Spinal fusion using tissue engineered bone-A prospective, randomized clinical pilot trial

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Abstract

Spinal fusion is performed using bone harvested from the ilium. However, graft harvest is associated with frequent complications and pain. If a tissue engineered bone (TEB) was available, spinal fusion could be performed without damaging normal tissues. In 8 patients, 10-20 mL of marrow fluid was collected from the iliac crest to fabricate. After primary culture in the standard medium, marrow mesenchymal cells were combined with porous tricalcium phosphate block and were cultured in osteogenic medium containing dexamethasone, β-glycerophosphate, vitamin C phosphate, and estriol. After 3 weeks of subculture, spinal fusion was performed using TEB. Nine patients who had undergone spinal fusion using iliac autografts served as the controls (AG group). In all patients, significant improvement in JOA score was seen in both TEB and AG groups. The radiographic fusion rate was 87.5% (7/8) in TEB group and 77.8% (2/9) in AG group at 6 months after surgery. The mean operating time in TEB group was shorter than in the AG group. Compared with the AG group, the patients receiving TEB graft had significantly less total blood loss. In the AG group, all of the patients complained of graft site pain for 2 to 4 weeks after the operation. Two patients (22.2%) still had graft site pain at 6 months postoperatively. Bone regeneration therapy using the TEB graft introduced in this report makes it possible to perform spinal fusion as is done using autogenous bone grafts, but with the minimally invasive procedure of bone marrow aspiration.

Introduction

Spinal fusion is a surgical technique used to join two or more vertebrae and involves placing autograft bone from pelvis. However, harvesting bone from the pelvis is associated with severe postoperative pain, and patients experience more pain at the harvest site than at the graft site, thus resulting in poor patient satisfaction. If a tissue engineering approach was used to produce autogenous bone *ex vivo* with culture techniques, spinal fusion could be performed without severe postoperative pain.

Bone marrow cells include hemopoietic cells and mesenchymal stem cells with an osteogenic capacity.[1,2] If the mesenchymal stem cells are cultured with bone growth factors (dexamethasone, beta-glycerophosphate, and vitamin C phosphate), bone-like tissue can be formed in the culture dish. It has been reported that such bone tissue contains osteoblasts and bone matrix, and that the process by which this tissue is formed resembles the early stages of bone formation *in vivo*.[3,4] The cultured cells have a high alkaline phosphatase (ALP) activity and express genes encoding ALP, osteocalcin, osteopontin, and other bone proteins. It has been found that the cultured bone matrix shows bone morphogenic protein (BMP) activity and is rich in the bone-specific protein osteocalcin as well as calcium, making it similar to bone *in vivo*.[5,6] Thus, the bone tissue obtained by culturing bone marrow cells can be expected to have high osteoblastic activity, a matrix rich in bone cytokines, and a potential for bone regeneration comparable to that of an autograft.[7,8]

We previously succeeded in binding cultured bone tissue to an artificial bone graft in order to endow the graft with regenerative potential.[9] Recently, further improvements in the culture technique have been added to create the tissue engineered bone (TEB) with a regenerative capacity.[10,11] The cancellous bone from ilium has a high cellular activity and can be used for bone reconstruction in situations such as spinal fusion and treatment of pseudoarthrosis. It has already been reported that our TEB has osteoblastic activity comparable to that of cancellous bone.[9,12-15] In the present study, the TEB was used for spinal fusion and good results were obtained.

Materials and Methods

Study subjects

The present bone regeneration therapy by TEB using marrow mesenchymal cells was submitted to the university ethics review boards was approved in 2000.

The subjects of the study in TEB group were 8 patients (3 men and 5 women) aged 59.8 years (range: 54-57 years) who were operated on at Nara Medical University Hospital between 2002 and 2005 for lumbar spinal stenosis with lumbar spondylolisthesis (Grade I) (n=5), pseudoarthrosis after a burst fracture of the second lumbar vertebra (n=1), atlantoaxial subluxation due to rheumatoid arthritis (n=1), and sacral cord tumor (n=1).

Patients (1 man and 8 women with an average age of 64.7 years; range: 52-79 years) who had undergone spinal fusion using iliac autografts between 1999 and 2002 for lumbar spinal stenosis associated with lumbar spondylolisthesis (Grade I) (n=8) or atlantoaxial subluxation associated with rheumatoid arthritis (n=1) served as the controls (AG group). In 4 of the 9 patients, internal fixation such as pedicle screws and rods was added (Table 1).

Cell culture and preparation of tissue engineered bone

The patients to be treated with TEB grafts gave informed consent, and 10-20 mL of marrow fluid was collected from the iliac crest (Figure 1). Mesenchymal cells from the bone marrow were cultured in T75 flasks containing standard culture medium of minimal essential medium containing antibiotics and 15% autogenous or fetal bovine serum. After 2 weeks, the cells were released with trypsin. 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) and cultured in the osteogenic medium of the standard medium containing osteogenic factors (10 nM dexamethasone, 10 mM Na β-glycerophosphate, 82 μg/mL vitamin C phosphate, and 10 nM estriol) for 3 weeks. One week before transplantation, mesenchymal cells from the bone marrow cultured in standard medium were reseeded to prepare TEB. The TEB was rinsed twice with physiological saline, packed under aseptic conditions, and refrigerated until use in the operating room.

Mesenchymal cells from the bone marrow fluid were cultured in T75 flasks containing standard culture medium. After 2 weeks, the

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cells were detached by trypsinization. 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) and cultured in medium containing bone growth factors (dexamethasone, beta-glycerophosphate, vitamin C phosphate, and estriol) for 3 weeks. At one week before transplantation, mesenchymal cells from the culture in standard medium were reseeded to prepare cultured artificial bone. The cultured artificial bone was rinsed twice with physiological saline, packed under aseptic conditions, and refrigerated until use in the operating room.

Immediately before the end of culture, the medium was tested for bacteria, fungi, mycoplasma in BML Inc. (Tokyo, Japan), and endotoxins to confirm that the bone graft was not contaminated. Endotoxin test was performed using assay kit (Endospecy ES-6 Set, Seikagaku Corp., Tokyo, Japan). Part of the cultured bone was tested to determine its ALP activity and osteocalcin content in order to evaluate osteogenic capacity. Briefly ALP activity was measured by using the supernatant was the enzyme solution and p-nitrophenyl phosphate as the substrate. Human osteocalcin was measured with a MID-TACT human osteocalcin enzyme immunoassay (EIA) kit (BT-480; Biomedical Technologies, Stoughton, MA, USA).[16,17]

The use of autogenous serum or fetal bovine serum was decided before the operation in consultation with the patient at the time of obtaining informed consent.

Transplantation of tissue engineered bone

In 8 patients, the TEB was transplanted to a posterior or posterolateral position (Figure 2). In 3 of the 7 patients, internal fixation such as pedicle screws and rods was added. In the patient with pseudoarthrosis, the TEB graft was transplanted to a posterior and transpedicular location to buttress the pseudoarthrosis.

The process of bone union after surgery was followed using X-ray, CT, and MRI, while symptoms were assessed using the JOA score (The Japanese Orthopaedic Association has developed a clinical symptom score for a patient. The JOA score can help determine the degree of improvement following surgical intervention).[18,19]

Japanese Orthopaedic Association (JOA) score[18]
**total score minimum score: -6, maximum score: 29*
**The higher the score the more normal the patient's overall status.*
**Parameters in the score:*
- subjective symptoms (9 points): low back pain (3-0) leg pain and/or tingling (3-0) gait (3-0)
- clinical signs (6 points): straight-leg test (2-0) sensory disturbance (2-0) motor disturbance (2-0)
- restriction in activities (14 points): turn over while lying (2-0) standing (2-0) washing(2-0) learning forward (2-0) sitting about 1 hour (2-0) lifting or holding a heavy object (2-0) walking (2-0)
- urinary bladder function (-6 points maximum).

Table 1. List of patients of tissue engineered bone group and iliac autograft group.

TEB Group No.	Age	Sex	Diagnosis
1.	65	M	Sacral nerve tumor
2.	58	M	Lumber fracture non union
3.	62	F	Lumbar canal stenois with spondylolisthesis
4.	75	M	Lumbar canal stenois with spondylolisthesis
5.	64	F	Lumbar canal stenois with spondylolisthesis
6.	54	F	Lumbar canal stenois with spondylolisthesis
7.	54	F	Lumbar canal stenois with spondylolisthesis
8.	46	F	Atlantoaxial subluxation (rheumatoid arthritis)
AG Group No.	**Age**	**Sex**	**Diagnosis**
1.	52	F	Lumbar canal stenois with spondylolisthesis
2.	58	F	Lumbar canal stenois with spondylolisthesis
3.	79	F	Lumbar canal stenois with spondylolisthesis
4.	67	M	Lumbar canal stenois with spondylolisthesis
5.	68	F	Lumbar canal stenois with spondylolisthesis
6.	68	F	Lumbar canal stenois with spondylolisthesis
7.	70	F	Lumbar canal stenois with spondylolisthesis
8.	66	F	Lumbar canal stenois with spondylolisthesis
9.	54	F	Atlantoaxial subluxation (rheumatoid arthritis)

TEB, tissue engineered bone; AG, iliac autograft.

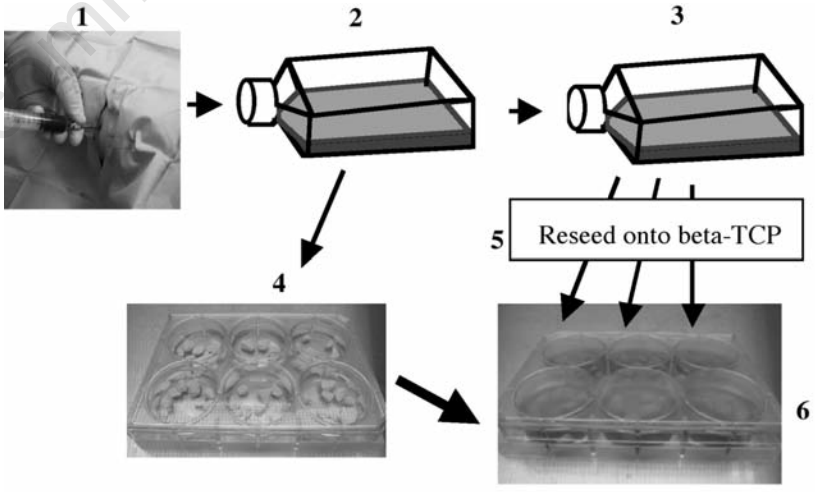

Figure 1. Preparation of tissue engineered bone. (1) After the patients all gave informed consent, 10-20 mL of bone marrow fluid was collected from the iliac crest. (2) Mesenchymal cells from the bone marrow fluid were cultured in T75 flasks containing standard culture medium. (3) After 2 weeks, the cells were detached by trypsinization. Then 1/10 of the cells thus obtained were cultured in T75 flasks containing standard medium. (4) The remaining cells were seeded onto porous beta-TCP (OSferion, G2, Olympus Co. Japan) and cultured in medium containing bone growth factors (dexamethasone, beta-glycerophosphate, vitamin C phosphate, and estriol) for 3 weeks. (5) At one week before transplantation, mesenchymal cells from the culture in standard medium were reseeded to prepare cultured artificial bone. (6) The fabricated TEB was rinsed twice with physiological saline, packed under aseptic conditions, and refrigerated until use in the operating room.

Results

Before transplatation, ALP stain of TEB showed high osteogenic capacity (Figure 3). TEB showed high osteogenic activity in *in vitro* situations, as previously reported.

The operating time did not differ significantly between the patients who were treated by transplantation of TEB and AG. However, the mean operating time was only half as long in the TEB group than in AG group (Table 2). This difference of the operating time was thought to be due to the different times required for nerve root decompression in each patient.

Compared with the AG group, the patients receiving TEB had significantly less intraoperative bleeding, less postoperative bleeding from the drain, and less total blood loss (Table 2). In the AG group, all of the patients complained of donor site pain for 2 to 4 weeks after the operation, and their symptoms were recorded in the nursing reports. Two patients (22.2%) still had bone-graft harvest site pain at 6 months postoperatively. In all patients of TEB group, pain improved or resolved by 3 months after surgery. There were no adverse reactions associated with transplantation. In all patients, good calcification was observed at 3 months after the operation. At 6 months postoperatively, beta-TCP was partly absorbed and remodeled, and imaging findings suggested the progress of ossification (Figure 4).

Patients with spinal stenosis and lumbar instability showed good posterolateral ossification, and their lower limb symptoms improved. The patient with pseudoarthrosis showed good ossification of the vertebra and posterolateral region. The radiographic fusion rate at 6 months after surgery was 87.5% (7/8) in the TEB group and 77.8% (7/9) in the AG group. The JOA scores for symptoms were significantly reduced in both groups of TEB and AG (Figure 5).

Discussion

Spinal fusion is usually performed using cancellous bone grafts taken from the ilium. The cancellous bone of the ilium has a rich blood supply and a high cellularity, making it very useful for bone reconstructive procedures such as spinal fusion. However, patients suffer from severe postoperative pain of pelvis, which it is difficult to control. Even laughing can cause bone-graft harvest site pain, and walking becomes difficult so that patients have to use a wheelchair. The complications associated with harvesting grafts from the ilium were reported, which include bleeding at the time of graft collection, postoperative pain, chronic pain, deformity of the pelvis, surgical scars, increased risk of pelvic fracture, and nerve damage.[20-33]

Because the ilium has a rich blood supply, graft collection causes considerable bleeding and careful hemostasis is necessary to prevent protracted postoperative hemorrhage. Bone-graft harvest site pain lasts for several months after the operation, and in some cases it may become chronic. In fact, chronic pain is reported in about 25% of patients,[21-24,27,33] and some reports mention a figure as high as 34%.[28] Harvest of bone from the iliac crest is also unsuitable in women with little subcutaneous fat in whom the graft site develops a cavity as well as because of the surgical scar. The ilium is more susceptible to fracture after graft collection, and a fall after surgery may cause a pelvic fracture or stress fracture.[29-31] During graft collection, the femoral cutaneous nerve may be damaged near the iliac crest, leading to meralgia.[34]

There have been reports about the countermeasures for complications associated with harvesting of grafts.[33-39] Reconstruction using TEB is free from the problems accompanying graft collection. It only involves the minimally invasive procedure of bone marrow aspiration and is less burdensome for patients. For the surgeon, the method has the advantage of shortening the operating time because there is no need for graft collection.

Spinal fusion can also be done without using autologous bone grafts by instrumentation. Because of the rigidity of metal instruments, firm fixation is achieved immediately after the operation. However, the metal components are foreign materials, so problems such as breaking and loosening of screws or migration of rods can occur over the long term. Accordingly, spinal fusion based on bone regeneration using autologous bone grafts is more desirable from a long-term perspective. However, spinal fusion using autografts has declined in popularity due to problems with graft collection, and the use of instrumentation is increasing. If it becomes possible to perform spinal fusion with the minimally invasive procedure of bone marrow aspiration, we can expect an increase in the use of posterolat-

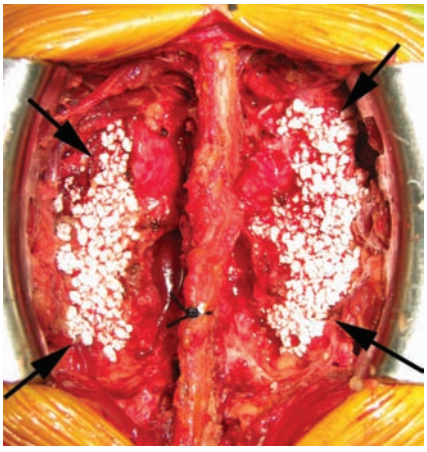

Figure 2. The intraoperative photograph by tissue engineered bone. Arrows indicate tissue engineered bone. (64 year-old female patient of lumbar canal stenosis with spondylolisthesis).

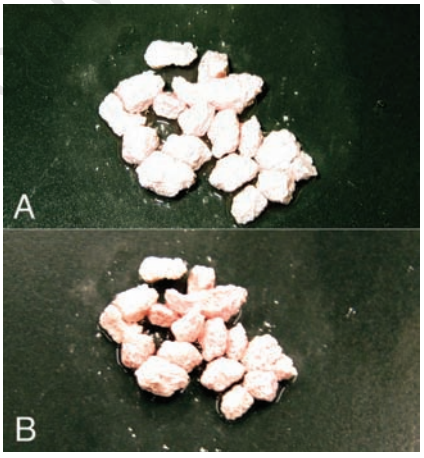

Figure 3. ALP stain of the tissue engineered bone (TEB, A) after culture in case 2 of TEB group. ALP stain showed significant osteoblastic activity of TEB (B) (method of ALP stain: TEB were washed twice with phosphate buffer saline (PBS), then rinsed with water and stained with 0.5 mg of naphtol-AS-MX phosphate sodium salt (Sigma, St. Louis, MO, USA) and 0.5 mg of Fast red violet B salt (Sigma, St. Louis, MO, USA)/mL in AMP buffer (1.0 mM $MgCl_2$, 10 mM p-nitrophenyl phosphate in 0.056M 2-amino-2-methylpropanol) for 10 min. After staining TEB were rinsed with tap water.)

Table 2. Surgery information: comparison of spinal fusion by tissue engineered bone (TEB, n=8) or iliac autograft (AG, n=9).

	TEB group mean (SD)	AG group mean (SD)	P
Operating time, min	168(26)	469(850)	0.319
Intraoperative bleeding, mL	127(120)	305(175)	0.027*
Postoperative bleeding from the drane, mL	230(85)	356(137)	0.043*
Total blood loss, mL	357(150)	693(326)	0.018*
Fusion rate	87.5%(7/8)	77.8(7/9)	

The data of operation time and intraoperating blood loss in case 1 of TEB group, were ruled out because the diagnosis was different.
*Statistical analysis: Measured values were analyzed using Microsoft Excel 2001 and expressed as the mean±standard deviation(SD). The unpaired Mann-Whitney U-test was used for comparisons between two groups. Statistical significance was established at the P<0.05 level.

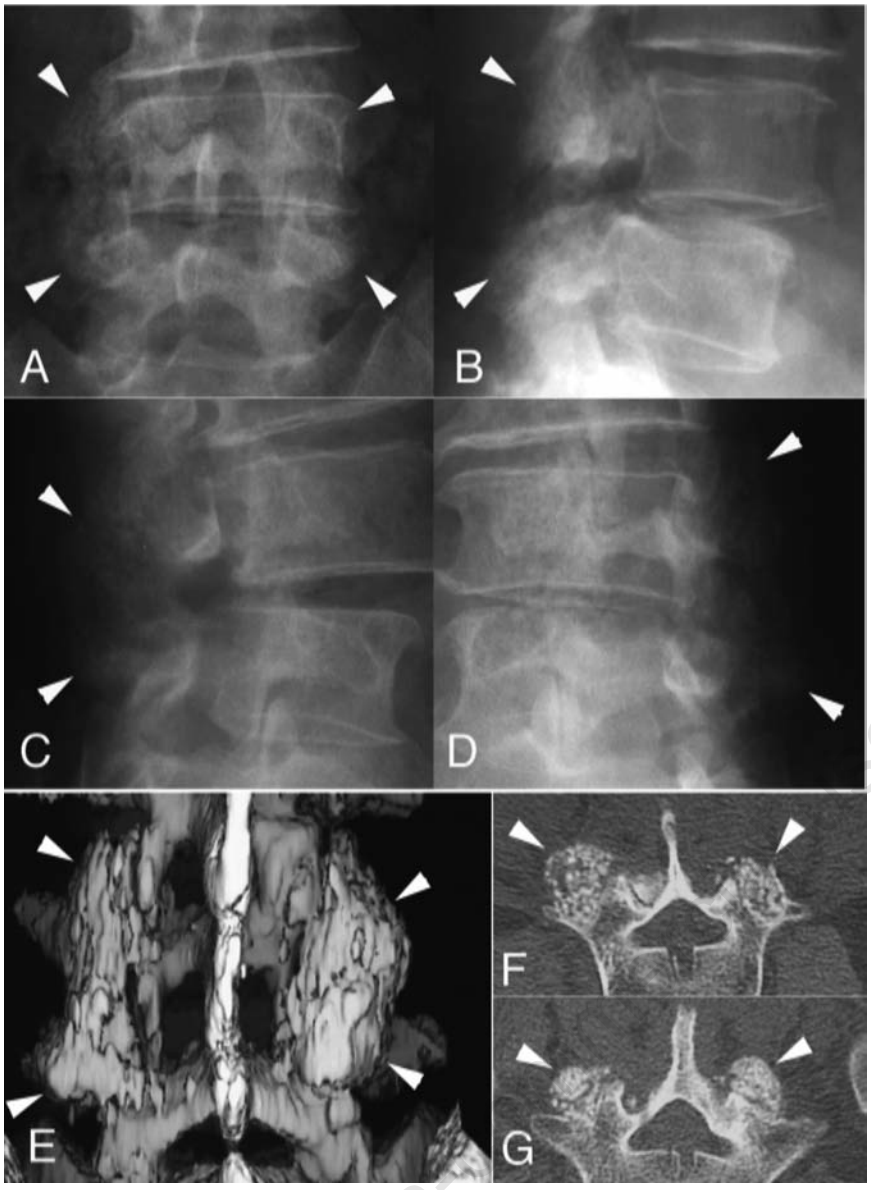

Figure 4. The X-ray and computed tomography findings of lumbar posterolateral fusion by tissue engineered bone at 6 months postoperation (62 years-old female patient of lumbar canal stenosis with spondylolisthesis). Arrowheads indicate tissue engineered bone. Bone fusion was observed and JOA scores improved 14 to 25. (A) Anteroposterior X-ray. (B) Lateral X-ray. (C, D) Oblique X-ray. (E) 3D-CT image at L4 and 5. (F) Computed tomography image of L4. (G) Computed tomography image of L5.

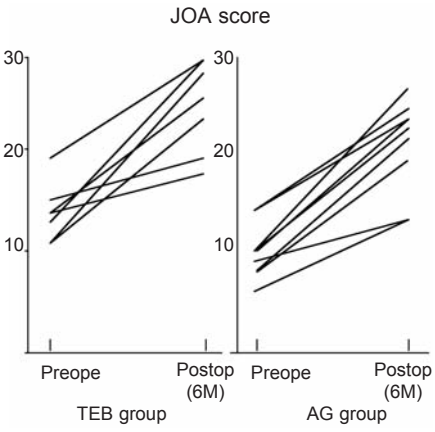

Figure 5. Japanese orthopedic association score. The Japanese orthopedic association scores for symptoms were significantly reduced in both groups of TEB and AG.

eral spinal fusion.

Recently, regenerative therapy using bone morphogenetic protein (BMP) has been reported.[40,41] Performance of bone regeneration using BMP is simple because it does not require cell culture, but a period of several weeks is needed for mature bone to regenerate, since bone regeneration occurs via endochondral ossification after osteogenic cells are derived from undifferentiated cells.

On the other hand, our TEB possesses higher osteogenic acitity. 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.[8,9] SEM study of rat TEB demonstrated that mineralized collagenous matrices together with osteogenic cells was observed on surface of the pore areas of TEB,[4,8,9] and that the differentiated osteogenic cells synthesized mineralized collagenous matrices and cement line on the artificial during culture.[8,9] 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 oseocalcin content could be detected at 1 week after transplatation.[9] Bone regeneration after transplantation of TEB was also demonstrated at the significant level of gene expression of ALP and osteocalcin,[12] and could be maintained for a long period.[15] Thus, bone regeneration by transplantation of TEB is considered to be a superior method.[4] Furthermore, TEB is reported to have superior bone regenerative potential compared with a bone marrow mesencymal cell/ceramic composites.[42]

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.[16,17] In SEM study of human TEB, mineralized collagenous matrices together with osteogenic cells was observed in the pore areas of TEB.[16,17] 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.[16] Bone regeneration due to autogenous transplantation of TEB was also confirmed in beagle dogs.[43]

However, when marrow fluid is collected from humans and cultured, there are individual differences with regard to the number of cells and the level of mitotic activity, so rapid bone regeneration is not certain, compared with our previous data of animal studies. Therefore, based on the technique of Maniatopoulos *et al.*,[3] 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 a new osteogenic factor in the culture medium.[10] Second, we found that a large quantity of osteogenic cells could be layered over artificial bone material, and we succeeded in preparing TEB with higher osteogenic activity.[11]

In the present study, bone regeneration was observed by imaging. More precise data would require biopsy to confirm regeneration of the bone histologically. However, performance of biopsy purely for the purpose of research raises ethical concerns and it is difficult to obtain patient consent. Because of the above-mentioned data that are already available, it is reasonable to confirm bone regeneration by imaging instead.

In the present study, patients who underwent posterolateral fusion with autogenous bone grafts were used as controls. From the scientific point of view, posterolateral fusion 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.[2,9,15] It has also been reported clinically that bone regeneration does not occur when artificial bone alone is transplanted for posterolateral fusion.[44] Under these circumstances, posterolateral fusion using artificial bone alone would not be ethical, even with the patient's consent.

In summary, bone regeneration therapy using the method introduced in this report makes it possible to perform spinal fusion as is done using autogenous bone grafts, but with the minimally invasive procedure of bone marrow aspiration. Use of iliac grafts is not necessary, so that the pain and complications associated with graft harvest can be avoided. Postoperative pain is reduced dramatically and the time needed for rehabilitation is shortened, leading to early discharge from hospital. If cell culture can be carried out on a commercial scale, this therapy is expected to come into wider use.

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