Macaque lumbar posterolateral spine fusion (PLF) was performed by using beta-TCP graft combined with bone marrow derived stromal cells (MSCs), to evaluate whether a beta-TCP/MSCs hybrid can be used for PLF instead of autogenous bone graft. Nine crab-eating macaque underwent bilateral PLF at L4-L5. The implants were divided into three groups: 1) beta-TCP/MSCs hybrid, 2) autogenous bone, and 3) beta-TCP. Six monkeys were sacrificed at 12 weeks and three monkeys were sacrificed at 24 weeks after implantation. Manual palpation, radiography, micro computed tomography, peripheral quantitative computed tomography (pQCT), and histology were used to assess bone formation. Manual palpation and X-ray showed that 83.3% of hybrid groups and 66.7% of autogenous groups achieved solid spine fusion, whereas none of other groups fused. Histological analysis showed that all of the hybrid groups achieved massive bone formation. Bone mineral density (BMD) evaluated with pQCT in the hybrid groups increased by additional new bone. Beta-TCP/MSCs hybrid can be used for PLF instead of autogenous bone graft. Thus it can be hypothesized that the monkey PLF can simulate human PLF.

Key words: posterolateral spine fusion, beta-tricalcium phosphate, bone marrow derived stromal cells, nonhuman primate, autogenous bone graft

Introduction

During spinal surgery, the use of bone grafts is a common practice. One such technique is posterolateral intertransverse fusion (PLF) which is the most popular method for achieving intersegmental fusion for lumbar instability. To obtain bony union, autogenous bone graft from iliac crest is recognized as the most successful bone graft material and is presently the “gold standard” against which all other graft materials are compared. However, donor site morbidity and some complications occur in a relatively high number of cases. Complications include increased blood loss, operative time, risks of nerve injury and additional incision sites. Additionally, pseudoarthrosis with the use of autogenous bone graft has been reported to occur in about 30% of the cases. The quantity of bone available to harvest may be not be sufficient for long, multi-level fusion or in patients with previous graft harvests. In order to overcome these problems, it is desirable to develop graft materials which have not only osteoconductivity but also osteoinductivity equal to that of natural bone and which can take the place of autogenous bone graft.

Osteoinductive growth factors, including bone morphogenetic proteins (BMPs), have also been a candidate for the restoration of graft materials. Boden and
Sandhu et al. have shown successful results when using rhBMP-2 with various scaffolds. However many reports have focused on their potential for either increased osteoclastic resorption or over-formation of bone. And in clinical usage, significant doses of BMP are necessary which are cost prohibitive. In addition, such a high dose of BMP may cause unexpected effects, even in sites distant from where BMP was directly used. Furthermore, BMP has not been approved to use in many countries.

Recently, it has been widely recognized that scaffold combined with cultured bone marrow derived stromal cells (MSCs) has the ability to induce bone formation. MSCs have multipotency and are easily harvested by aspiration and are easily proliferated. When sufficient numbers of cells are available, they are loaded into a porous ceramic scaffold and surgically implanted in vivo. Yoshikawa et al. improved the culture method for osteoblastic cells by introducing the method of Maniatopoulos. Uemura et al. further improved the culture method by applying a low-pressure system and a perfusion system. We referred to their method and further shortened the culture period by implanting graft materials just after cell-loading, even if they were cultured about 2 weeks after cell-loading.

As an appropriate ceramic, porous beta-TCP is a biodegradable material that can be replaced by bone tissue. Thus we selected porous beta-TCP as a scaffold and combined it with MSCs for bone regeneration.

The purpose of this experiment was to use this method for posterolateral fusion in nonhuman primates and evaluate whether it had potential fusion enhancing ability and autogenous bone substitutes.

Materials and Methods

Preparation of scaffold (porous beta-TCP): Many investigations have examined scaffolds by mixtures of beta-TCP and hydroxyapatite or polymer, because of the rapid degradation and weak mechanical properties of beta-TCP. However, the purity of beta-TCP also influences bone formation and biocompatibility. Accordingly, we selected a highly pure porous beta-TCP (Osferion, Olympus CO Ltd.) as a scaffold for bone tissue engineering. Its average pore size is 200-400μm in diameter, and almost all pores are interconnected via a 100-200μm path.

Preparation of MSCs and culture: The bone marrow derived stromal cells (MSCs) were obtained from male crab-eating macaque between 3 and 4 years of age, weighing between 3.3kg and 4.9kg. Under general anesthesia, the bone marrow was aspirated from the greater trochanter of the femur using a 20ml syringe with a 16-gauge bone marrow puncture needle. The marrow aspirates were suspended in Dubberco’s Minimal Essential Medium (DMEM), centrifuged at 1000 rpm for 5 min, and resuspended in a standard medium consisting of DMEM supplemented with 1% antibiotics/antimycotics (1× Antibiotic—Antimycotic, including 10,000 units/ml penicillin G sodium, 10,000μg/ml streptomycin sulfate and 25μg/ml amphotericin B in 0.85% saline, Gibco, USA) and 10% foetal bovine serum (Gibcobrl, Lot No. 32095241S) and incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO₂. The medium was changed 3 hours later to remove non-adherent cells. The adherent cells remaining were considered to be mainly MSCs. Subsequently, the medium was carefully replaced every three days. The colonies grew quickly within 14 days. When the cell density approached 80 to 90% confluence, they were released by treatment with 0.25% trypsin containing 1μM EDTA (Gibco, Invitrogen Corporation) for 5μmin at 37°C and then passaged. When second cell cultures became almost confluent within 7 days, they were differentiated by using the osteogenic medium. The osteogenic medium consisted of the standard medium supplemented with 0.25mM ascorbic acid phosphate (L-ascorbic acid phosphate magnesium salt n-hydrate, Wako Pure Chemical Industries, Osaka, Japan), 10 mM β-glycerophosphate (Sigma-Aldrich, Tokyo, Japan), and 100 nM Dexamethasone (Dex, Sigma-Aldrich, St. Louis, USA). The differentiation culture was maintained for 4 days, which was considered to be sufficient enough time to differentiate them into osteoblast-like cells. The cells were counted and adjusted by centrifugation at 1000 rpm for 5 min at 4°C, to 2×10⁶ cells/ml.

Preparation of beta-TCP/MSCs hybrid: The porous beta-TCP blocks were soaked in a cell suspension containing the standard medium using a low pressure system at 100mmHg for 1min and incubated for 3 hours at 37°C. The cells loaded into the beta-TCP blocks were observed using a scanning electron microscope (SEM, Hitachi, S-4500, Japan). They were confirmed to be attached to the pore surface of the beta-TCP (Figure 1). Just 3 hours after loading, each beta-TCP/MSCs hybrid was implanted at the operative sites in the macaque as described below.

Surgical procedure: Single level posterolateral
intertransverse process fusion was performed on nine crab-eating macaque at L4-5. General anesthesia was induced with intramuscular administration of ketamine (5mg/kg) and maintained with inhalational anesthesia of isoflurane (1.0-2.0%). After intubation, the macaque were placed in a prone position and then shaved. Subsequently, they were draped in the usual sterile manner. The position of L4-5 had been examined by palpation of the spinous process and iliac crests, and by comparison with a preoperative radiograph. A posterior midline skin incision was made after infiltration with 10ml of bupivacaine local anesthetic. Then, the transverse processes were exposed carefully to prevent bleeding, and an electric burr was used to decorticate the transverse processes. The supraspinous ligament and facet joints were left intact. The graft materials were prepared as described below and placed between the transverse processes. The fascial incisions were sutured with absorbable thread, and the skin incisions were sutured with surgical thread. Cefotetan (0.25g) was administered as an antibiotic for 2 days after surgery. The animals were allowed to move about their cages and then were fed ad libitum without restriction.

Experiments were performed in accordance with the guidelines of the Japanese Government for the care and use of laboratory animals.

Graft Materials: Nine macaque were bilaterally implanted, respectively. Graft materials were divided into three groups (n=6 each). One of the materials was implanted over the transverse process alternately.

1) The beta-TCP/MSCs hybrid group: MSCs were loaded into beta-TCP blocks (30×10×5mm) as described above. 2) The autogenous iliac crest bone group: Another skin incision was made parallel to the midline skin incision over the iliac crest. Posterior iliac crest bone was harvested partially (3.0ml). After removing the soft tissues, they were laid over the transverse process. 3) The beta-TCP group: beta-TCP blocks (30×10×5mm) alone were implanted.

So the hybrid graft was implanted to one side and the autograft was implanted to another side in three macaque. Additionally, the autograft and the beta-TCP in three macaque, the hybrid graft and the beta-TCP in three macaque.

Six macaque were sacrificed at 12 weeks and three monkeys were sacrificed at 24 weeks after implantation.

Evaluations: All monkeys were sacrificed with intravenous pentobarbital 12 or 24 weeks after surgery. The lumbar spines were harvested from L3-S1, and the attached tissue was removed to leave the posterolateral masses intact. Spine fusion status was evaluated blindly by manual palpation of the fusion segment, X-ray, micro CT, pQCT, and decalcified histologically.

Manual palpation: Specimens were further separated into two sagittal sections (because each section had different graft material). The lumber spines were manually palpated at the level of the fused motion segment proximally and distally. Each motion segment was graded as fusion if no motion was present, or not fusion if any motion was present.

Radiographic evaluation: Posteroanterior X-ray was performed at 3 week intervals up to the point of sacrifice. And soft X-ray was also performed after harvesting to examine the image detail. Bone formation was graded as fusion or not fusion.

Micro Computed Tomography (micro CT): Of the nine macaque, three underwent micro CT scans at the area of the posterolateral fusion after harvesting. Scans were performed on a high-speed CT scanner (SMX-130CT-SV, Shimadzu, Japan) with the following parameters: 34μA, 46kV, and 0.16mm slice thickness. Scans were evaluated for volume of fusion mass and presence of bone in any unintended areas.

Peripheral Quantitative Computed Tomography (pQCT): All specimens underwent pQCT scans (XCT Research SA+, Stratec Medizintechnik GmbH, Germany) at the level of L4, L5 transverse process and at their center with the following parameters: 0.12×0.12×0.77mm voxel size. Three slices were made on each specimen, and the bone mineral density (BMD) of the newly formed bone was measured to compare their average. The BMD of the unimplanted beta-TCP was also measured for comparison with the BMD of the newly formed bone.
beta-TCP/MSCs hybrid groups and the beta-TCP alone groups at 12 weeks after implantation.

Histological analysis: For light microscopic observation, the lumbar spine was fixed in 10% neutral-buffered formalin. After fixation, the specimens were dehydrated in ethanol. They were then decalcified (Decalcifying Solution A, Wako, Osaka, Japan), dehydrated and embedded in paraffin, and stained with hematoxylin and eosin. These samples were observed by optical microscope (Olympus, AX-70, Japan).

**Results**

All animals tolerated the surgical procedure well, and were observed to behave and feed normally.

Manual Palpation: Solid fusion was achieved in 5 of 6 (83.3%) macaque in the beta-TCP/MSCs groups and 4 of 6 (66.7%) in the autogenous bone graft groups, whereas none (0%) of the beta-TCP only groups fused, as judged by manual palpation. There was no statistically significant difference between beta-TCP/MSCs groups and autogenous bone graft groups.

Radiographic evaluation: The site of an autogenous bone grafting showed a homogenous fusion mass associated with some pieces of original bone. Beta-TCP/MSCs hybrid graft exhibited new bone formation, but it was difficult to assess bone formation because of TCP radiopacity. Only one case in the beta-TCP/MSCs groups showed “not union” because of the large defect of material due to the heavy damage of beta-TCP block.

Micro CT: Micro CT scans showed massive new bone formation directly connected with transverse process in the beta-TCP/MSCs groups at 12 weeks after implantation, which were greater than the bone formation of the autogeneous groups (Figure 2). And new bone formation continued even at 24 weeks after implantation.

pQCT: The pQCT scans revealed the same results as that of the micro CT. And the averages of bone mineral density (BMD) measured by pQCT increased more in the beta-TCP/MSCs groups than in beta-TCP block before implantation by additional new bone. Nevertheless the average value of the BMD decreased more in the beta-TCP groups than the

![Figure 2](imageURL)
BMD of the beta-TCP block before implantation, because of absorption of materials (Figure 3A, 3B).

Histological analysis: In the histologic study, the decalcified axial sections stained with HE showed spines which were implanted with beta-TCP/MSCs hybrid which confirmed the presence of normal bone ingrowth directly connected with the decorticated transverse processes in all specimens, which was parallel with the results of the micro CT scans (Figure 4). And the sagittal sections in the beta-TCP/MSCs groups proved that the new bone was induced throughout the carrier without one case with the presence of a fibrous tissue in the central area. Nevertheless the new bone was induced only at the edge of the carrier without bony bridging in the beta-TCP group (Figure 5, 6). Bone formation occurred only within the confines of the original ceramic, which still contained a slight amount of residual ceramic at 12 weeks. There was no evidence of any abnormal inflammatory response at the implant site.

Discussion

Beta-TCP/MSCs hybrids were capable of forming a sufficient amount of bone to obtain spinal fusion, whereas beta-TCP alone did not fuse. These results show the significance of MSCs in the application of..
porous beta-TCP. The dissolution of TCP may provide the Ca and P needed for bone formation.\textsuperscript{17,21} The osteoblast-like cells (MSCs differentiated into osteoblast-like cells) were distributed mostly throughout porous beta-TCP because of cell loading before implantation, which guarantees that osteogenesis will occur even in the center of the block. Furthermore, the porous beta-TCP used in this study has large interconnecting paths similar to that of cancellous bone, which makes it easier for blood vessels and cells to invade the center of the block. The blood supply may lead further to the proceeding of bone formation in porous TCP. This is because the beta-TCP/MSCs hybrids induce early bone formation in almost all the pores, and they cover the surface of the materials and limit exposure to the solution. This then leads to slow resorption of beta-TCP.\textsuperscript{22}

Yoshikawa and Ohgushi have improved the method of cultured bone.\textsuperscript{11,12,23} However, as the size of the scaffold increases, the introduction of cells into the central area of porous materials \textit{in vitro} becomes more difficult. MSCs (osteoblast-like cells) need a supply of oxygen and nutrients from surrounding blood vessels or tissue fluid \textit{in vivo}, however, the MSCs inside the porous materials can not obtain a supply of oxygen and nutrients during the culture \textit{in vitro}. To solve this problem, we implanted the beta-TCP/MSCs hybrid just after loading the cells.

In this study, graft materials were bilaterally implanted, respectively. It might have been adequate to implant the same materials bilaterally, in considering of the effect of another side. But it may be sufficient to discuss if hybrid can be used for PLF instead of autogenous bone graft.

Some reports say that ectopic bone formation using tissue engineering disappears with time by resorption.\textsuperscript{8,24} In this study, new bone formation in the beta-TCP/MSCs hybrid groups proceeded even at 24 weeks after implantation. One of the reasons for this result is that bone marrow cells were supplied from a decorticated transverse process by directly connecting with them. Furthermore, mechanical stress loaded to the implanted site may have played an important role in maintaining bone metabolism.\textsuperscript{25}

Several problems remain to be solved before any clinical applications can be undertaken. First of all, it is important to use auto-serum to culture MSCs, instead of foetal bovine serum which is thought to be unusable ethically. Another problem is that even if beta-TCP is good scaffold for this method, it is not ideal material because of its weak mechanical properties. Using fragile scaffold may lead to pseudoarthrosis, nevertheless, almost all the defects of damaged blocks were replaced by new bone formation in the beta-TCP/MSCs groups in this study. Great care must be taken when treating graft materials so as not to cause damage. However, these results were conducted without internal fixation including pedicle screws. Thus, co-usage of internal fixation may ensure further reliable fusion.

Beta-TCP/MSCs hybrid can be used for PLF instead of autogenous bone graft. Thus, it can be hypothesized that monkey PLF can simulate human PLF.
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