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Articular Cartilage Repair With Autologous Bone Marrow Mesenchymal Cells

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Articular cartilage defects that do not repair spontaneously induce osteoarthritic changes in joints over a long period of observation. In this study, we examined the usefulness of transplanting culture-expanded bone marrow mesenchymal cells into osteochondral defects of joints with cartilage defects. First, we performed experiments on rabbits and up on obtaining good results proceeded to perform the experiments on humans. Macroscopic and histological repair with this method was good, and good clinical results were obtained although there was no significant difference with the control group. Recent reports have indicated that this procedure is comparable to autologous chondrocyte implantation, and concluded that it was a good procedure because it required one step less than that required by surgery, reduced costs for patients, and minimized donor site morbidity. Although some reports have previously shown that progenitor cells formed a tumor when implanted into immune-deficient mice after long term in vitro culture, the safety of the cell transplantation was confirmed by our clinical experience. Thus, this procedure is useful, effective, and safe, but the repaired tissues were not always hyaline cartilage. To obtain better repair with this procedure, treatment approaches using some growth factors during in vitro culture or gene transfection are being explored. J. Cell. Physiol.

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Articular cartilage covers the ends of bones that form diarthrodial joints, and works as a lubricant and a shock absorber. Photomicroscopy reveals that histologically, articular cartilage is hyaline cartilage tissue because the intercellular matrix shows collapsed structure, and the tissue has no blood, lymphatic, or nerve supply.

Historically, articular cartilage has been considered to have only a weak capacity for repair, as reported by Hunter (1743) 250 years ago. It has been generally accepted that injuries that do not penetrate the subchondral bone (partial-thickness defects) are not repaired, while those that penetrate the subchondral bone (full-thickness defects) are repaired with the formation of various types of tissues, from a fibrous tissue to fibrocartilage. However, the reparative tissue, even that which is histologically like hyaline cartilage, lacks the biochemical capabilities to express some cartilage-specific molecules, and its biomechanical durability is substantially inferior to that of age-matched normal articular cartilage (Hunziker, 2002). The cartilage repair responses are different for individuals of different ages and for different species of animals, and such responses depend upon the physiological status of the animal, as well as the nature and extent of the injury.

What happens when such articular cartilage are left untreated? Until recently, many clinicians have been thinking that articular cartilage defects were not a major problem because they caused few clinical problems, at least during short observation periods. However, recently, some reports revealed that clinical symptoms or radiological changes due to articular cartilage defects become prominent when observed for more than 10 years (Messner and Gillquist, 1996; Shelbourne et al., 2003). Thus, it is now generally thought that articular cartilage should be repaired to prevent subsequent osteoarthritic changes. Articular cartilage defects are a major clinical problem; however, presently there is no treatment that is widely accepted to regeneratively repair these lesions. Currently, there is no satisfactory clinical technique for repairing articular cartilage defects. Current clinical practice usually involves bone marrow stimulation technique, in which subchondral bone is broken to facilitate cartilage repair from bone marrow-derived cells and cytokines, and consists of multiple perforations (Pridie, 1959), abrasions (Johnson, 1986), and micro-fractures (Steadman et al., 2003). However, with this procedure, cartilage defects are most often repaired with fibrocartilage, which is known to be biochemically and biomechanically different from normal hyaline cartilage and this tissue subsequently undergoes degeneration (Hunziker, 2002). Recent studies explored the usefulness of autologous chondrocyte implantation (ACI) (Brittberg et al., 1994) and mosaicplasty (Hangody et al., 2004; Matsusue et al., 1993) were explored. We can repair small articular cartilage defects using these techniques, although their effectiveness is still controversial. Even after ACI and mosaicplasty, some defects continued to persist in the articular cartilage, albeit not in the main weight-bearing portions of the joint. In ACI, no evidence of effectiveness has been reported so far (Nakamura et al., 2009), and we have to perform another operation to obtain autologous cells.

It has been reported that cells isolated from postnatal mammalian bone marrow have the potential for differentiation into specific cells of mesenchymal tissues, such as bone and cartilage, when implanted in vivo (Ashton et al., 1980; Goshima et al., 1991); thus, adherent cells in bone marrow blood contain progenitor cells for bone and/or cartilage. We assumed that these cells were suitable to repair osteochondral defects of joints because they could differentiate into both bone and cartilage. Thus, we performed autologous culture-expanded bone marrow mesenchymal cell (BMMC) transplantation in a rabbit model.

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Cartilage Repair With BMMC

Rabbit experiment (Wakitani et al., 1994)

We collected autologous osteochondral progenitor cells from bone marrow. They were culture-expanded and embedded into a collagen gel. These cellular grafts were then transplanted into large (3 mm × 6 mm, 3 mm in depth) full-thickness defects in the weight-bearing articular surfaces of 68 rabbits. These transplants were then observed for up to 6 months after surgery.

As early as 2 weeks after the transplantation, the defect was mostly replaced with cartilage. The replacement of this repaired cartilage began in the deeper portion of the defect with vascularized bone. By 4 weeks after transplantation, the deeper portion of the defect had been almost completely replaced by bone, and 24 weeks after transplantation, subchondral bone was completely repaired without loss or alteration of the overlying articular cartilage. We assume that BMMC preparations rapidly and quantitatively differentiate into chondrocytes in the rabbit distal medial femoral condyle defect, as has been observed in subcutaneous implantation samples. We hypothesize that these donor chondrocytes and the cartilage tissue that they form is replaced by host-derived vascular and bone-forming cells up to the bone articular cartilage junction.

Repair of articular cartilage defect in humans

Because the usefulness of BMMC transplantation in repair of osteochondral joint defects has been confirmed in a rabbit model, we thought that this technique could be applied in humans. BMMC have a number of suitable properties. First, it is easy to obtain autologous cells. This can be achieved by the aspiration of blood from the bone marrow using local anesthesia, without major side effects. Another reason for our interest in using these cells is that because we can cause them to proliferate without their capacity for differentiation being lost, this technique can be applied to large articular cartilage defects.

Two patients presented in our clinic because their knee pain prevented them from walking normally (Wakitani et al., 2004b). After thorough examination, we concluded that the knee pain was due to the injured articular cartilage, because there was no other abnormality in their knees. There were no improvements in clinical symptoms despite conservative treatment for a few months, and we decided to repair the defect with BMMC transplantation. Three weeks before transplantation, bone marrow was aspirated from the iliac crest of each patient. After erythrocytes had been removed using dextran, the remaining nucleated cells were placed in culture. When the attached cells had reached subconfluence, they were subcultured to expand in culture. Adherent cells were subsequently collected, embedded in a collagen gel, and then transplanted into the articular cartilage defect in the patellae and covered with autologous periosteum. Six months after transplantation, clinical symptoms (pain and walking ability) improved considerably, and the improvement persisted for 9 years post-transplantation in one case and 7 years in the other; both patients have been satisfied with the outcome. As early as 2 months after transplantation, the defects were covered with tissue that showed slight metachromatic staining. Two years after the first and 1 year after the second transplantation, arthroscopy was performed and the defects were found to have been repaired with fibrocartilage. We confirmed that autologous BMMC transplantation was an effective approach for promoting the repair of articular cartilage defects. Now, 12 years in the first and 10 years in the second case have passed, and there has been no clinical problem.

In order to apply this technique to the repair of articular cartilage defects in human osteoarthritic knees, we transplanted autologous culture-expanded BMMC into the cartilage defect of osteoarthritic knee joints when the patients were undergoing high tibial osteotomy (HTO), and observed the repair tissue when they were undergoing surgery for removal of the Steinmann pins and staples that fixed the separated proximal tibia (Wakitani et al., 2002). Twenty-four patients with knee osteoarthritis (OA) who underwent HTO were included in this study. Fifteen were female and nine were male. The patients' average age was 63 years (range 49–70 years). Twelve received autologous bone marrow cell transplants, and 12 were cell-free controls. All subjects enrolled in this study gave their informed consent, as approved by the institutional committee on human research; this committee also found this protocol to be acceptable. BMMC were prepared in the same manner as in the former two cases. The mean transplanted cell number was 1.3×10^7. HTO was performed using dome osteotomy, fixed with two pins with a Charnley clamp and two staples. At the time of HTO for OA of the knee, we transplanted these cells embedded in collagen gels into the medial femoral condyle, where articular cartilage was lost and subchondral bone was eburnated. We abraded the eburnated subchondral bone, transplanted cells in collagen, and covered the bone with autologous periosteum collected from the antero-medial surface of tibia. The mean size of the abraded area was 14 mm × 35 mm. The mean follow-up period was 16 months. Although the clinical improvement was not significantly different, the arthroscopic and histological grading score was better in the cell-transplanted group than in the cell-free control group. As early as 6.3 weeks after transplantation, the defects were covered with white soft tissue, in which metachromasia was partially observed, and 42 weeks after transplantation, the defects were covered with white soft tissue that was much harder than that observed at 6.3 weeks but was still softer than the surrounding normal cartilage. In almost all areas of the repair tissue, metachromasia was observed, and the repair tissue appeared similar to hyaline cartilage. This repair was found to occur much earlier and to be better than that reported in HTO only or HTO with abrasion (Fujisawa et al., 1979; Akizuki et al., 1997). The untreated tibial articular cartilage defects were not repaired at all.

We analyzed the clinical results 64 months after transplantation (Wakitani et al., 2008). The clinical scores were not significantly different between the cell-transplanted and the control groups. Longer observation might be necessary to see the effect of cell transplantation. Another possibility is that BMMC transplantation is not so effective in the osteoarthritic knee because the environment of the OA knee may not be good for cells or because the age of the patients was high.

Other reports of cartilage repair with BMMC transplantation

Kuroda et al. (2007) reported that transplantation of BMMC into 20–30-mm, full-thickness articular cartilage repair defect in the weight-bearing area of the medial femoral condyle of a 31-year-old judo player was effective.

We reported BMMC transplantation into osteochondral defects in five knees (femur and patella) from three patients. A 31-year-old female (bilateral knees), a 46-year-old male, and a 42-year-old male (bilateral knees) underwent BMMC transplantation in their patellofemoral joints (Wakitani et al., 2007b). All patients had suffered from pain and clicking in their patellofemoral joints on motion. Because magnetic resonance imaging (MRI) revealed articular cartilage abnormalities in the patellofemoral joints, we performed arthroscopy to confirm the lesions. After arthroscopy, we decided to transplant autologous BMMC. In the case of the 31-year-old female patient, we found articular cartilage damage in both the femur and the patella. We removed the damaged articular cartilage, transplanted BMMC embedded in the collagen gel, and covered

the transplanted tissue with autologous periosteum. Improvements in clinical symptoms were observed in all patients.

Recently, we applied this technique to repair osteochondral defects in three elbows (humeral capitellum; Wakitani et al., 2006). BMMC transplantation in humeral capitellum was performed on three 14-year-old boys. All patients were throwing athletes and had been suffering from elbow pain during throwing motion. Range of motion was slightly restricted. As shown in X-ray film, separated bone fragment was observed in capitellum and diagnosed osteochondral dissecans. Because the separated fragment was large, unstable, and divided into small pieces, we decided to remove the fragment and to transplant autologous BMMC. Clinical symptoms were much improved in all patients.

Nejadnik et al. (2010) reported BMMC transplantation into 36 articular cartilage defects and followed up for 24 months. They compared the results with those of 36 ACI and concluded that BMMC transplantation showed comparable results with ACI. They reported that it was a good procedure because it required one step less of surgery, reduced costs for patients, and minimized donor site morbidity.

These were all reports of the BMMC transplantation for articular cartilage defects that we could find presently.

Discussion of BMMC transplantation

Autologous culture-expanded BMMC transplantation was shown to be effective in the repair of articular cartilage defects, although no evidence has been shown. Important advantages of the techniques described herein are obvious from the data provided. Although these progenitor cells are not abundant, we have been able to mitotically expand them in culture. These approaches have considerable relevance to the treatment of human cartilage defects, and provide the starting point for the refinement of a repair technology capable, in principle, of regenerating large areas of articular cartilage.

The number of reports of BMMC transplantation in human is limited. The reports of BMMC transplantation that we could find are shown above. Besides these, we could find some reports in scientific meetings in the world. However, the total number of BMMC transplantations is much less than that of ACI. The reason for this is that ACI was explored first and made available very early. Even in ACI, evidence of the effectiveness is still controversial (Knutsen et al., 2004, 2007). There is only one report of randomized controlled trial in BMMC transplantation, which is mentioned above (Hui et al., 2010). This report showed that the clinical effectiveness of BMMC transplantation is comparable to results with ACI, although BMMC transplantation had superiority in some procedures. We have to explore more to show the evidence of effectiveness of BMMC transplantation.

The repair tissues were not completely composed of hyaline cartilage. Theoretically, hyaline cartilage is preferable. These cells could be driven in vitro into the chondrogenic lineage using cytokines (Sekiya et al., 2001; Yamamoto et al., 2004; Nawata et al., 2005) or gene transfection (Ikeda et al., 2004; Katayama et al., 2004), and the resultant autogenetic chondrocytes would be transplanted into cartilage defects.

It has been reported that cells isolated from human marrow aspirates could be induced to differentiate into other mesenchymal lineages, such as adipocytic, chondrocytic, or osteocytic lineages in vitro (Johnstone et al., 1998; Pittenger et al., 1999). Furthermore, they are reported to differentiate into cells other than mesenchymal tissues, ectodermal (neurocyte; Kopen et al., 1999) and endodermal tissues (hepatocyte; Petersen et al., 1999) (transdifferentiation). Recently, these cells are considered to be a useful cell source to repair some kinds of tissues, such as bone, cartilage, tendon, muscle, heart, small vessel, liver, nerve, and so on.

Other Progenitor Cells

Further investigations have been performed throughout the world for the repair of articular cartilage defects with hyaline cartilage, using certain other types of cells. Osteochondral progenitor cells or mesenchymal stem cells have been reported to exist in many kinds of tissues, such as the synovium, muscle, and fat. Autologous cells of these tissues are easily obtained. Within these cells, synovial cells are reported to have the best capacity for differentiating into cartilage, and are expected to be used clinically (Sakaguchi et al., 2005). Fat-derived mesenchymal cells are noteworthy (Mochizuki et al., 2006). These days, much of the population has excess fat, making it relatively easy to collect a large quantity of autologous cells.

Allogeneic cell transplantation has been explored in animal models. We have reported that cartilage-like tissue, generated ectopically by muscle-derived cells in a diffusion chamber using bone morphogenetic protein (BMP)-2, is effective in repairing articular cartilage defects in rats (Nawata et al., 2005). We have also reported that cartilage-like tissue, generated ectopically by amnion-derived cells using BMP-2 is effective in repairing articular cartilage defects in rats (Wei et al., 2009). These methods might be a new technique of tissue engineering for the repair of articular cartilage defects. Embryonic stem (ES) cells or inducible pluripotent stem (iPS) cells are one of the most promising cell sources for many kinds of tissue repair. These cells can be used to repair osteochondral defects, but it is difficult to induce these cells to differentiate exclusively into chondrocytes. We have reported that when we transplant ES cells into joint spaces they form a teratoma and subsequently destroy the joint (Wakitani et al., 2003). However, we also reported that when they are transplanted into osteochondral defects they form cartilage and promote the repair process (Wakitani et al., 2004a). The mechanism of this phenomenon is unclear; the use of ES cells is expected to increase in the future.

Problems With Cell Transplantation

Tumorigenesis

It has been reported that human adult stem cells from fat tissues can transform after long-term culture (Rubio et al., 2005). To our knowledge, this is the first report of transformation of cultured mesenchymal cells from adult humans. Some reports that supported tumorigenesis (Røsland et al., 2009). In these reports, cells were cultured for extraordinarily long periods, several months. Cells reached senescence or crisis phase, and cells appeared afterwards that had karyotype abnormality and formed tumor when injected into mice. Extremely long culture in vitro may injure the karyotypes and promote tumorigenesis. However, there are some reports that denied tumorigenesis. (Bernardo et al., 2007; Meza-Zepeda et al., 2008). Transformation of cultured cells is a major problem in cell therapy. We have never observed tumor formation in any of our extensive number of animal experiments or in clinical cases of BMMC transplantation. Human somatic cells have limited capacity for cell division. The possibility cannot be excluded, but the transformation of cultured adult human BMMC is considered to be rare.

To confirm the safety of BMMC transplantation, we investigated the patients with BMMC transplantation. Between January 1998 and November 2008, 41 patients received 45 transplantations. Neither tumors nor infections were observed in between 5 and 137 months (mean 75 months) of follow-up. From this result, we concluded that autologous BMMC transplantation is a safe procedure (Wakitani et al., in press).

Assessment of articular cartilage repair

As we explained in this article, the effectiveness of ACI has not yet been shown. One of the reasons for this is that it is difficult to estimate the effectiveness of articular cartilage repair. Clinical symptoms of articular cartilage are not resolved. Some patients feel nothing, while others suffer from pain. Although clinical symptoms are very important, they are not objective.

We have to objectively estimate the effectiveness of articular cartilage repair. One of the means for performing objective assessments of repair is MRI. Using MRI, we can estimate the extent of repair of the defect with different materials, but it is difficult to estimate the quality of the repair tissue. In the near future, we will be able to estimate its quality by MRI, because studies to develop such techniques are ongoing.

Arthroscopic biopsy is currently the most reliable procedure for estimating the repair tissue quality. However, this is an invasive procedure. Arthroscopy itself is invasive, and the biopsy is even more so. Therefore, even if we consider arthroscopy to be acceptable, it is not acceptable to perform biopsy. Thus, arthroscopic assessment methods using ultrasound, etc. are now being explored (Hattori et al., 2005).

Biological markers for OA have been explored. Many researchers have tried to detect the metabolic products of articular cartilage components (proteoglycans, type II collagen, and non-collagenous proteins) in joint fluid or blood and thereby develop a marker of OA. Keratan sulfate (KS), chondroitin 6 sulfate (C6S), cartilage proteoglycan aggrecan turnover epitope (CS846), hyaluronan (HA), and cartilage oligomeric protein (COMP) were candidate markers, and have been reported to be markers of OA to some extent. We measured KS levels using high-performance liquid chromatography, which has been reported to be more accurate than enzyme-linked immunosorbent assay (ELISA), and 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 (Wakitani et al., 2007a). We have reported that newly explored highly sensitive ELISA kit is effective in detecting the serum KS, for screening early OA in humans (Wakitani et al., 2010).

Xenogenic proteins

We usually add fetal calf serum (FCS) into the medium for cell culture. For BMMC culture, prior to 2001, we added FCS. Cows with bovine spongiform encephalopathy (BSE) have been found in the USA, so some investigators are now using FCS from Australia as BSE is not found in that country. However, it is possible that in future, cows in Australia will carry BSE. If possible, we should not use FCS when we culture human cells for transplantation. Following confirmation that BMMC could be multiplied with autologous serum, we used autologous serum, not FCS, in human cell culture for transplantation.

Subsequently, an additional problem has been pointed out. It has been reported that a nonhuman molecule (silica acid Neu5Gc) is expressed on human ES cells when they are cultured on mouse feeder layers, and that antibodies specific for this molecule kill the cells (Martin et al., 2005). This report indicated that it was possible that cultured human cells expressed molecules from animals when they were in contact with animal-derived materials. Both FCS and collagens sometimes used as delivery vehicles may also be associated with induction of the same phenomenon.

Conclusion

We transplanted autologous culture-expanded bone marrow mesenchymal cells into articular cartilage defects. Clinical symptoms were improved but the repair cartilage was not hyaline cartilage. To regenerate articular cartilage by cell transplantation, it is essential that cells proliferate without losing their capacity for differentiation. To find appropriate conditions, different culture conditions, mechanical stresses, growth factors, and gene transfection have all been explored, but these have not yet been applied clinically.

The safety of cells is important. It has been reported that long-term culture may induce karyotype abnormality and tumorigenesis; we think these are due to the extraordinarily long culture periods. Usual culture periods are not dangerous for cell transplantation, as we showed in clinical experience.

Thus, this procedure is one of the most useful, effective and safe, but the repaired tissues were not always hyaline cartilage. To obtain better repair with this procedure, treatments with some growth factors during in vitro culture or gene transfection are being explored.

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Clinical Outcome of Microsurgical Bilateral Decompression *via* Unilateral Approach for Lumbar Canal Stenosis

Minimum Five-Year Follow-up

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Study Design. A retrospective study.

Objective. To evaluate minimum 5-year clinical outcome and radiologic changes in patients who underwent microsurgical bilateral decompression *via* a unilateral approach.

Summary of Background Data. Some authors have reported satisfactory short-term results of minimally invasive decompressive procedures such as microscopic or microendoscopic decompressive laminotomy for lumbar spinal stenosis (LSS). However, there have been a few reports on the long-term clinical outcome of these procedures.

Methods. The study consisted of 57 patients who underwent this surgery and had been followed for at least 5 years. The preoperative diagnoses were LSS without instability in 27 patients, degenerative lumbar spondylolisthesis (DS) in 20 patients, and degenerative lumbar scoliosis (DLS) in 10 patients. The mean duration of follow-up was 6 years. Clinical outcome was evaluated by Japanese Orthopedic Association (JOA) score. Complications, rate of reoperation, and radiographic changes after surgery on plain radiograph were evaluated.

Results. The mean JOA score was 13.8 ± 3.6 points before surgery, and improved to 24.9 ± 3.1 points at 3 months and 22.6 ± 4.7 points at the latest follow-up. There were no significant differences in JOA score at the latest follow-up among patients with LSS, DS, and degenerative scoliosis (22.3 ± 5.3, 23.3 ± 4.4, and 21.6 ± 2.6, respectively). Four patients (7%) underwent reoperation; 2 had DS and 2 had DLS. The preoperative percentages of slippage in patients with LSS, DS, and DLS were 0.4% ± 2.2%, 13.2% ± 5.9%, and 0.0% ± 1.3%, respectively, whereas degrees of progression of slippage at latest follow-up were 1.2% ± 3.1%, 2.4% ± 4.7%, and 0.0% ± 0.0%, respectively. There were no significant differences in progression of slippage among these 3 disease groups.

Conclusion. Microsurgical bilateral decompression *via* a unilateral approach is a minimally invasive technique that yielded satisfactory surgical outcomes even on minimum 5-year follow-up.

Key words: minimally invasive surgery, long-term, lumbar spinal stenosis, degenerative lumbar spondylolisthesis, unilateral approach. **Spine 2010;XX:000–000**

Decompressive laminectomy has been widely used as a treatment for lumbar spinal stenosis. Although satisfactory surgical outcomes have been reported with it, instability following the procedure has become the greatest concern among surgeons as a cause of deterioration of symptoms.[1,2] Whether this procedure is indicated for patients with degenerative lumbar spondylolisthesis (DS) and degenerative lumbar scoliosis (DLS) is another important concern.

Less invasive surgery using microsurgical and endoscopic procedures has come to be more commonly used for the treatment of lumbar spinal stenosis (LSS) over the last decade. The point of these procedures is maximal preservation of structural components such as midline structures, facet joints, and paravertebral muscle to prevent postoperative instability. Microsurgical bilateral decompression *via* a unilateral approach was first described by Poletti.[3] The procedure was modified by McCulloch and Young and described in detail in 1998.[4] In this technique, the dural sac and bilateral nerve roots can be decompressed with preservation of the supra- or interspinous ligament complex as well as the contralateral paraspinal muscles and facet joints. Enlargement of the central parts of the spinal canal is achieved by dome-shaped undercutting of the laminae and resection of the ligamentum flavum on the contralateral side.[3–7] The technique of limited osteoplastic laminectomy by spinous process osteotomy preserves the midline osseoligamentous structures and limits the instability created by standard lumbar decompressive laminectomy.[8] In our institution, microsurgical bilateral decompression *via* a unilateral approach, a modified version of McCulloch's method, has been performed since 1998 for the treatment of degenerative lumbar disorders such as LSS, DS with less than 10° of angular instability, and DLS with less than a 25° Cobb angle. The purpose of this study was to investigate the clinical outcomes of this surgical procedure over longer than 5-year follow-up.

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Table 1. Patients Demographics

Average age	69.6 yrs (range, 46–86)
Gender	Men:women = 27:30
Average follow-up	6.0 yrs (range, 5–8 yrs)
Diagnosis	
Lumbar spinal stenosis (LSS)	27
Degenerative lumbar spondylolisthesis (DS)	20
Degenerative lumbar scoliosis (DLS)	10
No. levels decompressed	
1 level	33
2 level	23
3 level	1

■ Materials and Methods

Patients

Fifty-seven patients operated on by 1 senior author (H.M.) and with longer than 5-year follow-up were included in this study. The clinical indications for this surgical procedure were leg pain and/or leg numbness inducing intermittent claudication rather than back pain. Radiologic evaluation included radiograph examination, magnetic resonance imaging, myelography, dynamic radiograph examination, and CT-myelography. The radiologic indications for use of this surgical procedure were LSS without instability, DS with less than 10° of angular instability, and DLS with less than a 25° Cobb angle. There were 27 men and 30 women. The age at surgery ranged from 48 to 86 years, with a mean of 69.6 years, and the duration of follow-up ranged from 5 to 8 years, with a mean of 6 years. The preoperative diagnoses were LSS in 27 patients, DS in 20 patients, and DLS in 10 patients. When the radiologic degenerative changes were more extensive than expected based on the clinical findings, we routinely used selective nerve root block to decide the level of decompression. Thirty-three patients underwent single-level, 23 patients underwent 2-level, and 1 patient underwent 3-level decompression (Table 1). The level of surgery was L2–L3 in 3 patients, L3–L4 in 24 patients, L4–L5 in 49 patients, and L5–S1 in 4 patients.

Surgical Procedure

Microscopic bilateral decompression *via* a unilateral approach was modified from the method reported previously to complete decompression on the contralateral side.[3–7] The laminotomy was performed on the side of approach in the area of the ligamentum flavum insertion, and resection of the articular process was performed in trumpeted manner until the inner aspect of the pedicle, with slight tilting of the microscope laterally. After the side of approach had been completely decompressed, the operating table and the microscope were tilted about 15° to observe the contralateral side. The basal part of the spinous process of the caudal half of the cranial lamina and a small cranial portion of the caudal lamina were removed with a high-speed drill. Then the contralateral lamina was undercut with a high-speed air drill leaving the ligamentum flavum in place as protection for the dural sac and the nerve root. Following sufficient resection of the bony segment, the ligamentum flavum was removed *en bloc* with a curette, while protecting the dural sac and contralateral nerve root with a patty. With recognition of the inner aspect of the pedicle on the contralateral side, we confirmed adequate decompression of the contralateral side (Figure 1).

Clinical Evaluation

Two authors (H.T. and S.D.) not involved in the care of these patients reviewed all records. Operative time, blood loss, Japanese Orthopedic Association score (JOA score), complications, rate of reoperation, and deterioration of symptoms in the follow-up period were investigated. Clinical outcomes were evaluated based on JOA score before surgery, 3 months after surgery, 1 year after surgery, and at latest follow-up (Table 2). Rate of improvement was calculated as follows, as suggested by Hirabayashi *et al.*[9] The overall result was classified as excellent in the case of greater than 75% improvement ratio in score, and good for 50% to 75%, fair for 25% to 49%, and poor for 0% to 24% improvement. Anteroposterior (AP) and lateral preoperative plain radiographs, postoperative radiographs, and radiographs at latest follow-up were examined. On lateral radiographs, slippage ratio was measured by the Boxall method.[10] On the AP view, the angle of scoliosis was measured by the Cobb method and the lateral slippage ratio was measured by the Boxall method.[11]

Statistical Analysis

Values are the mean ± standard deviation. The degree of significance was determined by *post hoc* testing using the Bonfer-

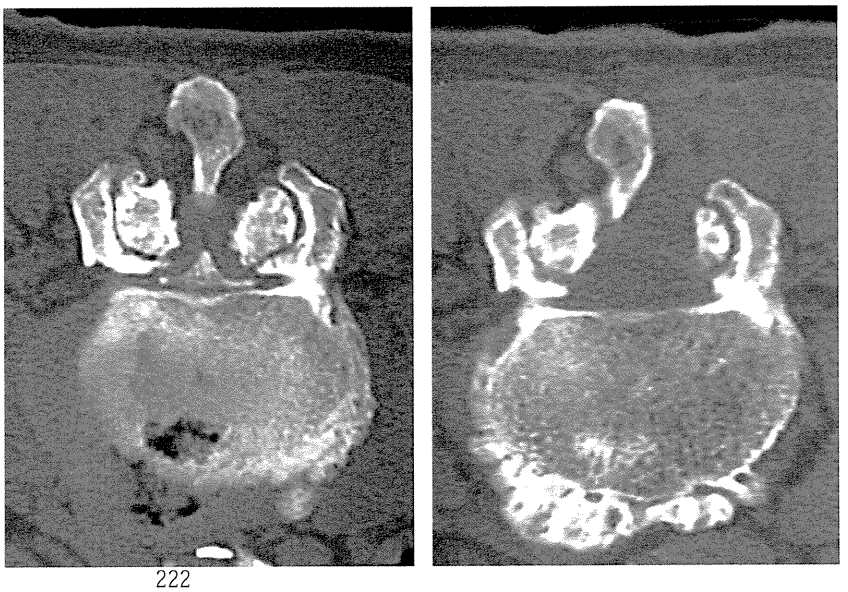

Figure 1. CT myelogram before operation and CT after operation. The lumbar spinal canal was adequately decompressed on the side of approach as well as the contralateral side.

Table 2. Criteria of the Japanese Orthopedic Association Lumbar Scores (JOA Score)

Item	Score
Subjective symptoms (9 points)	
Low-back pain	
None	3
Occasionally mile	2
Always present or sometimes	1
Always sever	0
Lower-limb pain and/or tingling	
None	3
Occasionally mile	2
Always present or sometimes	1
Always sever	0
Gait	
Normal	3
Able to walk at least 500 m, pain/numbness/ weakness present	2
Unable to walk at least 500 m, pain/numbness/ weakness present	1
Unable to walk at least 100 m, pain/numbness/ weakness present	0
Clinical signs (6 points)	
Strait leg raising (SLR)	
Normal	2
30°–70°	1
Less than 30°	0
Sensory disturbance	
Normal	2
Mild sensory disturbance	1
Apparent sensory disturbance	0
Motor disturbance	
Normal (MMT: normal)	2
Slightly weakness (MMT: good)	1
Markedly weakness (MMT: less than fair)	0
Restriction of activities of daily living (14 points)	
Turning over while lying	
None/moderate/severe	2/1/0
Standing	
None/moderate/severe	2/1/0
Washing face	
None/moderate/severe	2/1/0
Leaning forward	
None/moderate/severe	2/1/0
Sitting	
None/moderate/severe	2/1/0
Lifting or holding heavy object	
None/moderate/severe	2/1/0
Walking	
None/moderate/severe	2/1/0
Urinary bladder function (−6 points)	
Normal	0
Mild dysuria	−3
Severe dysuria	−6

MMT indicates manual muscle test.

roni method for continuous data and the χ^2 test for categorical data. An associated probability (P value) of <0.05 was considered significant.

■ Results

Clinical Results

The mean blood loss per level was 113.4 ± 74.8 mL, and the mean operative time per level was 134.2 ± 28.7 minutes.

The mean JOA score was 13.8 ± 3.6 points before surgery, but improved to 24.9 ± 3.1 points at 3 months, 25.6 ± 2.5 points at 1 year, and 22.6 ± 4.7 points at latest follow-up. The mean rate of improvement was 71.5% at 3 months, 73.5% at 1 year, and 57.9% at latest follow-up. On evaluation at latest follow-up, 11 patients were categorized as excellent (19.3%), 26 patients as good (45.6%), 17 patients as fair (29.8%), and 3 patients as poor (5.3%). In total, 64.9% of patients were rated as excellent or good. Nine patients exhibited gradual deterioration of symptoms during the follow-up period, and have undergone conservative treatment such as epidural block and root block. Of the 9 patients with symptom deterioration, 3 had LSS, 4 had DS, and 2 had DLS. Four patients (7%) underwent reoperation due to deterioration of symptoms. On evaluation by preoperative diagnosis, patients with LSS exhibited a 71.9% mean rate of improvement at 3 months and 54.1% at latest follow-up. For patients with DS, the corresponding percentages were 72.3% and 64.1%, whereas for patients with DLS, they were 75.1% and 51.6%. Deterioration of score was greatest for DLS, although there were no significant differences in JOA score at latest follow-up among LSS, DS, and DLS (Figure 2).

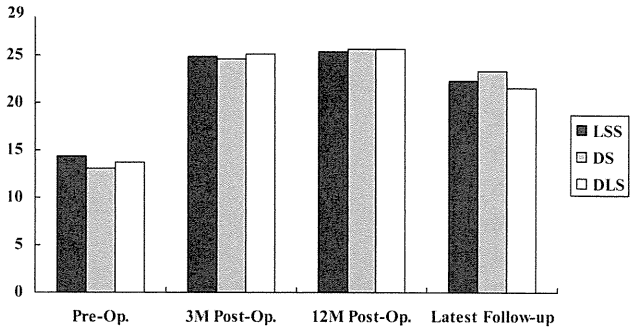

Figure 2. Average JOA scores before surgery, after surgery, and at the time of latest follow-up. Neither the ratio of improvement nor the score at latest follow-up differed among the preoperative diagnoses.

The 4 patients requiring reoperation included 2 with DS and 2 with DLS (Figure 3). No patient with LSS required reoperation. By type of reoperation, 1 patient with DS underwent repeat decompression and another with this condition underwent herniotomy. One patient with DLS underwent posterior lumbar interbody fusion at the level of operation during the study period, and another patient with DLS underwent second surgery at another

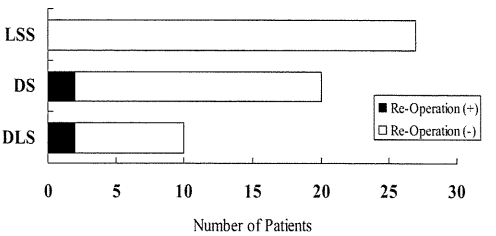

Figure 3. Numbers of patients requiring reoperation. The 4 patients requiring reoperation included 2 patients with degenerative lumbar spondylolisthesis (DS) and 2 patients with degenerative lumbar scoliosis (DLS). The rate of reoperation was 0% for lumbar spinal stenosis (LSS), 10% for degenerative lumbar spondylolisthesis, and 20% for degenerative lumbar scoliosis.

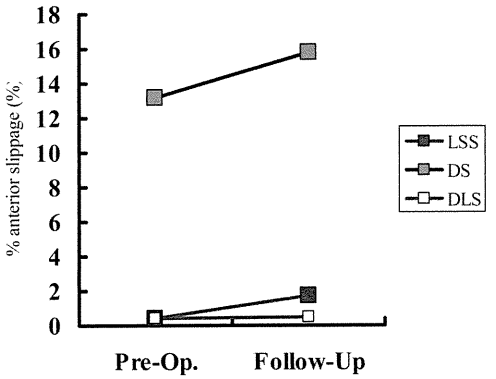

Figure 4. Progression of anterior slippage during the follow-up period. Progression of anterior slippage after this surgical procedure was 1.8% on average for longer than 5-year follow-up. The preoperative percentages of anterior slippage in patients with lumbar spinal stenosis (LSS), degenerative lumbar spondylolisthesis (DS), and degenerative lumbar scoliosis were 0.4% ± 2.2%, 13.2% ± 5.9%, and 0.0% ± 1.3%, and the percentages of slippage at latest follow-up were 1.8% ± 3.8%, 15.6% ± 7.9%, and 0.5% ± 1.4%, respectively.

level. Reoperation was performed a mean of 3.6 years (range, 1–7.3 years) after initial operation.

Radiologic Evaluation

The level of operation was L2–L3 in 3 patients, L3–L4 in 24 patients, L4–L5 in 49 patients, and L5–S1 in 4 patients. Progression of slippage was evaluated at the L4–L5 level. The preoperative percentages of anterior slippage ranged from 0 to 28.3, with a mean of 5.1% ± 7.3%. The percentages of anterior slippage at latest follow-up ranged from 0 to 37.5, with a mean of 6.9% ± 8.9%. Progression in anterior slippage after this surgical procedure was 1.8% during follow-up. The preoperative percentages of anterior slippage in patients with LSS, DS, and DLS were 0.4% ± 2.2%, 13.2% ± 5.9%, and 0.0% ± 1.3%, and the percentages of slippage at latest follow-up were 1.8% ± 3.8%, 15.6% ± 7.9%, and 0.5% ± 1.4%, respectively (Figure 4). The degrees of progression of slippage were 1.2% ± 3.1%, 2.4% ± 4.7%, and 0.0% ± 0.0%, respectively. There were no significant differences in progression of anterior slippage among these diseases. Number of cases of progression in slippage of more than 5% are shown in Figure 5. Progressive anterior slippage was found in 8 of 49 patients (16.3%), including 3 with LSS (3/23 13.0%) and 5 with DS (5/17 29.4%). No correlations were found between changes in clinical symptoms and progression of spondylolisthesis. The preoperative Cobb angle in DLS was 14.2° ± 7.4°, and progressed to 17.8° ± 9.6° at the latest follow-up. Preoperative lateral slippage in the coronal plane was found only in DLS; the preoperative value of 4.7% ± 6.2% increased slightly to 6.5% ± 6.8% after surgery, though this change was not significant. There were no patients with progression of lateral slippage of more than 5% after this procedure.

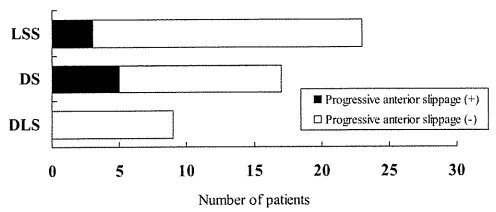

Figure 5. Numbers of patients with progression of slippage of more than 5%. Three such patients had lumbar spinal stenosis (LSS), with a frequency of new slippage of 13.0%, whereas 5 such patients had degenerative lumbar spondylolisthesis (DS), with a frequency of progression of slippage of 29.4%.

■ Discussion

LSS is a common lesion in elderly patients suffering from low back and leg pain with intermittent claudication. When conservative treatment fails, surgical treatment of this lesion becomes necessary, the most common of which is expansive laminectomy. Some authors have reported satisfactory results with decompressive laminectomy.[12–17] However, iatrogenic instability following laminectomy has become a problem.[1,2,18] Johnsson *et al* reported that postoperative slippage occurred in 18 of 45 patients (40%) who underwent laminectomies.[1] In their study, 65% of patients with DS exhibited a high risk of further slippage after operation and 20% of patients with LSS exhibited additional slippage. Mardjetko *et al* reviewed the incidence of progression of slippage after decompression and reported it to be 31%.[18] In some cases, spinal fusion combined with adequate decompression is therefore required.[19–21] A randomized trial and a study with alternating treatment assignments revealed better outcomes with decompression plus fusion than with conventional decompression alone.[20,22] However, some authors have suggested that patients treated with spinal fusion have a higher likelihood of greater blood loss, a longer operative time, and a higher rate of complications, and thus require more extensive revision surgery.[23–26] Thus, decompression surgery with clinical outcome equivalent to that of conventional decompression and less postoperative instability would be desirable.

McCulloch and Young developed unilateral laminotomy for bilateral ligamentectomy and reported a good or excellent outcome in 90.9% of 22 patients with acquired degenerative spinal stenosis.[4] Weiner *et al* reported limited osteoplastic laminectomy with spinous process osteotomy preserving the midline osseo-ligamentous structures, and found that 87% of patients reported high rates of satisfaction at 9 months' follow-up.[8] Thome *et al* reported that clinical outcome after unilateral laminectomy was equivalent to that with conventional laminectomy with a minimum follow-up period of 12 months.[27] As regard the long-term clinical outcome of less invasive decompression procedures, Oertel *et al* reported that 85.3% of 102 patients had excellent to fair results of surgery over 4 to 10 years (mean, 5.6 years) of follow-up, with a rate of reoperation of 11.8%.[28] Costa *et al* reported that 87.9% of 374 patients experienced clinical benefit and only 8% of patients suffered from segmental

instability at the treated level at a mean duration of follow-up of 30.3 months (range, 16–53 months).[29] Cavusoglu *et al* reported good results in 68% patients at 4 years, and noted that reoperation was not required for recurrent spinal stenosis at the same segments within 4 to 7 years.[30] In the present study, we evaluated clinical outcome and radiographic changes over a minimum 5-year follow-up, which ranged from 5 to 8 years with a mean of 6 years. The present study is thus the longest follow-up study of less invasive decompression procedures.

In our study, the rate of reoperation was 0% for LSS, 10% for DS, and 25% for DLS. The mean rate of reoperation was 7.0%. Katz *et al* reported that 23% of patients had undergone reoperation after 7- to 10-year follow-up for conventional decompressive surgery.[31] Iguchi *et al* reported that 3 of 37 patients (8.1%) who underwent decompression alone with longer than 10-year follow-up required additional surgery because of disc herniation at segments subjected to laminectomy.[15] Atlas *et al* found that 23% of patients who underwent decompression alone had required at least 1 additional lumbar spine operation by 10 years after their original procedure.[32] Compared with these previous reports on expansive laminectomy, the rate of reoperation was relatively low in our study. Whether this procedure should be performed for the treatment of DLS must be carefully determined.

In the present study, postoperative slippage occurred in 13% of cases of LSS and 29.4% of cases of DS. Matsunaga *et al* reported that progressive spondylolisthesis was observed in 34% of nonsurgically managed patients with DS during 10- to 18-year follow-up.[33] Yoshida *et al* reported that the rate of progression of slippage was 33.3% while that of new slippage was 12.0% over 11-year follow-up.[34] Mardjetko *et al* reviewed the incidence of progression of slippage after decompression and reported it to be 31%.[18] Compared with these reports, we found little progression of spinal slippage with the present procedure over a minimum 5-year follow-up. The radiographic changes after this procedure were similar to those described in other reports on the natural course of LSS and DS.

There are a few limitations to this study. First, this was a retrospective study without any control group. Second, the indications for this surgical procedure were limited to patients with less than 10° of angular instability in the case of DS and those with less than a 25° Cobb angle in the case of DLS. The usefulness of this procedure for patients with severe deformity or less stability is thus still unclear. However, the present study yielded the important finding that microsurgical bilateral decompression *via* a unilateral approach yielded satisfactory long-term outcome in patients with LSS and in some patients with DS or DLS.

■ Conclusion

Microsurgical bilateral decompression *via* a unilateral approach is a minimally invasive technique that yielded satisfactory surgical outcomes even with a minimum 5-year follow-up period. Good clinical outcome was obtained not only for LSS but also for DS and DLS. Spinal stability was superior to that with other expansive procedures even in cases of DS and DLS.

■ Key Points

- Microsurgical bilateral decompression *via* a unilateral approach is a minimally invasive technique that provided satisfactory clinical outcome for longer than 5-year follow-up.
- Good clinical outcome was obtained not only for LSS but also for DS and for DLS.
- Radiographic changes after this procedure were similar to those described in other reports on the natural course of LSS and DS.

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Synthesis, characterization of calcium phosphates/polyurethane composites for weight-bearing implants

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Abstract: Calcium phosphate (CaP)/polymer composites have been studied as an alternative graft material for the treatment of bone defects. In this study, lysine-triisocyanate-based polyurethane (PUR) composites were synthesized from both hydroxyapatite (HA) and β-tricalcium phosphate (TCP) to reduce the brittleness of CaP and increase the bioactivity of the polymer. The mechanical properties and *in vitro* cellular response were investigated for both HA/PUR and TCP/PUR composites. The composites were implanted in femoral defects in rats, and *in vivo* bioactivity was evaluated by X-rays, micro-computed tomography (μCT), and histological sections. In biomechanical testing, PUR improved the mechanical properties of the CaP, thus rendering it potentially suitable for weight-bearing applications. *In vitro* cell culture studies showed that CaP/PUR composites are biocompatible, with β-TCP enhancing the cell viability and proliferation relative to HA. CaP/PUR composites also supported the differentiation of osteoblastic cells on the materials. When implanted in rat femoral defects, the CaP/PUR composites were biocompatible and osteoconductive with no adverse inflammatory response, as evidenced by X-rays, μCT images, and histological sections. Additionally, a histological examination showed evidence of cellular infiltration and appositional remodeling. These results suggest that CaP/PUR composites could be potentially useful biomaterials for weight-bearing orthopaedic implants.
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INTRODUCTION

Calcium phosphates (CaP) have been extensively investigated for treating osseous defects. Hydroxyapatite (HA) and tricalcium phosphate (TCP) have been shown to be osteoconductive.[1] The composition of bone is approximately 70% mineral content, which is primarily HA.[2] Thus because of its natural presence in bone, synthetic HA is an attractive bone substitute. HA is prepared from the hydrothermal conversion of bone or naturally occurring coralline apatite, and it can be synthesized with variable porosity.[3] TCP is a biocompatible and bioactive ceramic that has been demonstrated to bond to bone directly.[4,5] Whereas HA bone cements exhibit compressive strengths in the range of 4–50 MPa,[6,7] TCP has a significantly lower strength than HA.[3] Despite their favorable biocompatibility and osteoconductivity, both HA and TCP are subject to brittle fracture and graft migration, potentially requiring additional surgeries for repair or removal.[3,8,9]

CaP/polymer composites have been synthesized to reduce the brittleness of CaP as well as to increase the bioactivity of the polymer.[8] Multiple polymeric systems have been used to prepare CaP composites with varying porosities and compositions.[10] HA/chitosan/PLA composites synthesized using *in situ* precipitation with 50–80 wt % HA exhibit compressive elastic modulus and strength values in the range of 416–857 MPa and 166–256 MPa, respectively.[11] HA/PLA composites synthesized using solvent casting at lower HA contents (30–40 wt %) can have a bending strength and modulus as high as 269 MPA and 7.6 GPa, respectively.[12–15] These composites remodeled almost completely when implanted as intramedullary rods in the distal femur of rabbits after five to seven years.[12]

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Two-component biodegradable polyurethanes (PUR) offer several advantages in the synthesis of CaP composites. PUR systems based on lysine polyisocyanates are biocompatible and degrade to nontoxic breakdown products.[16–21] Furthermore, they comprise a reactive system that is suitable for injectable applications.[20,22] TCP/PUR composites (10 wt % β-TCP) prepared from lysine ethyl ester diisocyanate (ELDI) exhibit compressive modulus and strength of 2.3 and 139 MPa, respectively,[21] and support appositional bone growth and remodeling when injected into femoral cortical defects in sheep.[22] PUR chemistry also enables interfacial binding between the polymer and filler phases, as we have shown in composites prepared from lysine triisocyanate (LTI) and allograft bone particles.[19] Although CaP/polymer composites incorporating relatively low volume fractions of CaP support cellular infiltration and new bone formation, remodeling of these materials proceeds slowly (e.g., requiring five to seven years for complete remodeling).[23] In contrast, PUR composites incorporating mineralized allograft bone particles at concentrations above the random close packing (RCP) limit of 64 vol % support rapid (e.g., 6 weeks) infiltration and remodeling by providing a pathway for cellular infiltration as osteoclasts resorb the mineralized filler phase.[19] Furthermore, allograft/PUR composites showed compressive modulus and strength approaching values reported for cortical bone. However, the limited availability of allograft, as well as the risk of complications,[24] motivates the search for synthetic alternatives. Another advantage of synthetic osteoconductive matrices such as calcium phosphates is the relative ease (compared with allograft) with which the surface composition can be modified.[25] In the present study, we fabricated CaP/PUR composites with the CaP filler loading exceeding the RCP limit to promote cellular infiltration and remodeling. PUR composites were synthesized from both HA and β-TCP to investigate the mechanical properties, *in vitro* cellular response, and *in vivo* bioactivity when implanted in femoral defects in rats.

MATERIALS AND METHODS

Materials

Lysine triisocyanate (LTI) was purchased from Kyowa Hakko (New York, NY). Tegoamin 33, a tertiary amine catalyst, was received from Goldschmidt (Hopewell, VA). Glycerol, stannous octoate, and ε-caprolactone were purchased from Sigma-Aldrich (St Louis, MO), and glycolide and DL-lactide were supplied by Polysciences (Warrington, PA). HA (50–150 μm) and TCP (100–300 μm) were purchased from Berkley Biomaterials.

Fabrication of CaP/PUR composites

A polyester polyol (600 MW) with a backbone of 60% caprolactone, 30% glycolide, and 10% lactide was synthesized using known methods.[26] The components of the composite were mixed using a one-shot method, wherein the appropriate amounts of Tegoamin 33, polyester triol, CaP, and LTI were added to a 10 mL cup and mixed using a Hauschild SpeedMixer (FlackTek, Landrum, SC). The mixing speed was gradually ramped to 3300 rpm for one minute and mixing continued at 3300 rpm for 30 s. The composites incorporated 79.0 wt % (66.2 vol %) CaP; composites incorporating 70.0 wt % (56.8 vol %) CaP were used in biomechanical testing for comparison. The reactive paste was transferred to a cylindrical mold, compressed to ~63,000 lbf for 50 min, demolded to yield a green cylinder (6.1 mm diameter), and cured at 37°C for 12 h in a vacuum oven. Mechanical properties and remodeling in a rat femoral plug model were investigated for four composite formulations: HA79 (79 wt% HA), TCP79 (79 wt% TCP), HA70 (70 wt% HA), and TCP70 (70 wt% TCP).

Mechanical properties, scanning electron microscopy, *in vitro* degradation

Cylindrical CaP/PUR rods, approximately 6.3 × 12.6 mm^2 (n = 3), were fabricated by compression molding. The rods were hydrated in PBS for 24 h prior to testing. The cylinders were placed between two fixed compression platens of an MTS 898 equipped with a 13 kN load cell, preloaded to ~12 N, and loaded at 24 mm min^{-1} until failure. Significant differences between the treatment groups were determined by one-way ANOVA with the Bonferroni correction ($p < 0.05$). Sample composites were mounted on a SEM pin stub mount and sputter-coated for 60 s using a Cressington Q108 sputter coater, which deposited gold at a 30 mA current. A Hitachi S-4200 scanning electron microscope was used to acquire images at a voltage of 10 kV.

The *in vitro* degradation rates of PUR (control) and CaP/PUR composites were evaluated by measuring the mass loss at various time points during 7 weeks of incubation for 10 mg samples (n = 5) in 1 mL of phosphate buffered saline (PBS; pH 7.4) at 37°C. At each time point, the samples were rinsed in deionized water, dried under vacuum for 48 h at room temperature, and weighed.

In vitro cell proliferation and osteogenic differentiation on CaP/PUR composites

Discs ~250 μm thick were cleaned and sterilized by sonication in both deionized (DI) water and ethanol, followed by washing with additional DI water. We further washed the composites with serum-free alpha minimum essential media (α-MEM, Fisher Scientific). We used serum-free media to avoid possible effects of growth factors contained in the serum.[27] After washing, 2T3 cells (the clonal osteoblastic cell-line isolated from murine calvaria[28]) were cultured on the composite discs to evaluate the effects of composite composition on cellular activity *in vitro*. The clonal osteoblastic cells, which have similar osteogenic differentiation ability as primarily isolated calvarial osteoblasts, can provide useful and reproducible models for investigating *in vitro* cellular activity.[28] A total of 5 × 10^3 cells were seeded on each composite in 12-well tissue-culture polystyrene plates. Cells were cultured with α-MEM containing 10% fetal bovine serum (FBS, HyClone) and 1% penicillin/streptomycin (HyClone) at 37°C in a humidified incubator supplemented with 5% CO_2. The medium was changed every 2 days.

After 2 and 5 days, cell proliferation on CaP/PUR composites was evaluated. The cell-seeded scaffolds were washed with PBS, and 4 μ*M* Calcein AM (Live/Dead Viability/Cytotoxicity Kit, Invitrogen-Molecular Probes) was added to the

samples. Calcein AM dye is retained within live cells, imparting green fluorescence (excitation/emission: 495/515 nm). Cell proliferation was assessed qualitatively by fluorescent images acquired with an Olympus DP71 camera attached to a fluorescent microscope (Olympus CKX41, U-RFLT50). Osteoblastic cell proliferation on CaP/PUR composites was quantitatively evaluated using PicoGreen assays ($n = 4$). After the cells were removed from the discs using 0.25% trypsin and 1 m*M* ethylenediaminetetraacetic acid (EDTA, Invitrogen), DNA content was measured using the Quant-iT PicoGreen dsDNA assay kit (Invitrogen-Molecular Probes) according to the manufacturer's instructions. The fluorescence intensity was measured at excitation and emission wavelengths of 495 and 515 nm^2.

The *in vitro* osteogenic differentiation of osteoblastic cells cultured on CaP/PUR composites was evaluated ($n = 4$). A cell number of 5×10^4 was seeded on CaP/PUR composites. After confluence, the cell-seeded scaffolds were cultured with osteogenic medium containing 2.5% FBS, 10 m*M* β-glycerophosphate (Sigma-Aldrich), and 100 μg/mL ascorbic acid phosphate (Wako, Osaka, Japan) for 1, 4, and 7 days. The cells were removed from the CaP/PUR discs, washed with PBS, and lysed with 0.1% Triton X-100. The cells were then subjected to three freeze/thaw cycles and sonication. The lysates (20 μL) were added to 100 μL of substrate buffer (2 mg/mL disodium p-nitrophenylphosphate hexahydrate and 0.75*M* 2-amino-2-methyl-1-propanol). After incubation of the mixtures at 37°C for 30 min, absorbance at 405 nm was measured. Alkaline phosphatase (ALP) activity was determined from a standard curve generated by employing the reaction of a *p*-nitrophenol solution. ALP activity was normalized by DNA content determined using the PicoGreen assay. The Student's *t* test and one-way ANOVA with the Bonferroni correction was performed for statistical comparison ($P < 0.05$).

In vivo rat study

All surgical procedures were reviewed and approved by the Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (Harlan Labs) aged 8 weeks (200–250 g) were used for this study. A mono-cortical plug bone defect with a diameter of 3 mm was created in the distal region of the femur diaphysis, and a cylindrical CaP/PUR composite (3×5 mm^2) was implanted into the defect. After 4 weeks, the rats were sacrificed and the femurs removed and fixed in 10% phosphate-buffered formalin.

μCT analysis

Radiological analysis of the defect in the distal femur at week 4 was performed using a Scanco μCT40 (SCANCO Medical) at a voxel size of 24 μm. The X-ray source settings were 55 kVp and 145 mA with an integration time of 300 ms.

Histology

Rat bones were decalcified with 10% EDTA, dehydrated, embedded in paraffin, and sectioned at 5 μm thickness. The coronal slice sections were stained with hematoxylin and eosin (H&E). The specimens were examined with light microscopy. Tartrate resistant acid phosphastase (TRAP) staining was used to confirm the presence of osteoclasts.

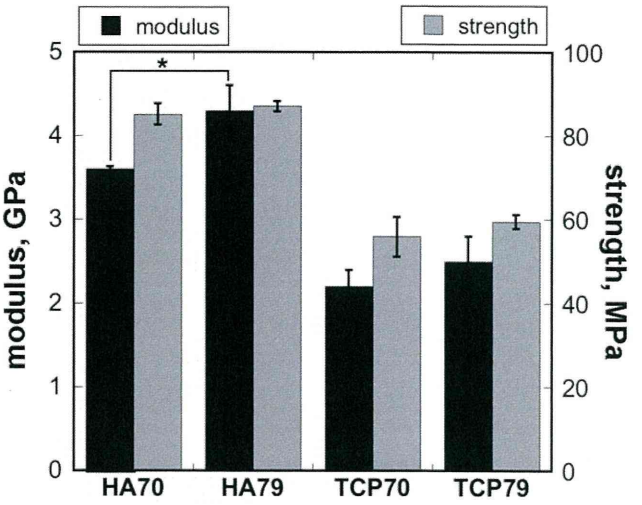

FIGURE 1. Compressive properties of PUR/HA and PUR/TCP composites. HA70: 70 wt % HA, HA79: 79 wt % HA, TCP70: 70 wt % TCP, TCP79: 79 wt % TCP.

RESULTS

Mechanical properties, particle size, *in vitro* degradation

Figure 1 summarizes the compressive modulus and strength values for the CaP/PUR composites, which ranged from 2.5 to 3.6 GPa and 59.6 to 87.0 MPa, respectively. HA/PUR composites exhibited significantly greater compressive modulus and strength values than the TCP/PUR composites at both examined filler contents. However, the volume fraction of filler did not have a significant effect on compressive strength for either type of filler. Increasing the filler content for the β-TCP groups had no significant effect on the modulus unlike the effects seen for the HA group, where the modulus increased with filler content.

SEM images of the CaP/PUR composites are shown in Figure 2. After compression molding, the particle size was reduced from 50–150 μm to <10 μm. Higher magnification views of the HA/PUR (79 wt %) material reveal a large number of particles smaller than 1 μm (the bottom panels in Figure 2). These observations suggest that the process of compression molding resulted in attrition of the CaP particles and accompanied by a significant reduction in size.

The degradation rates of the CaP/PUR composites (79 wt %) and the PUR control are presented in Figure 3 ($n = 5$). The mass of the HA/PUR composites decreases with time and is less than that measured for the TCP/PUR composites and PUR control at each time-point. These observations suggest that dissolution of the HA phase is the primary mechanism of composite degradation at the early time points investigated. In contrast, the mass of the TCP/PUR composites does not decrease with time until day 21, and the mass of the PUR control does not decrease until day 42. The mass of the TCP/PUR composites was less than that of the PUR control after day 30.

In vitro cell proliferation and osteogenic differentiation on CaP/PUR composites

Calcein staining (Figure 4) showed favorable cell growth on the surface of CaP/PUR composites (79 wt %). The density

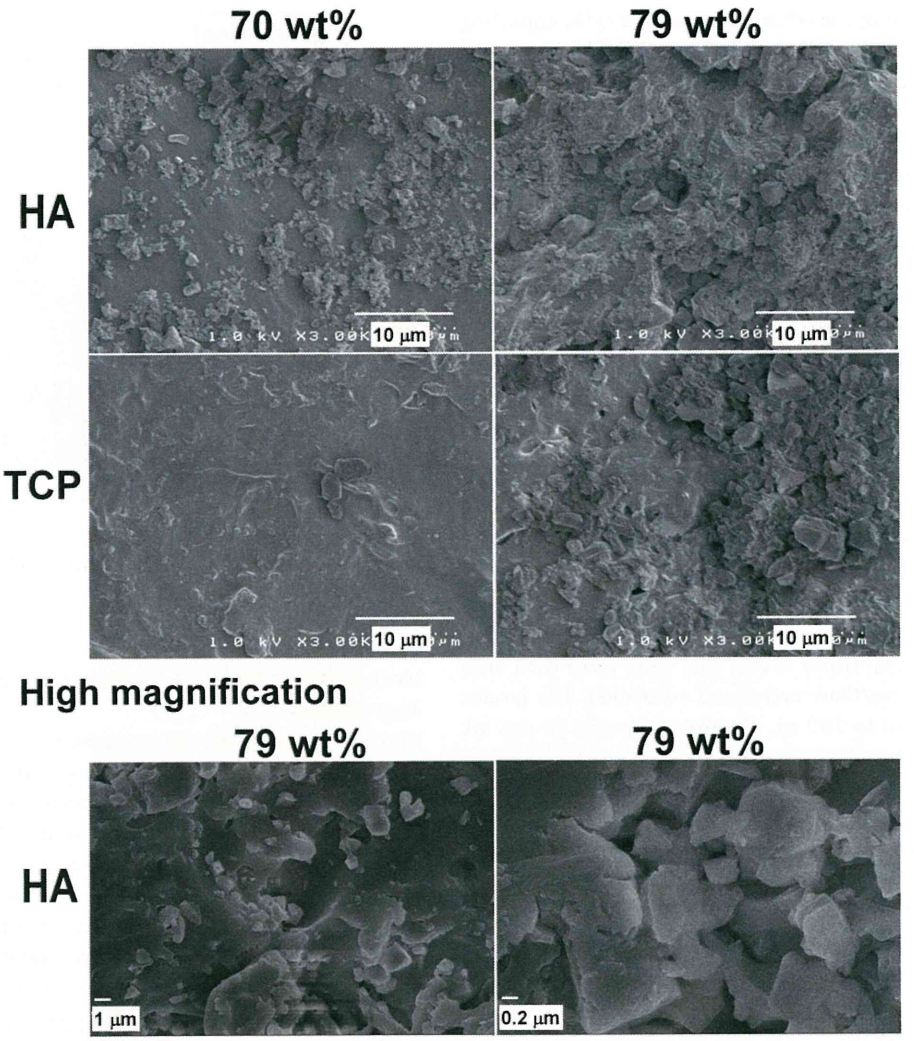

FIGURE 2. SEM images of HA70, HA79, TCP70, and TCP79 composites. The bottom panels show higher magnification images of the HA79 composites.

of live cells at day 5 increased relative to day 2 on both HA/PUR and TCP/PUR composites, which suggests the biocompatibility of CaP/PUR composites. A quantitative analysis with a PicoGreen assay also showed that the amount of cellular DNA significantly increased at day 5 on both HA/PUR and

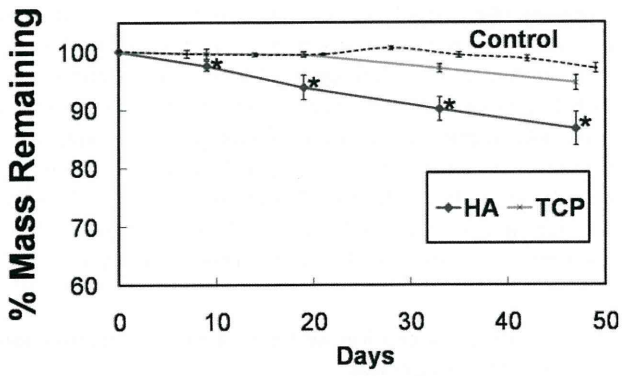

FIGURE 3. *In vitro* degradation of PUR/HA, PUR/TCP composites and Control (PUR). $*p < 0.05$, compared with PUR/TCP.

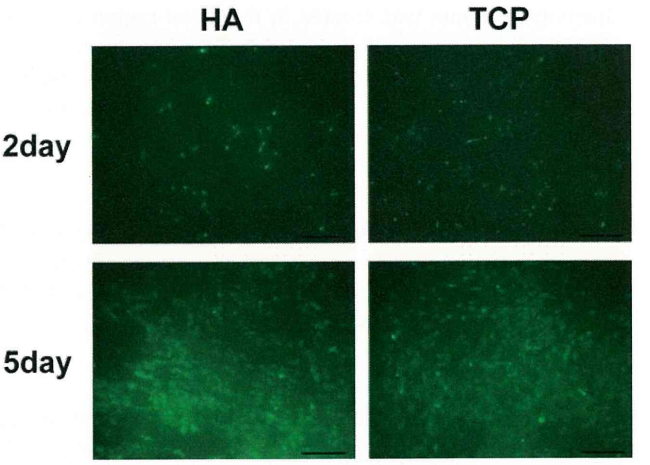

FIGURE 4. Proliferation of osteoblastic cells seeded on the surface of PUR/HA and PUR/TCP composites. The cells were stained by calcein at days 2 and 5. The bars: 250 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

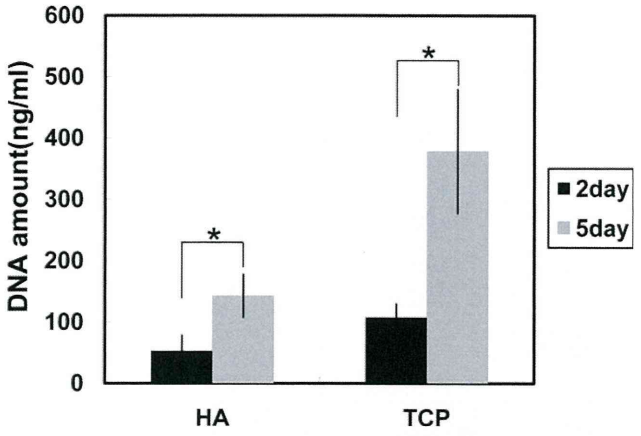

FIGURE 5. DNA amount of osteoblastic cells cultured on PUR/HA and PUR/TCP composites surfaces at days 2 and 5. $*p < 0.05$.

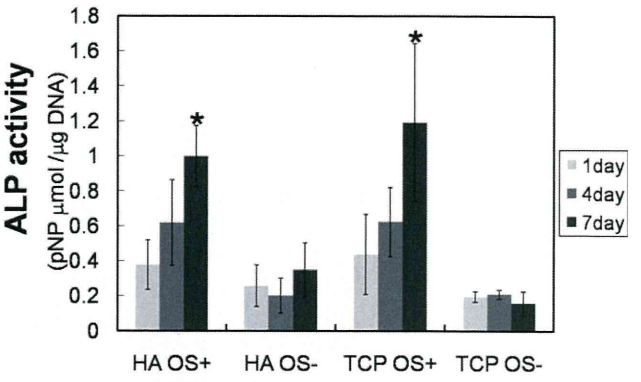

FIGURE 6. Osteogenic differentiation of osteoblastic cells seeded on PUR/HA and PUR/TCP composites. ALP activity was measured at days 1, 4, 7 after culture on the composites with osteogenic supplements (OS). $*p < 0.05$, compared with day 1.

TCP/PUR composites (79 wt %) (Figure 5). The rate of proliferation on the TCP/PUR composites was greater than the rate of cell growth on HA/PUR composites.

ALP activity of the cells seeded on CaP/PUR composites (79 wt %) significantly increased when cultured with osteogenic medium (Figure 6). ALP activity showed time-dependent increases in both HA/PUR and TCP/PUR composites, suggesting that the cells can differentiate on the surface of the composites. There was no significant difference in ALP activity between HA/PUR and TCP/PUR composites.

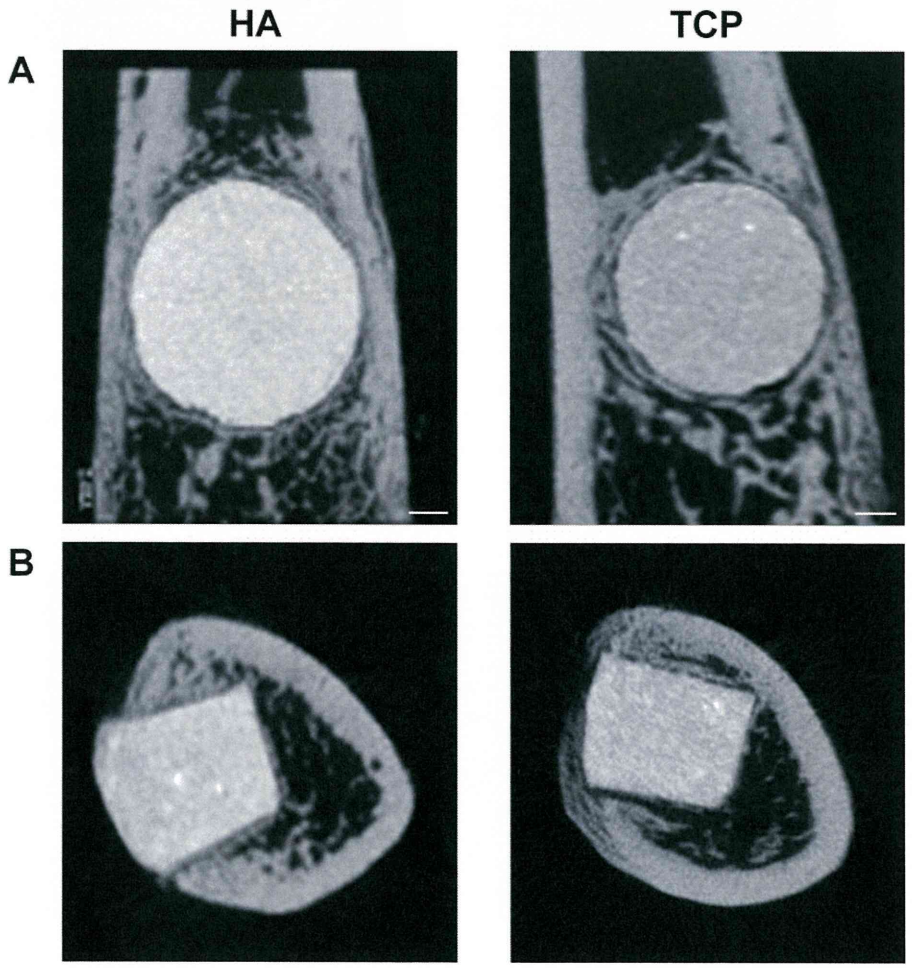

FIGURE 7. Micro CT of PUR/HA and PUR/TCP composites at week 4. A: Coronal view. B: Axial view. Scale bars: 500 μm.

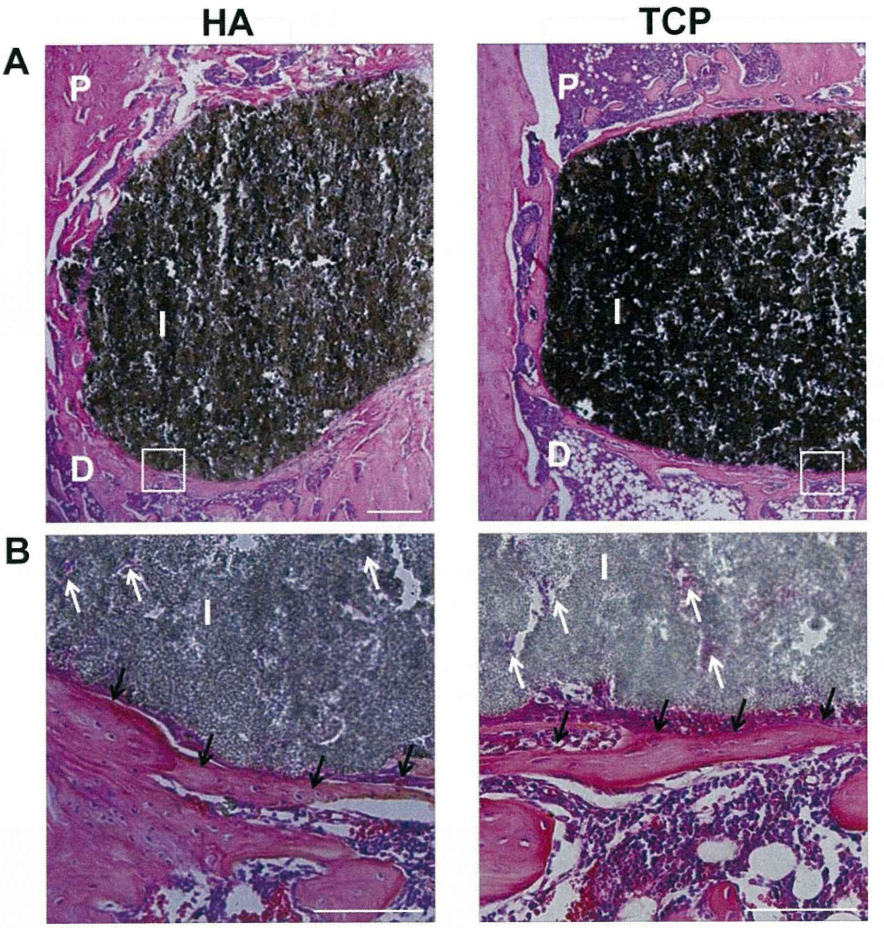

FIGURE 8. Histological pictures (HE staining) of PUR/HA and PUR/TCP composites at week 4. A: P, proximal; D, distal; I, implants. The bars: 500 μm. B: High magnification. The white arrows: cell infiltration to the scaffolds. The black arrows: New bone formation. Scale bars: 100 μm.

μCT analysis

μCT images from the extracted femurs at week 4 (Figure 7) showed new bone formation around both HA/PUR and TCP/PUR composites (79 wt %). The material shape became irregular at the boundary between the implant and newly formed bone in the μCT images. These findings show that the composites are osteoconductive and support appositional bone growth.

Histology

Histological sections of the implanted CaP/PUR composites (79 wt %) (Figure 8) showed extensive bone matrix

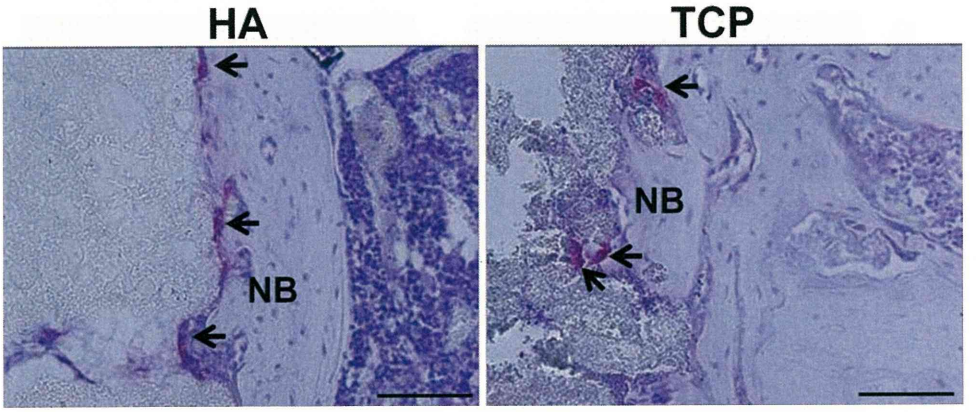

FIGURE 9. Histological pictures (TRAP staining) of PUR/HA and PUR/TCP composites at week 4. I, implants; NB, new bone formation, The black arrows: TRAP positive multinucleated cells. Scale bars: 100 μm.

formation at the surface of both HA/PUR and TCP/PUR composites, which is consistent with μCT images. Higher magnification images revealed cellular infiltration into the materials. No inflammatory response was observed at week 4. As observed in Figure 8(A), HA/PUR composites showed evidence of remodeling near the base of the implant. However, the size of the original implants for both treatment groups changed only minimally, suggesting that the extent of remodeling in the composites was low. Histological sections stained for TRAP (Figure 9) showed osteoclast-mediated resorption at the boundary between the implants and newly formed bone.

DISCUSSION

Multiple CaP/polymer composites with varying porosities and filler contents have been studied as biomaterials.[10] These systems typically incorporate filler contents far below the random close packing limit (RCP) of spheres (~64 vol%),[29] and β-TCP/polymer composites have been reported to decrease in strength as the amount of β-TCP increases.[10] However, another study has shown that varying the filler content of HA/chitosan (CS) composites has a minimal effect on the strength of the composites at loadings under 80 wt % (~64 vol %).[11,29] In this study, varying the filler content from 70 to 79 wt % (56.8–66.2 vol %) for the CaP/PUR composites had no significant effect on strength. Consistent with previous reports that HA is stronger than TCP,[3] HA/PUR composites exhibited greater compressive modulus and strength values compared to the β-TCP/PUR composites. At the 70 wt % filler content, there were no significant differences in the compressive modulus in the treatment groups. However, when the filler content was increased to 79 wt %, there was a significant difference, suggesting a greater contribution of the filler composition at the higher loading. Therefore, we focused on 79 wt % CaP/PUR composites in the subsequent *in vitro* and *in vivo* experiments. The strength of the HA/PUR composites (87.0 MPa) was lower than values reported for chitosan (CS)/HA composites, which were also prepared at 80 wt % HA (166 MPa).[11] However, the compressive modulus of HA/PUR composite materials (4.3 GPa) was an order of magnitude higher than that of the CS/HA composites (416 MPa). Considering that compressive modulus and yield strength of the PUR alone were 0.99 GPa and 40 MPa, respectively, the CaP particles appear to be providing substantial mechanical reinforcement of the composites.

The *in vitro* degradation rate of CaP/polymer composites can vary substantially depending on the polymers and the ceramic components, as well as the manufacturing methods.[30–32] The CaP/PUR composites in this study degraded slowly *in vitro*, with the degradation rates in PBS ranging from 0.8 to 2.0 wt %/week. The *in vitro* degradation rates of HA/PUR and TCP/PUR composites were similar to those measured in other CaP degradation studies,[33,34] as evidenced by the fact that both materials retained 85–95% of their original mass after 7 weeks. Another study has reported that CaP/polymer composites degraded more slowly and maintained their shape longer than the pure polymer.[35] However, in the present study, both HA/PUR and TCP/PUR composites degraded faster than the PUR control. While TCP is more water-soluble than HA,[36,37] HA/PUR degraded faster than both TCP/PUR and the PUR control in this study, suggesting that at early time points the primary mechanism of degradation of the HA/PUR composites is dissolution of the HA phase. Additionally, high HA content may influence the pH of the surrounding microenvironment,[38] which can influence the polymer degradation rate.[39]

Cellular proliferation was higher on the surface of the β-TCP composites. Previous studies have suggested that β-TCP can enhance osteoblast viability and proliferation, because calcium and phosphate ions stimulate osteoblastic activity.[3,21,40] In contrast, the dissolution of crystalline HA is slow and reduces the pH of the surrounding microenvironment, thereby slowing cell growth.[38] Similarly, in this study, the β-TCP/PUR composites supported a significantly higher proliferation rate of osteoprogenitor cells compared with the HA/PUR composites, which is thought to result from the dissolution of β-TCP particles exposed on the surface of the composites. Interestingly, the filler type did not have a significant effect on ALP activity of the cells.

The remodeling of CaP/polymer composites *in vivo* has been observed in several studies. HA/PLLA composites implanted in rabbit femoral plug defects have taken up to seven years to resorb and remodel.[12] In this study, both radiographs and histological sections show appositional bone growth at the surface of the CaP/PUR composites, which has also been observed for allograft/PUR composites implanted in the rabbit distal femur.[19] However, CaP/PUR composites showed substantially less resorption and cellular infiltration compared with allograft/PUR composites. Osteoclasts infiltrated and resorbed the CaP/PUR composites near the bone-implant interface, as confirmed by TRAP staining (Figure 9). Although there is limited evidence of remodeling at the early time point investigated (4 weeks), infiltration of osteoclasts near the implant-bone interface suggests that at later time points the CaP/PUR composites may remodel via slow reverse creeping substitution,[41–43] as reported previously for allograft/PUR composites. However, the rates of cellular infiltration and resorption were substantially less than those observed for allograft/PUR composites at similar filler loadings.[19] The SEM images (Figure 2) indicate that the CaP particles were fractured by the compression molding process, which reduced the size of many of the particles to <10 μm. In contrast, these results were not observed for compression-molded allograft bone/polymer composites.[19] The size of allograft bone particles dramatically affects the potential of the particles to remodel, which is highest for particles ranging from 90 to 300 μm[44]; particles < 100 μm are only slowly resorbed. Thus the relatively slow osteoclast-mediated resorption of the CaP composites may be due, at least in part, to the small size of the particles. Alternatively, previous studies have suggested that cortical allograft bone particles are more rapidly resorbed and replaced by living bone in the rabbit distal femur than HA particles because of the organic components in the allograft bone.[45] Allograft bone particles, which have been

reported to undergo up to 70% resorption by osteoclasts after 14 days,[46] resorb faster than HA particles (0.02 μm^3 μm^{-2} day^{-1})[47] *in vitro*. These observations suggest that the slower resorption rate of CaP composites could also be attributed to the differences in composition between CaP and allograft.

In this study, we examined the *in vivo* bioactivity of CaP/PUR composites using a rat femoral plug defect model with a short-term observation period. Large animal models with a long-term observation may be required in the future to further investigate the osteoconductive ability and full remodeling of the materials. However, the data from this study suggest the potential of CaP/PUR composites for weight-bearing implants as a biocompatible, osteoconductive, and resorbable material.

CONCLUSION

CaP/PUR composites have been synthesized using a two-component polyurethane derived from LTI. The mechanical properties of the composites suggest that they could be useful for weight-bearing applications because the PUR increased the compressive strength of the CaP. Cell culture studies showed that CaP/PUR composites were biocompatible, with β-TCP further enhancing the cell viability and proliferation. CaP/PUR composites also supported the osteogenic differentiation of the osteoblastic cells. When implanted in the distal femurs of rats, CaP/PUR composites were shown to be biocompatible and osteoconductive with no adverse responses observed. Histological sections revealed evidence of infiltration of osteoclasts and resorption of CaP near the bone-implant interface, as well as appositional remodeling via slow reverse creeping substitution. The current study suggests that CaP/PUR composites could be a potentially useful option for weight-bearing implants.

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