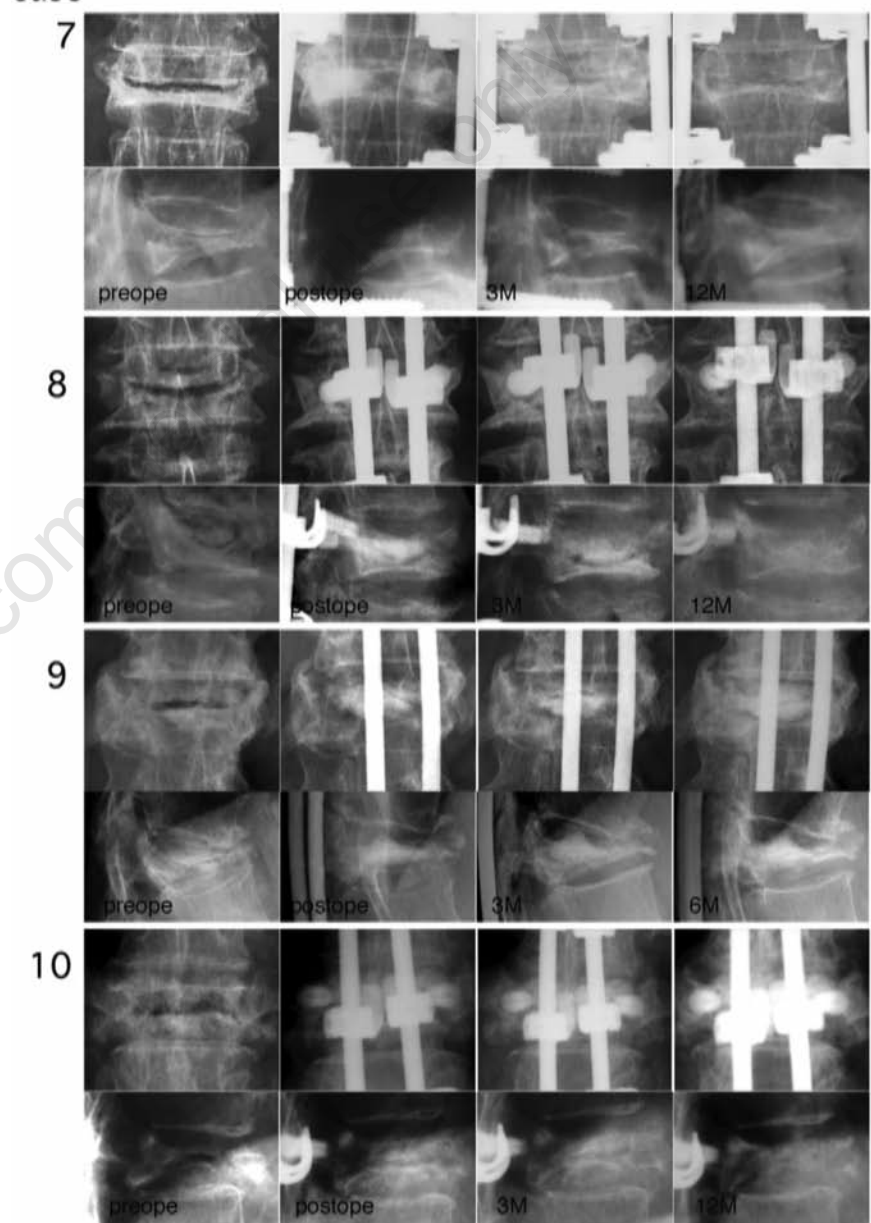


Figure 11-B. X ray of case 11-13 who underwent grafting with instrumentation.

TCP, eliminating the radiographic appearance of excessive calcification.

Treatment of fractures using BMP was reported to achieve good results. Bone regeneration therapy using BMPs does not require cell culture and thus is simple.^{41,42} However, BMPs are cytokines that induce undifferentiated cells to differentiate into osteogenic cells, thereby initiating bone regeneration. According to the osteoregenerative therapy, bone regeneration occurs promptly following transplantation of TEB because the TEB has a bone matrix containing cytokines such as BMPs as well as cells with osteogenic capacity.^{43,44} The early phase of bone regeneration is completed during culture on artificial bone, so new blood vessels are promptly formed to induce bone regeneration after grafting.

In elderly patients with thoracolumbar pseudoarthrosis caused by osteoporosis, injection of artificial bone and bone cement into the vertebral body has been reported to achieve early improvement of pain. However, reconstruction of the vertebral body with artificial materials seems to be associated with loosening between the host bone and artificial materials, so the long-term results are unclear. With the present method, the vertebral body undergoes regeneration using autogenous cultured bone, indicating that better mid-term and long-term results can be expected.

For reconstruction of extensive bone defects, autogenous bone, allogeneic bone, and artificial bone are now used as well as bone cement. Reconstruction with autografts is ideal, but it poses problems such as invasion of healthy tissues harvest bone and the limited amount that can be harvested. Allograft bone, like autogenous bone, is derived from humans; the difference is that allograft is harvested from an individual other than the one receiving the graft. Allograft bone is taken from cadavers that have donated their bone so that it can be used for living people who are in need of it; it is typically sourced from a bone bank. Allografting allows large grafts to be used without invasion of the patients. Unlike biologically inactive artificial bone, the allografts possess bone matrix containing cytokines that stimulate bone regeneration, and therefore have a high osteoregenerative potential. However, there is no cellular activity and the osteoregenerative potential is inadequate in some patients.⁴⁵ Bone banks are still not sufficiently developed in Japan and it is often impossible to obtain grafts. Bone cement and artificial bone can be used to fill extensive bone defects, but the long-term results with these artificial materials (which have no biological functions) are variable.⁴⁶ Our reconstruction method was performed by utilizing advantages of these grafting materials.

From the scientific point of view, treatment of pseudoarthrosis with artificial bone alone

should be the control. However, animal experiments have shown that bone regeneration does not occur when artificial bone is transplanted alone.^{17,29,30,43,43} It has also been reported clinically that bone regeneration does not occur when artificial bone alone is transplanted for posterolateral fusion.⁴⁶ Under these circumstances, treatment of pseudoarthrosis using artificial bone alone would not be ethical, even with the patient's consent.

The present paper presents osteoregenerative therapy using TEB grafting as a new option for bone reconstruction. With this technique, TEB that resembles autogenous bone can be prepared by minimally invasive bone marrow aspiration, which allows osteoregenerative therapy to be performed without causing injury to autogenous bone. If the culture phase could be done on an industrial scale, this technique could become widespread. Combining cell culture with recently developed biomaterials allows the performance of various types of osteoregenerative therapy. Above all, it is effective for pseudoarthrosis and is a promising less invasive treatment for the future.

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