Histological findings from the implants

All the experimental specimens exhibited similar histological features. With standard hematoxylin and eosin staining, specimens 2, 4, and 8 weeks after injection were composed of bone in the MSCs/β-TCP fibrin glue admixture implants (Fig. 5). Histological examination at 2, 4, and 8 weeks showed a calcified bone matrix with occasional small remnants of biodegraded fibrin glue β-TCP in the implants. Two weeks after implantation, an osteoblast lining, and at 4 weeks, a little mature bone was observed together with cuboidal, active osteoblasts. Eight weeks after implantation when the osteocalcin content was significantly higher than before, bone formation was still progressing and an increase in the mature lamellar bone areas was observed. It was confirmed that the bone areas increased with time. Despite the bone formation in vivo, no cartilage was observed in the porous areas.

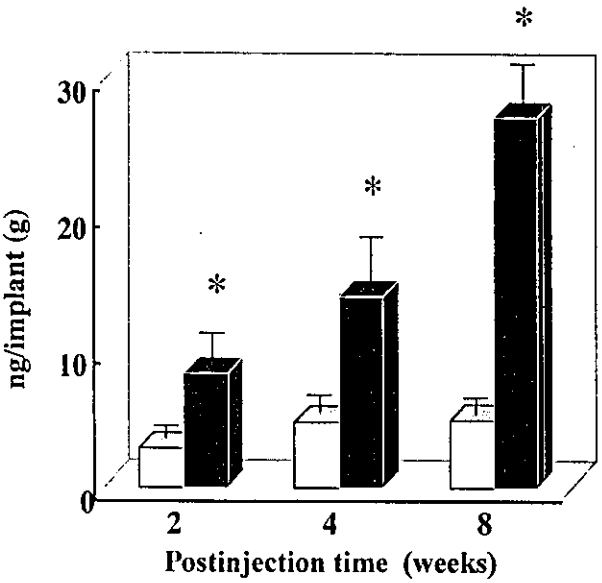

Fig. 4 – Osteocalcin content for mineral detection. Experimental samples (MSCs/β-TCP fibrin glue admixture ■) and control (fibrin glue β-TCP admixture □) harvested at 2, 4, and 8 weeks after injection. Each point represents the mean value of osteocalcin content ± SED ($n = 5$ at each point). Asterisks indicate significant differences at $p < 0.05$.

Control implants with fibrin glue β-TCP admixtures alone exhibited none of these histological features. They did not show any bone formation in the implants 2, 4 and 8 weeks after implantation. Only fibrous tissues were observed (Fig. 5). With time, the fibrin glue β-TCP was gradually resorbed, resulting in implants that became smaller and flatter, and contained numerous pores and fibrous tissue.

Osteopontin, a protein important in bone development, was identified in the experimental groups, but was not detected in the control groups. The osteocytes were positive for the antibody. These results were consistent with the osteocalcin content, radiographic findings, and histological evaluations (Fig. 6).

Biochemical findings of the implants

In MSCs' β-TCP fibrin glue admixtures, the osteocalcin content was detected 2 weeks after implantation, followed by a steady increase with time in the groups with MSCs/β-TCP fibrin glue admixtures. The osteocalcin content of experimental groups was

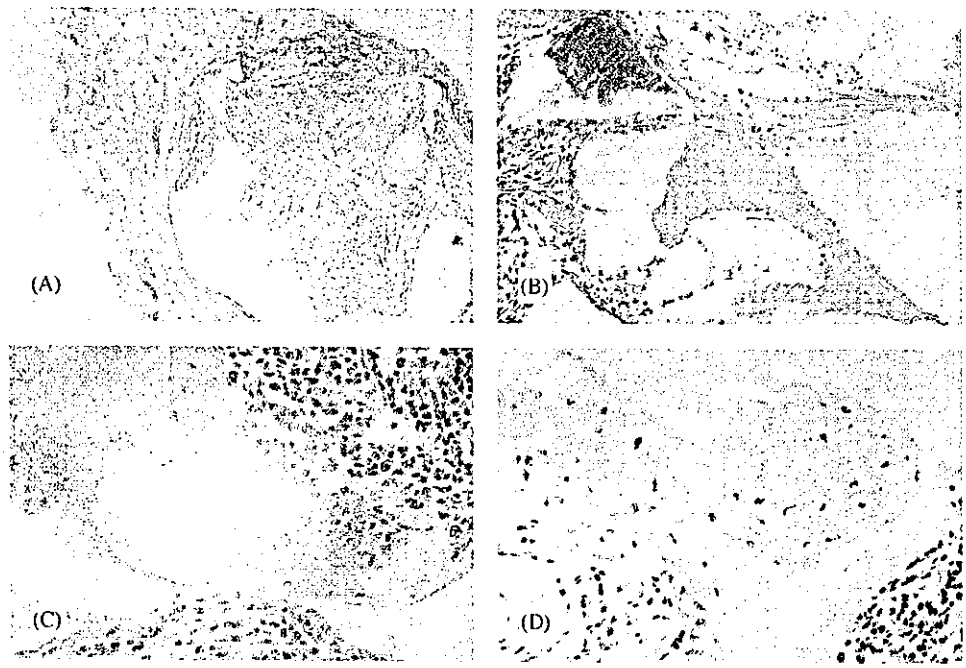

Fig. 5 – Histological comparison of the implants developed from MSCs/β-TCP fibrin glue admixtures or fibrin glue β-TCP admixtures. Sections of representative implants from the control group (fibrin glue β-TCP admixture, A), and MSCs/β-TCP fibrin glue admixture harvested at 2 (B), 4 (C), and 8 (D) weeks. (Original magnification, A: × 50; B: × 100; (C and D: × 200).

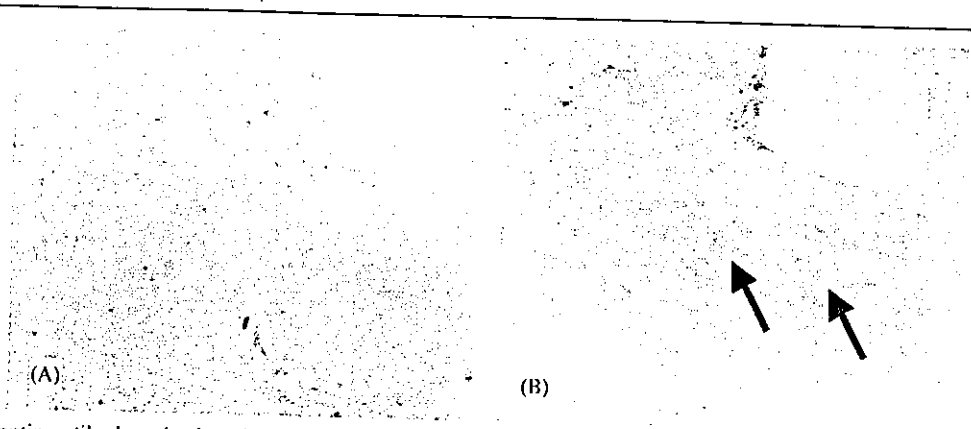

Fig. 6 – Osteopontin antibody-stained sections of the implants. Representative implants from the control group (fibrin glue β-TCP admixture, A), and MSCs/β-TCP fibrin glue admixture (B) harvested at 8 weeks. Arrows point to osteocytes. Original magnification, ×200.

significantly higher than in the control groups (Fig. 4). In fibrin glue β-TCP admixtures, osteocalcin content was almost undetectable.

DISCUSSION

Treatment of bone loss has been traditionally managed by open implantation of solid materials. However, the invasive nature of this approach is its main drawback, and injectable delivery systems have now been developed. They hold the promise that tissue losses can be reduced without problems associated with direct implantation. It has been reported that a simple combination of cells with HA or HA/TCP was capable of inducing osteogenesis at ectopic sites (*Yoshikawa* et al., 1992, 1996). In a previous study, we reported that β-TCP composites loaded with MSCs had excellent osteogenic characteristics (*Boo* et al., 2002). However, these delivery substances did not have good plasticity, and the cellular implantation procedure was complicated by problems associated with delivery systems. Previous studies of injectable delivery systems with the capacity to accommodate cell proliferation and matrix production have been performed experimentally using collagen gel (*Kimura* et al., 1984; *Wakitani* et al., 1989), hyaluronic acid (*Robinson* et al., 1990) and calcium alginate (*Paige* et al., 1995) to induce both cartilage and bone. The efficacy of these, and other delivery agents were found to depend on their respective biocompatibility, cytotoxicity, and biodegradability. Optimally, delivery substances used for bone or cartilage replacement or repair through tissue engineering would provide the same environment as the bone or cartilage matrix in vivo. An appropriate rate of biodegradability should be considered with the capacity of the respective cells to multiply. The data presented in this paper demonstrate that stem cells migrate effectively into fibrin glue.

Fibrin, the final product of normal blood coagulation, is recognized as one of the most important elements not only in the clotting process, but also in wound healing (*Staindal* et al., 1981). *Young* and *Medawar* (1940) initially reported its application as a suture material for experimental peripheral nerve repair. Most recently, pasteurization of fibrin glue has extended its clinical use in many fields of surgery (*Fricke* and *Lamb*, 1993), and it can be used as a cell carrier. In this regard, this study demonstrates that MSCs/β-TCP matrix composites can be spatially transferred with fibrin glue to recipient sites in animal models without loss of viability of the cultured tissue. Also, fibrin glue allows MSC proliferation without deforming the cell's structure, making it an appropriate delivery substance. In this study, the fibrin glue was applied following the manufacture's protocol; the operation time was about 10 s and the glue hardened relatively quickly (Table 1). We did the preliminary studies with the combination of MSCs with only fibrin glue, but this did not result in bone regeneration, only fibrous tissue. Therefore the MSCs, were applied in combination with fibrin glue and β-TCP. The increase in osteopontin in admixture implants correlated with the structural appearance of minerals over the same experimental time course. From the results of this study, fibrin glue provides an environment appropriate for the proliferation and differentiation of cells in vivo.

Bone formation in implants occurs by two major processes namely endochondral ossification and intramembranous ossification (*Caplan* and *Pechak*, 1987) in this study, however, direct bone formation occurred in mesenchymal tissue without prior formation of cartilage. *Isogai* et al. (2000) reported that a combination of fibrin glue and cultured periosteal cells resulted in new bone formation at heterotopic sites in nude mice. In their study, bone formation began at implant sites where cells and the extracellular matrix together lead to events closely resembling an endochondral ossification model: Cartilage develops and is progressively replaced by bone. As we could not find the formation of cartilage-like tissue, in our own experiments, this bone formation may have occurred by intramembranous ossification, as bone marrow–derived MSCs were used. It is known that bone marrow- and periosteum-derived cells have intrinsically different responses to osteo-inductive agents (*Solchaga* et al., 1998). Recent advances in the culturing of multipotential MSCs

from bone marrow (*Haynesworth* et al., 1992), and the repeated demonstration that their differentiation can be directed to the osteoblastic lineage suggest that the clinical use of MSCs for bone regeneration is possible. By definition, MSCs are able to undergo many cycles of cell division without losing their osteogenic capacity (*Bruder* et al., 1997). The proliferative expansion of MSCs (not periosteal cells) generates large numbers of potentially osteogenic cells that may be used in clinical settings to direct bone formation and repair (*Bruder* et al., 1998).

CONCLUSION

The ability to inject MSCs/β-TCP fibrin glue admixtures that solidify within the host and are replaced over time by bone has powerful implications for the future of oral, maxillofacial, reconstructive and orthopaedic surgery. The methods detailed in these studies are the first steps towards custom-made autogenous bone grafts.

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Autogenous Injectable Bone for Regeneration with Mesenchymal Stem Cells and Platelet-Rich Plasma: Tissue-Engineered Bone Regeneration

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ABSTRACT

We have attempted to regenerate bone in a significant osseous defect with minimal invasiveness and good plasticity, and to provide a clinical alternative to autogenous bone grafts. Platelet-rich plasma (PRP) may enhance the formation of new bone and is nontoxic, nonimmunoreactive, and accelerates existing wound-healing pathways. We have used a combination of PRP as an autologous scaffold with *in vitro*-expanded mesenchymal stem cells (MSCs) to increase osteogenesis, compared with using the scaffold alone or autogenous particulate cancellous bone and marrow (PCBM). The newly formed bones were evaluated by radiography, histology, and histomorphometric analysis in the defects at 2, 4, and 8 weeks. According to the histological observations, the dog MSCs (dMSCs)/PRP group had well-formed mature bone and neovascularization compared with the control (defect only), PRP, and PCBM groups at 2 and 4 weeks. Histometrically, at 8 weeks newly formed bone areas were 18.3 ± 4.84% (control), 29.2 ± 5.47% (PRP), 61.4 ± 3.38% (PCBM), and 67.3 ± 2.06% (dMSCs/PRP). There were significant differences between the PCBM, dMSCs/PRP, and control groups. These results demonstrate that the dMSCs/PRP mixture is useful as a osteogenic bone substitute.

INTRODUCTION

MULTIPLE METHODS have been studied for the restoration of bone defects in the fields of craniomaxillofacial reconstructive, plastic, and orthopedic surgery, but augmentation and manipulation of biocompatible material still remains a difficult clinical problem with respect to soft and hard tissues. Excised autogenous tissue, including fat, fascia, cartilage, and bone chips, has frequently been used. However, each of these alternative treatments causes specific problems. The preferred autogenous material is limited in supply, has attendant donor site morbidity, and is occasionally not suitable for the proposed reconstruction because of poor tissue quality or is extremely difficult in shaping the graft. Allografts are also in limited supply because of a scarcity of tissue donors. Synthetic prostheses suffer from increased susceptibility to infection, incidences of extrusion, and an uncertain long-term interaction with the host's physiology.

To address many of these concerns in the field of solid organ transplants, Vacanti *et al.*[1–3] described a new technology called *tissue engineering* that involves the morphogenesis of new tissues using constructs formed from isolated cells with biocompatible scaffolds and growth

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factors. As currently described, this technology requires the open implantation of the cell–scaffold constructs. Extending these techniques to the osteoblasts and formulating a system whereby the cell–scaffold constructs could be delivered less invasively would greatly expand the applicability of tissue engineering to fields such as craniomaxillofacial reconstruction, plastic, and orthopedic surgery.

Mesenchymal stem cells (MSCs) have been thought to be multipotent cells that can replicate as undifferentiated cells and that have the potential to differentiate in to lineages of mesenchymal tissue, including bone, cartilage, fat, tendon, muscle, and marrow stroma,[4,5] and received widespread attention because of their potential utility in tissue-engineering applications. Previous work has demonstrated that MSCs alone can be used in the tissue repair of bone.[5,6] But if a defect was filled with MSCs alone, it needed a great many MSCs, and it was difficult to keep MSCs in the defect region for filling bone defects. So more recently many workers have attempted to use a ceramic as a scaffold.[7]

In our previous study, we investigated the osteogenic potential of a new biodegradable β-tricalcium phosphate (β-TCP) combined with MSCs as a scaffold and compared it with hydroxyapatite (HA).[8] The results of this study showed that the scaffolds had potential, but were brittle and made it difficult for new bone to invade the defect because of the lack of completely interconnected pores, slow resorption of the ceramic, and absence of osteoinductive properties. Subsequently, we used a technique that involves minimal invasiveness and a material with jelly-like flexibility that could be used to deliver autogenous bone to correct or reconstruct bone defects such as osteoporotic fractures, periodontitis, and tumor resections. Fibrin glue was used as the injectable scaffold; the injectable MSCs/β-TCP–fibrin glue admixtures can provide a three-dimensional scaffold for successful transplantation and engraftment of osteoblasts.[9] The use of fibrin as an osseoconductive material and a medium for compacting grafts, as well as a graft material, has been reported.[10,11] Several authors[12–14] underlined the importance of growth factors found in the autologous fibrin adhesive. So we thought of using the platelet-rich plasma (PRP) gel, which was a mixture of growth factors and an autologous modification of fibrin glue. PRP gel is formed by mixing platelet-rich plasma, derived by differential centrifugation of autologous whole blood, with thrombin and calcium chloride. The critical difference in composition between PRP gel and fibrin glue is the presence of a high concentration of platelets and a native concentration of fibrinogen in the PRP gel. Platelets, once activated in the presence of thrombin, release a myriad of factors and begin to form the scaffold for the developing fibrin clot. PRP can introduce several advantages, including the enhancement and acceleration of bone regeneration and more rapid and predictable soft tissue healing. It has been reported[12–14] that, using a monoclonal antibody technique, receptors for the transforming growth factor β_1 (TGF-β_1), transforming growth factor β_2 (TGF-β_2), and platelet-derived growth factor (PDGF) were found in medullar bone. A high concentration of PDGF and TGF-β, indicated by monoclonal antibodies, was present in the plasma obtained, thus providing evidence of the presence of these growth factors in the original plasma used to obtain autologous fibrin.

Therefore in this article we explore the ability of a PRP scaffold combined with MSCs to increase the rate of bone formation and to enhance the bone regeneration rate and amount in the bone defect with clinically relevant volume, compared with particulate cancellous bone and marrow (PCBM).

MATERIALS AND METHODS

Canine animal models

All animal experiments undertaken in this study were performed in strict accordance with protocols approved by the Institutional Animal Care Committee. After a period of housing, four adult hybrid dogs with a mean age of 2 years were operated on under general anesthesia. The first molar, premolars, and the second and third premolars in the mandible region were extracted and the healing period was 2 months. Bone defects on both sides of the mandible were prepared with a trephine bar with a diameter of 10 mm, and these were made perpendicular to the lateral cortex. The defects were implanted with graft materials as follows: PRP, PRP and dog MSCs (dMSCs), PCBM, and control (defect only), and investigated for osteogenesis. Without any differences in bone regeneration in the various grafted areas in terms of bone healing, we created three defects and implanted four materials randomly without being specific to the sites. PCBM was also harvested from the iliac crest (Fig. 1).

MSC isolation and cultivation

The dMSCs were isolated from dog iliac crest marrow aspirates (10 ml) according to the reported method.[6] Briefly, the basal medium, low-glucose DMEM and growth supplements (50 mL of mesenchymal cell growth supplement, 10 mL of 200 mM L-glutamine, and 0.5 mL of penicillin–streptomycin mixture containing 25 units of penicillin and 25 μg of streptomycin), were purchased from BioWhittaker (Walkersville, MD). Three supplements for inducing osteogenesis, dexamethasone (Dex), sodium β-glycerophosphate (β-GP), and L-ascorbic acid 2-phosphate (AsAP), were purchased from Sigma (St. Louis, MO). The cells were incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO_2.

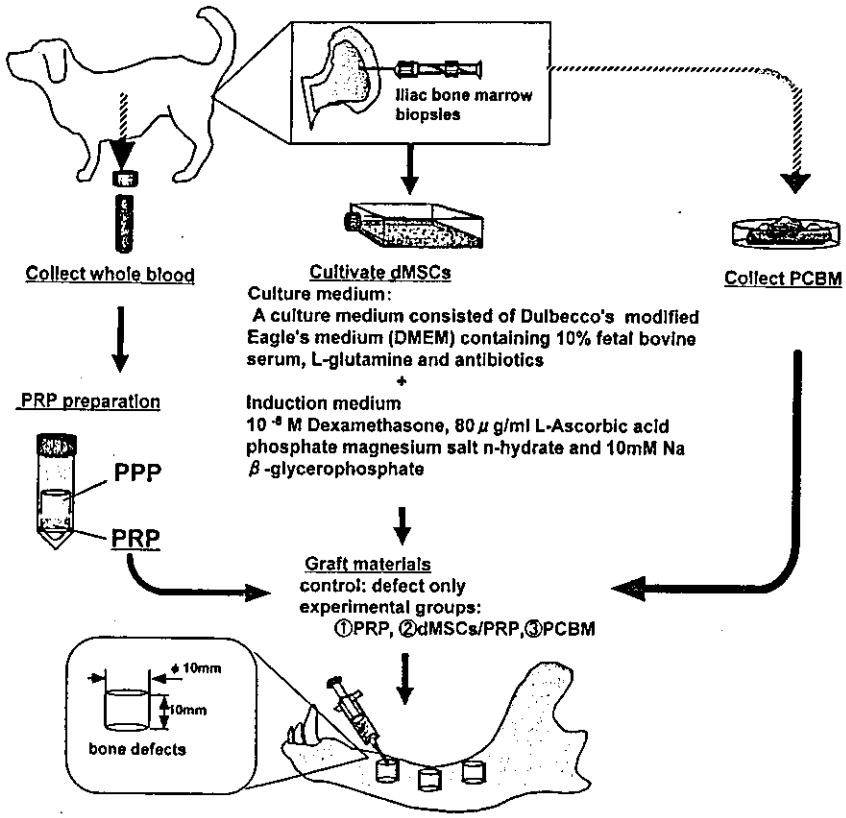

FIG. 1. Scheme of experimental protocol.

We replated the dMSCs at densities of 3.1×10^3 cells/cm^2 in control medium (0.2 mL/cm^2). The differentiated dMSCs were confirmed by detecting alkaline phosphatase activity using *p*-nitrophenylphosphatase as a substrate and alkaline phosphatase (ALP) staining. The dMSCs grown in control medium had low ALP activity and the ALP levels in the medium containing osteogenic supplements (OS medium) from day 6 showed a dramatic increase over time. In culture, dMSCs were trypsinized and used for implanting.

PRP, PRP gel preparation, and injection of MSCs/PRP admixture

Approximately 50 mL of whole blood was drawn from the canine into centrifuge tubes containing 10 mL of the culture medium with preservative-free heparin (250 U/mL). The blood was first centrifuged in a standard laboratory centrifuge machine (Himac CT; Hitachi, Tokyo, Japan), for 5 min at 1100 rpm. Subsequently, the yellow plasma (containing the buffy coat, which contained the platelets and leukocytes) was taken up into a neutral monovette with a long cannula. A second centrifugation at 2500 rpm for 5 min was performed to combine the platelets into a single pellet and the plasma supernatant, which is platelet-poor plasma (PPP) and contains relatively few cells, was removed. The resulting pellet of platelets, the buffy coat/plasma fraction (PRP), was resuspended in the residual 5 mL of plasma and used in the platelet gel. The platelet counts in the PRP and PPP were measured in a Sysmex XE-2100 (Sysmex, Tokyo, Japan). Platelet counts yielded a mean value of 295,000, with a range of 224,000 to 333,000. The PRP mean platelet count was 1,293,400, with a range of 935,000 to 1,840,000. These values confirmed the platelet sequestration ability of the process, which shows that the concentration was 438% above the baseline platelet counts. The PRP was stored at room temperature in a conventional shaker until use. Bovine thrombin in a powder form (10,000 units) was dissolved in 10 mL of 10% calcium chloride in a separate sterile cup. Next, 3.5 mL of PRP, dMSCs (1.0×10^7 cells/mL), and 0.5 mL of air were aspirated into a 5-mL syringe, and in a second 2.5-mL syringe 500 μL of the thrombin–calcium chloride mixture was aspirated. Here the cells resuspended directly into PRP. The two syringes were connected with a T connector and the plungers of the syringes were pushed and pulled alternatively, allowing the air bubble to traverse the two syringes. Within 5 to 30 s, the contents assumed a gel-like consistency as the thrombin effected the polymerization of the fibrin to produce an insoluble gel. The gel was injected into the bone defect field, using a 16-

gauge needle attached to the 5-mL syringe. Samples were analyzed 2 ($n = 6$), 4 ($n = 6$), and 8 ($n = 6$) weeks after injection. For radiographic assessment, radiographs were taken in the lateral planes under general anesthesia.

Histological and histomorphometric analysis

Each implantation site was excised with a trephine bar with a diameter of 2 mm at 2, 4, and 8 weeks after implantation, and each was assessed by histological and histomorphometric methods. The specimens were fixed in 10% buffered formalin, decalcified (K-CX; Falma, Tokyo, Japan), and stained with hematoxylin and eosin. These specimens were examined under a light microscope and analyzed by a pathologist blinded to the identity of each specimen and who determined the presence or absence of bone formation. The primary author, who agreed with the above pathologist in all cases, reviewed all sections.

Histomorphometric findings were analyzed with a microcomputer for image analysis. Each image of the specimens at the implantation site excised with a trephine bar (diameter of 2 mm) was copied on color reversal film, digitized as a 256 × 256 array of 8-bit density values, and transferred to a microcomputer for analysis (NIH Image, version 1.61; National Institutes of Health).[15] The augmented area was defined as the area that was enclosed within the mandible bone excised with a trephine bar (diameter of 10 mm). The volume of newly formed bone in the augmented area was quantified with this computer-based image analysis system. It is calculated as the percentage of bone present, deducting some normal bone areas from the measurement areas in the section based on 2-mm biopsies.

Statistical analysis

Group means and standard deviations were calculated for each measured parameter. Differences in newly formed bone between the control, PRP, dMSCs/PRP, and PCBM groups were analyzed by analysis of variance (ANOVA). The Mann–Whitney test was used to evaluate the difference in newly formed bone for each healing period. A p value of <0.05 indicated statistical significance.

RESULTS

Establishment of a bone defect model in a dog mandible

Figure 1 shows the experimental design of 10-mm-long defects created in a dog mandible to obtain an environment that does not regenerate bone naturally. The bone-regenerating ability of the implants was subsequently evaluated by radiographic and histological examination and by histomorphological analysis.

In vivo *macroscopic findings, radiographic assessment, and histological evaluation of PRP, dMSCs/PRP, PCBM implants compared with control*

PRP, dMSCs/PRP, and PCBM were implanted into 10-mm defects in a dog mandible. Macroscopic findings showed that the bone regeneration by dMSCs/PRP and PCBM was to a natural marginal bone level, but the regeneration by PRP and the control (defect only) was not complete. The dMSCs/PRP scaffold had almost completely disappeared without infection after implantation (Fig. 2A, panels a–c).

Bone regeneration and implant resorption were also monitored by taking X-rays and performing histological evaluation every 2 weeks. Osteogenesis spread slowly through the defect base in PRP and the controls. Defects filled with PCBM were radiolucent at 8 weeks, indicating PCBM resorption. In contrast, defects filled with implants of dMSCs/PRP were found to show good bone formation, suggesting that the bone formation occurred at about the same rate as implant resorption (Fig. 2B). Implanted and nonimplanted control regions were collected at 2, 4, and 8 weeks, and processed and decalcified for histology. Radiographs confirmed the histological observations. In the controls and defects filled with PRP, the cortical continuity was never restored and the cavities were invaded by a vascular, fibrous tissue and little new bone formation (Figs. 3 and 4, panels A–C and D–F) was seen. On the other hand, cavities filled with dMSCs/PRP resulted in new bone formation even after 2 weeks, with a tubular pattern at 8 weeks and abundant vascularization (Figs. 3 and 4, panels G–I). This pattern reflected normal bone macrostructure, with well-differentiated marrow cavity and cortices, compared with cavities filled with PCBM, which showed dead space by PCBM resorption (Figs. 3 and 4, panels H and K).

Histomorphometric analysis

The bone-regenerating ability of all implants was assessed by measuring the cortical and medullary bone surface areas by image analysis (Table 1). Adding PRP to the cavity did not significantly increase the cortical or medullary bone surface area compared with the control. In contrast, the dMSCs/PRP and PCBM groups showed a significant increase in the surface area at all weeks compared with the control ($p < 0.001$ at 2 and 8 weeks, $p < 0.005$ at 4 weeks, ANOVA) or PRP ($p < 0.05$ at 2 amd 4 weeks, $p < 0.001$ at 8 weeks, ANOVA), confirming the radiological and histological data. However, there was no significant difference in newly formed bone between dMSCs/PRP and PCBM over time.

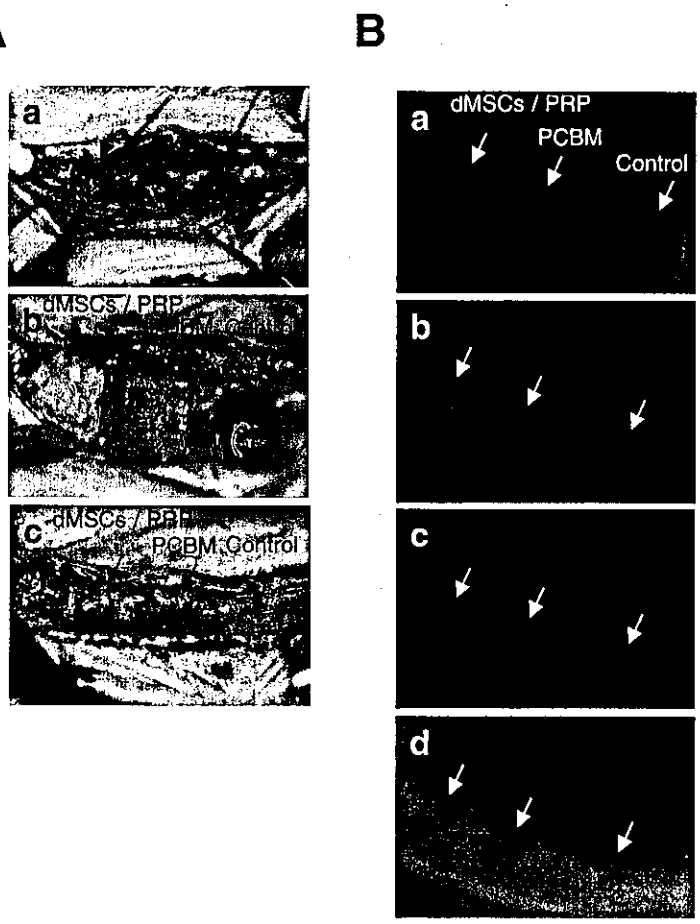

FIG. 2. (**A**) Macroscopic observations for bone regeneration: (a) The experimental design in dog mandible, prepared with a trephine bar 10 mm in diameter. (b) Implanted materials in bone defects. (c) Bone regeneration with dMSCs/PRP, PCBM, and control groups at 8 weeks. Bone regeneration by dMSCs/PRP and PCBM achieved a natural level, but regeneration by PRP and the control (defect only) was not complete. (**B**) The radiographic follow-ups: (a) X-rays after implantation. (b) X-rays at 2 weeks. Note the absence of bone formation within the control group defect. We found implanted PCBM in the PCBM group and bone formation in the dMSCs/PRP group. (c) X-rays at 4 weeks. Note the bone formation within the defect in the dMSCs/PRP group and the PCBM group compared with the control group. (d) X-rays at 8 weeks. Defects filled with PCBM were radiolucent at 8 weeks, indicating PCBM resorption. In contrast, defects filled with implants of dMSCs/PRP showed good bone formation.

DISCUSSION

Tissue-engineering approaches have attempted to create new bone based on MSCs seeded onto porous ceramic scaffolds.[7,16,17] These attempts have given suboptimal results that are due to the slow resorption rate of the hydroxyapatite-based ceramics. In our previous study, we used a biodegradable material, a β-TCP block loaded

TABLE 1. HISTOMORPHOLOGY DATA[a]

	2 weeks (%)		*4 weeks (%)*		*8 weeks (%)*	
Control	9.69 ± 4.97	**	14.9 ± 4.41	*	18.3 ± 4.84	**
PRP	8.01 ± 3.67		19.9 ± 5.13		29.2 ± 5.47	
PRP/MSCs	29.2 ± 3.93	**	36.8 ± 4.79	*	67.3 ± 3.38	**
PCBM	34.1 ± 6.86		38.7 ± 5.93		61.4 ± 2.06	

[a]Significance: *$p < 0.005$; **$p < 0.001$.

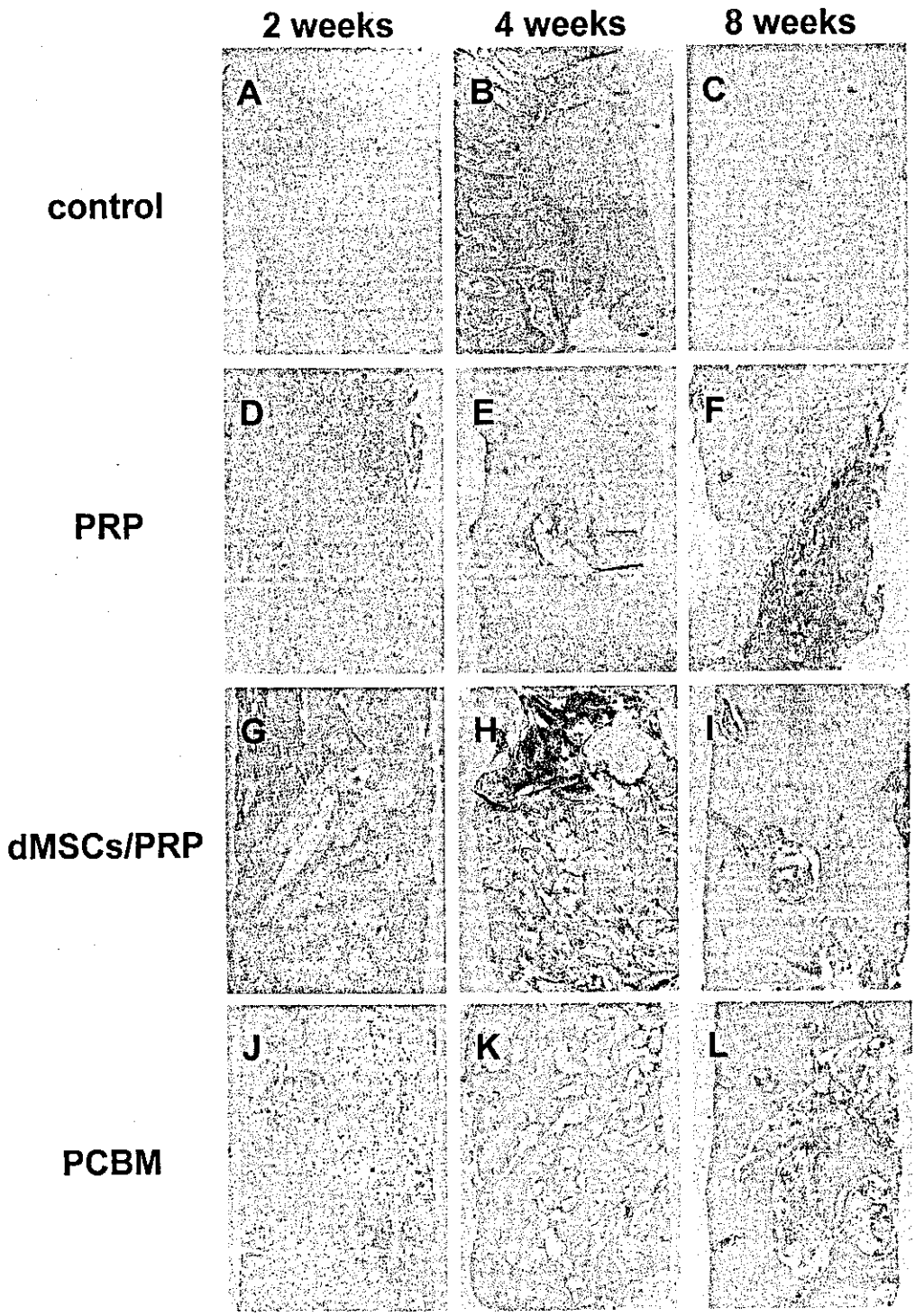

FIG. 3. (A–L) Histologic evaluation of control, PRP, PCBM, and dMSCs/PRP implantations at 2, 4, and 8 weeks: lower magnification. Sections of representative implants are shown from the respective groups. The sections were stained with hematoxylin and eosin. Original magnification: (A–L) ×40. (A) Two weeks in control group; (B) 4 weeks in control group; (C) 8 weeks in control group; (D) 2 weeks in PRP group; (E) 4 weeks in PRP group; (F) 8 weeks in PRP group; (G) 2 weeks in dMSCs/PRP group; (H) 4 weeks in dMSCs/PRP group; (I) 8 weeks in dMSCs/PRP group; (J) 2 weeks in PCBM group; (K) 4 weeks in PCBM group; (L) 8 weeks in PCBM group.

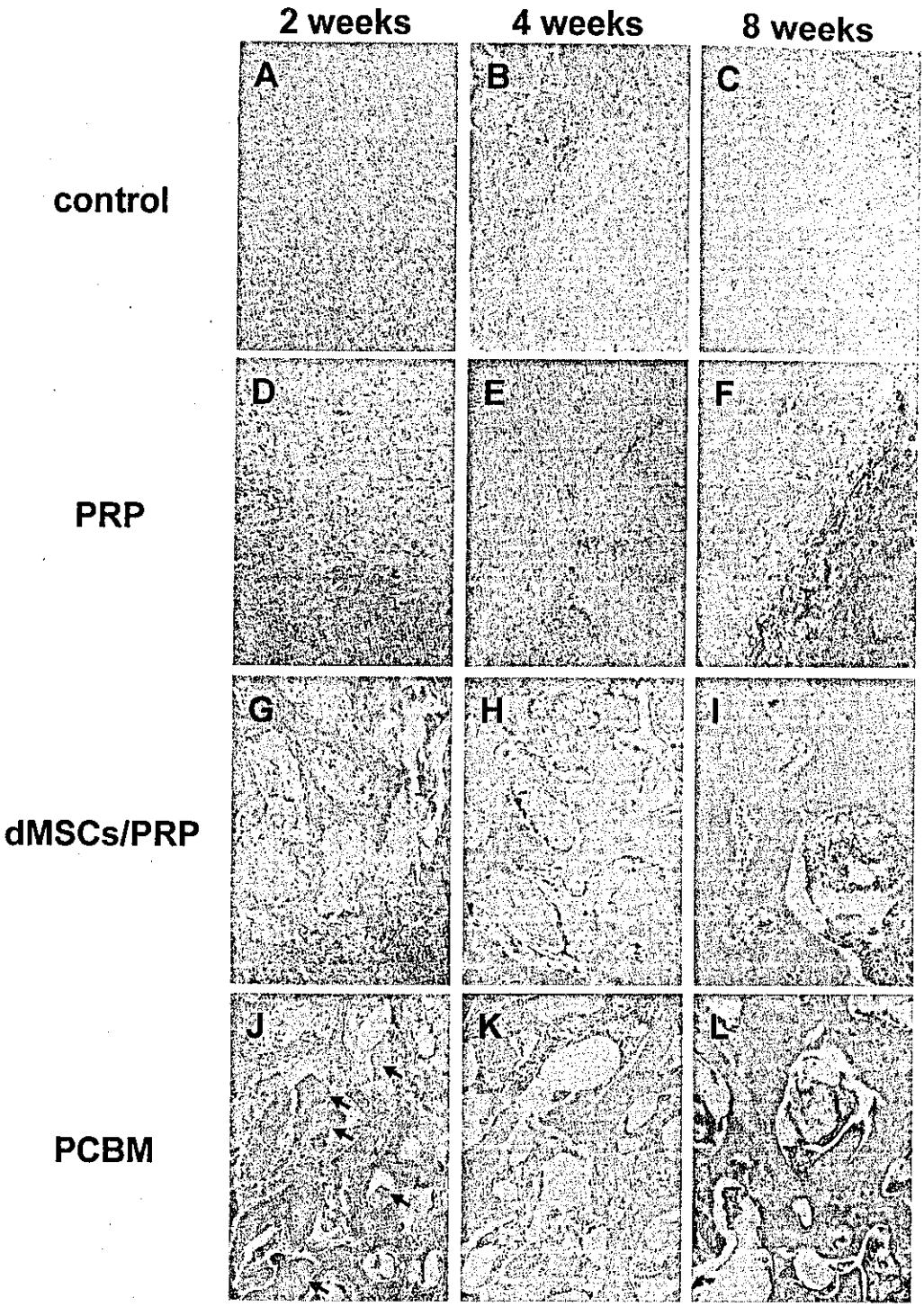

FIG. 4. Histologic evaluation of control, PRP, PCBM, and dMSCs/PRP implantations at 2, 4, and 8 weeks: higher magnification. Sections of representative implants are shown from the respective groups. The sections were stained with hematoxylin and eosin. Original magnification: (A–L) ×200. (A) Two weeks in control group; (B) 4 weeks in control group; (C) 8 weeks in control group; (D) 2 weeks in PRP group; (E) 4 weeks in PRP group; (F) 8 weeks in PRP group; (G) 2 weeks in dMSCs/PRP group; (H) 4 weeks in dMSCs/PRP group (active vascularization found); (I) 8 weeks in dMSCs/PRP group (laminar bone observed); (J) 2 weeks in PCBM group (arrows, implanted PCBM); (K) 4 weeks in PCBM group (dead space found, from resorption by implanted PCBM); (L) 8 weeks in PCBM group.

with MSCs, which had excellent osteogenic characteristics.[8] But these delivery substances did not have good plasticity and the cellular implantation procedure was complicated by problems associated with the delivery vehicles. Optimally, these should combine an appropriate rate of biodegradability with the capacity for the respective cells to multiply. In this study, we have used a combination of PRP with MSCs and found a progressive, complete resorption of the scaffold, leaving relatively mature remodeled bone. To our knowledge, there was an almost complete disappearance of the biomaterial when used in conjunction with MSCs and was replaced by mature bone with the appropriate architecture at an early stage, hence representing true bone regeneration. This has not been demonstrated previously. However, the control group was surrounded by soft tissue that never healed, thereby confirming the critical size of this defect. We also found that the extent of healing differed significantly, depending on the source of the cells. Filling a defect with PRP alone did not allow osteogenesis to occur in the affected areas. Advances in the culturing of multipotent MSCs from bone marrow[18] and the repeated demonstration that their differentiation can be directed to the osteoblastic lineage suggest that the clinical use of MSCs for bone regeneration is possible. By definition, MSCs are able to undergo many cycles of cell division without a loss of their osteogenic capacity.[19] The proliferative expansion of MSCs, but not periosteal cells, generates large numbers of potentially osteogenic cells that may be used in clinical settings to direct bone formation and repair.[7] On the other hand, the tissue-engineered bone by dMSCs/PRP performed better, suggesting a positive influence of PRP on the MSCs. The PRP scaffold for MSCs would encourage MSCs adhesion, proliferation, and differentiation to elicit bone formation. The implanted scaffold would become vascularized, because osteogenesis requires a well-developed vascular supply.[20] We found that the dMSCs/PRP group vascularized well. Ideally, the scaffold should be resorbed at a rate commensurate with new bone formation, within a few weeks. This makes it different from most hydroxyapatite, β-TCP ceramics,[7,16] or coral scaffolds,[17] which virtually do not degrade during the first few weeks of implantation. Presumably, the disappearance of the dMSCs/PRP left in place induced bone tissue formation, which then self-organized according to the surrounding environment.

The average rate of vascularization in the rabbit ear chamber was estimated at 0.09–0.25 mm/day.[21] If one assumes a similar rate of vascularization in dogs, blood vessels should reach the center of the implant within at least 20 days. Although it is still possible that there is massive cell death within the core of the implant due to a lack of vascularization, the results obtained with the tissue-engineered bone suggest good cell viability and the direct participation of MSCs in osteogenesis. Therefore we might speculate that PRP activity promotes vascularization.

Bone formation results from a complex cascade[22,23] of events that involve the proliferation of primitive mesenchymal stem cells, differentiation to osteoblast precursor cells (osteoprogenitor, preosteoblast), maturation of osteoblasts, formation of a matrix (type I collagen), and finally mineralization.[22,23] The initial event must be the chemotactic attraction of the osteoblasts. Owen and Friedenstein proposed that marrow derived and periosteal-derived progenitor cells had been shown to produce bone and cartilage in numerous *in vivo* and *in vitro* studies and the differentiation process appeared to depend heavily on the influences of numerous cytokines.[5] In this time the use of dMSCs/PRP provides conditions for obtaining more rapid and effective bone regeneration. The PRP contains an autologous source of platelet-derived growth factor (PDGF), transforming growth factor β (TGF-β), and so on. And this PRP gel, which is a coagulated mass, is also easy to manipulate, but it must be applied without delay to preserve growth factor activity. The life span of a platelet in a wound and the period of the direct influence of its growth factors were less than 5 days.[24] In addition to these growth factors, other proteins carried within platelets[25] may act in concert with cytokines released from other cellular sources, thus modulating hemostasis. These results suggested that reinforcing growth factor concentration through the application of PRP in the wound improved soft tissue repair and bone regeneration.

Khouri *et al.* were able to experimentally generate *in vivo*, autogenous, well-perfused bones of various desirable shapes by tissue transformation, which is the transformation of mesenchymal tissues, such as muscle, cartilage and bone induced by the osteoinductive factor osteogenin, which is identical to BMP-3, and by its parent substratum, demineralized bone matrix (DBM).[26] Despite the soundness of the concept and the validity of the laboratory data, the method is still not widely used. Because it is difficult to obtain a routine supply of DMB approved for clinical use, which must be prepared from cadaveric human bone, it is not accepted in Japan. More importantly, the batch-to-batch variation in inductive potency of different DBM preparations has led most surgeons to abandon its use,[27] even in distant muscle flap transfer and tissue molding. And the method requires added invasiveness at other sites. It is also difficult to purify osteogenin easily and without toxicity or immunoreactivity. On the other hand, as our method involves autogenous bone regeneration by tissue engineering, it is nontoxic, nonimmunoreactive, with minimal invasiveness and good plasticity.

In conclusion, our findings demonstrated that dMSCs/PRP implants can elicit true bone regeneration as well as autogenous bone (PCBM) grafts, with complete

disappearance of the biomaterial and formation of PRP in a bone defect of clinically relevant volume. And the fact that PRP is an autologous preparation, introduced at the time of surgery, eliminates concerns about disease transmission and immunogeneic reactions associated with allogeneic or xenogeneic preparations, and the possibility of mislabeling a sample, which might occur through laboratory error. Moreover, the ability to inject dMSCs/PRP mixtures that solidify within the host and are replaced over time with bone has powerful implications for the future of oral–maxillofacial and reconstructive surgery.

The methods detailed in these studies are the first steps toward customized autogenous bone grafts. Theoretically, one could obtain a host's MSCs by biopsy with minimal invasiveness, induce the cells to proliferate as osteoblasts *in vitro*, and then reimplant them in a controlled manner to produce a direct contour augmentation, reconstruction, periodontosis, or dental implant. These data presented from MSCs/PRP admixtures show that MSCs, migrate effectively into and through PRP. PRP allows MSC proliferation without deforming cell structure and is an appropriate delivery substance. It may hold promise as a highly suitable vehicle for delivering cells by injection to correct or reconstruct bony defects in a clinical setting.

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Ultrasound Enhances Transforming Growth Factor β-Mediated Chondrocyte Differentiation of Human Mesenchymal Stem Cells*

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ABSTRACT

In clinical studies and animal models, low-intensity ultrasound (US) promotes fracture repair and increases mechanical strength. US also promotes cartilage healing by increasing glycosaminoglycan synthesis of chondrocytes. As mesenchymal stem cells (MSCs) have the ability to differentiate into chondrocytes, US may promote their differentiation. Here, we evaluated the effects of US on the differentiation of MSCs toward chondrocytes and cartilage matrix formation. When human MSCs cultured in pellets were treated with transforming growth factor β(TGF-β, 10 ng/mL), they differentiated into chondrocytes as assessed by alcian blue staining and immunostaining for aggrecan, but nontreated cell pellets did not. Furthermore, when low-intensity US was applied for 20 min every day to the TGF-β-treated cell pellets, chondrocyte differentiation was enhanced. Biochemically, aggrecan deposition was increased by 2.9- and 8.7-fold by treatment with TGF-β alone, and with both TGF-β and US, respectively. In contrast, cell proliferation and total protein amount appeared unaffected by these treatments. These results indicate that low-intensity US enhances TGF-β-mediated chondrocyte differentiation of MSCs in pellet culture and that application of US may facilitate larger preparations of chondrocytes and the formation of mature cartilage tissue.

INTRODUCTION

CARTILAGE IS RESTRICTED to specific regions including the joint surface, ears, nose, and ribs, and mainly helps physical movement. Its destruction in joint diseases such as rheumatoid arthritis and osteoarthritis, and its hypoplasia such as in microtia in infants, cause severe problems. Because of the lack of blood vessels and the presence of a unique extracellular matrix composed of specific structural molecules,[1] cartilage is one of the most difficult tissues to regenerate. Cartilage defects are currently treated by several different techniques, including total joint replacement[2] performed for larger defects, and subchondral drilling[3] and tissue transplantation for

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smaller ones.[4,5] To maintain the quality of life of patients, treatment that limits surgical invasion is desirable, and therefore cartilage tissue engineering[6] is required.

Cartilage tissue engineering comprises three factors: cell source, growth factors, and scaffolds. Chondrocytes from other cartilage such as rib cartilage are most commonly used for the formation of cartilage tissue.[7] However, their cell number is limited and it is difficult to construct a tissue of large size. Differentiation of embryonic stem cells toward chondrocytes has been accomplished,[8] but its clinical application is impractical at present from ethical points of view.[9] In contrast, mesenchymal stem cells (MSCs) are promising because they can easily be prepared from patients without invasive surgery. These cells grow rapidly, retaining their capacity to differentiate into chondrocytes under certain conditions.[10,11] Several growth factors such as transforming growth factor β (TGF-β), bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), and insulin-like growth factors (IGFs) are involved in chondrocyte differentiation, proliferation, and maintenance.[12] These molecules are used for cartilage tissue engineering. The application of scaffolds has two advantages in this type of engineering. It enables three-dimensional culture, which is a necessary microenvironment for maturing the chondrocyte phenotype. It serves as an artificial matrix that gradually becomes replaced with native cartilage matrix. Although several methods have been attempted, with consideration of these factors, no cartilage tissue has been engineered that fulfills clinical requirements.

There has been accumulating evidence that stimulation of chondrocytes facilitates cartilage matrix formation.[13] For instance, hydrostatic pressure on bovine chondrocytes is known to enhance their matrix synthesis and accumulation.[14,15] Direct compression on bovine chondrocytes embedded in agarose gel increases glycosaminoglycan and collagen composition.[16] Thus, stress may serve as another important factor in cartilage tissue engineering.

Studies have shown that low-intensity ultrasound (US) accelerates fracture healing and shortens the period of treatment in patients[17,18] and animal models.[19,20] In this process, chondrocytes may respond to US signals. It was demonstrated that treatment of mature chondrocytes with low-intensity US enhances their matrix gene expression, such as the aggrecan gene.[21,22] *In vitro* culture studies support this observation. Nishikori *et al.*[23] also demonstrated that US upregulated chondroitin sulfate formation by chondrocytes embedded in collagen gel. Thus, US may work as a "bioreactor" that enhances cartilage matrix formation and maintains chondrocyte differentiation.

Here, we examined the effects of US on cartilage matrix formation in MSCs cultured in pellets. We demonstrate that US significantly accelerated TGF-β-mediated chondrocyte differentiation as assessed by aggrecan deposition.

MATERIALS AND METHODS

Pellet culture

Human mesenchymal stem cells (hMSCs) were obtained from Cambrex Bio Science Walkersville (Walkersville, MD). Frozen-preserved hMSCs were thawed, suspended with MSC growth medium (MSCGM; Cambrex Bio Science Walkersville), and plated in a 75-cm^2 culture flask and cultured for 7 days. The cells were expanded into a 182-cm^2 culture flask. After trypsinization, approximately 3.0×10^5 hMSCs were transferred into a 15-mL polypropylene tube and pelleted by centrifugation at $300 \times g$ for 5 min. The pellet was cultured at 37°C with 5% CO_2 in 2 mL of basic medium containing high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10^{-7} M dexamethasone, ascorbate 2-phosphate (50 μg/mL), 0.35 mM proline, 1 mM pyruvate, and ITS+ Premix (50 mg/mL [BD Biosciences, Franklin Lakes, NJ]: insulin [6.25 μg/mL], transferrin [6.25 μg/mL], sodium selenate [6.25 μg/mL], bovine serum albumin [BSA, 1.25 mg/mL], and linoleic acid [5.35 μg/mL]).

Treatment with TGF-β and application of ultrasound

After 24 h, samples of the pellets were divided into four groups: cultured in basic medium (no treatment), cultured in basic medium with US treatment every day (US), cultured in chondrogenic medium containing TGF-β_3 (10 ng/mL), and cultured in the above-described chondrogenic medium with US treatment every day (TGF-β + US). All the pellets were cultured at 37°C with 5% CO_2 and the culture medium was changed every 3 days until the pellets were harvested.

The US apparatus (Teijin Pharma, Tokyo, Japan) was used to deliver a US signal with spatial and temporal average intensities of 15, 30, 60, and 120 mW/cm^2. The frequency was 1.0 MHz with a 200-μs tone burst repeating at 1.0 kHz (Fig. 1). The polypropylene tube was set on the US transducer and the US wave was transmitted through the bottom of the tube. The US was applied for 20 min/day every day. The nontreated groups were subjected to the same conditions as the treated groups, except that the US apparatus was not turned on.

Measurement of pellet size

After culture for 10 days, photographs of the pellets were taken, using a stereomicroscope (SZX12; Olympus, Tokyo, Japan). The pellet size was defined as the largest cut surface, which was measured with NIH Image software.

Histological examination

After taking photographs, the pellets were fixed with 10% buffered formalin (Wako, Osaka, Japan) and buried

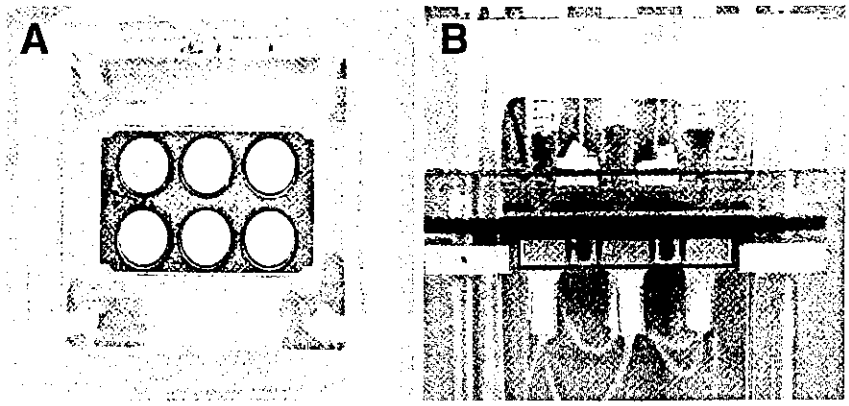

FIG. 1. The apparatus for low-intensity US. (**A**) Upper view; (**B**) side view. In (**B**) polypropylene tubes containing cell pellets are placed on a transducer.

in fixed mouse liver tissue that supports the pellets. The sample was then dehydrated, embedded in paraffin, sectioned, and stained with alcian blue.

Sections were deparaffinized, hydrated, and digested with chondroitinase ABC (1 unit/mL; Seikagaku, Tokyo, Japan) in distilled water at room temperature for 30 min. Endogenous peroxidase was inactivated by treatment with 0.3% hydrogen peroxide for 15 min. The slide was then washed with phosphate-buffered saline (PBS). The section slides were treated with goat serum for blocking, followed by incubation with a rabbit anti-aggrecan antibody (gift from T. Yada, Aichi Medical University, Nagakute, Japan) at 4°C for 16 h. After washing three times with PBS, peroxidase-conjugated goat anti-rabbit IgG antibody (Nichirei, Tokyo, Japan) was applied for 30 min. A substrate reagent containing 3,3′-diaminobenzidine (DAB) chromogen and 0.6% hydrogen peroxide (Nichirei) was applied to the section for 2 min.

Quantification of protein, aggrecan, and DNA

The pellets were washed twice with PBS, followed by extraction with 4 M guanidinium hydrochloride, 0.1 M Tris-HCl (pH 7.2), 1 mM phenylmethylsulfonyl fluoride, and 10 mM sodium ethylenediaminetetraacetate at 4°C for 24 h using a shaker (Taitec, Tokyo, Japan). Soluble fractions were used for protein and aggrecan quantification. The insoluble fraction was treated with proteinase K in ATL solution supplied by Qiagen (Valencia, CA) and used for DNA quantification.

Protein was measured with a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). Samples were incubated with BCA reagent at room temperature for 30 min and measured by absorbance at 562 nm. A standard curve was obtained with serial dilutions of BSA. Aggrecan was measured with a proteoglycan (PG) enzyme-linked immunosorbent assay (ELISA) kit (Biosource Europe, Nivelles, Belgium). Samples were reacted with a monoclonal anti-IgG1 domain antibody coated on the microtiter well and a monoclonal anti-keratan sulfate antibody labeled with horseradish peroxidase (HRP). After the incubation period, a sandwich (coated anti-IgG1 domain antibody–aggrecan–anti-keratan sulfate antibody–HRP) was formed. After treatment with the substrate, the microtiter plate was read at 450 nm.

DNA was measured on the basis of the enhancement of fluorescence that occurs when bisbenzimide binds to intact DNA.[24] Samples were excited at 412 nm, and fluorescence was measured at 507 nm. A standard curve was obtained by serial dilutions of calf thymus DNA (Sigma, St. Louis, MO).

Statistical analysis

Sample quantities are presented as means ± standard deviation (SD). Differences among experimental groups were analyzed by Student t test, with the level of significance at $p = 0.05$.

RESULTS

Several chondrogenic cell lines, such as ATDC5[25] and N1511,[26] are able to differentiate in monolayer culture. We tested whether hMSCs similarly exhibit differentiation in response to TGF-β. hMSCs grew on monolayer in a spindle-like shape. When treated with TGF-β, these cells became plump morphologically. However, these cells were barely stained with alcian blue, suggesting that monolayer culture of hMSCs is not suitable for their chondrocytic differentiation. Therefore, we employed a pellet culture system as previously reported.[27] MSCs were centrifuged in a 15-mL tube at 300 × g for 5 min and cultured in 2 mL of serum-free medium (Fig. 2A). After 24 h, the cell pellets became spherical (Fig. 2B). One milliliter of medium was replaced every 3 days with

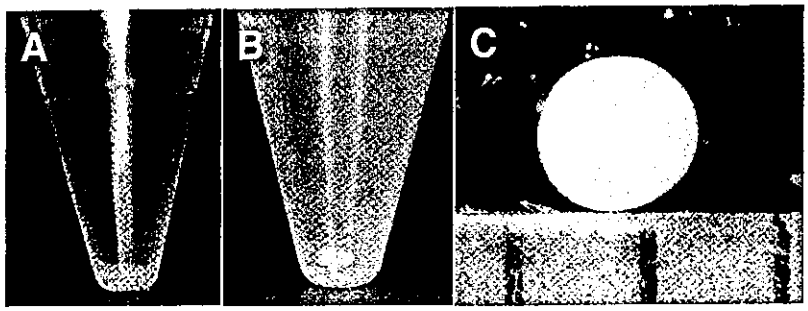

FIG. 2. Pellet culture. (**A**) Cell pellet of hMSCs immediately after centrifugation appears quadrilateral. (**B**). The cell pellet after 24 h of incubation appears oval. (**C**) Stereomacroscopic view of a pellet on day 10. The pellet appears spherical. Scale indicates 1 mm.

a fresh aliquot. The pellets gradually became larger and their size reached 1 mm in diameter with a smooth surface by day 10 (Fig. 2C). The pellets continued to grow to 1.5 mm but did not grow further, and remained this size even after 3 weeks. A large number of cells, such as 1×10^6 or 2×10^6 cells, did not form solid pellets and remained fragile. Histologically, the fragile tissue showed central necrosis (data not shown).

Next, we examined the effects of TGF-β on chondrocyte differentiation of the cells in the pellets. When the pellets were histologically examined on day 10, TGF-β-treated pellets were strongly stained with alcian blue,

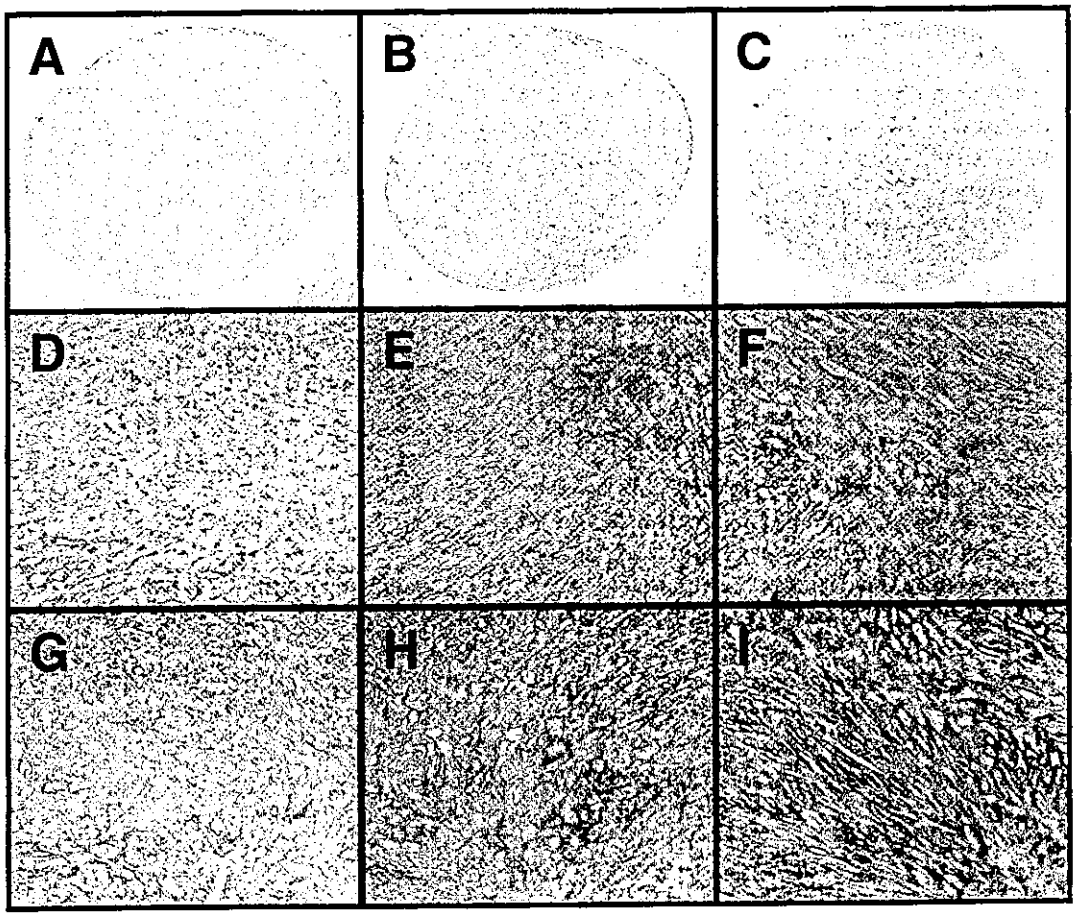

FIG. 3. Patterns of alcian blue staining (**A–F**) and immunostaining for aggrecan (**G–I**). Cell pellets without treatment (**A, D.** and **G**), treated with TGF-β (**B, E,** and **H**), those treated with both TGF-β and US (**C, F,** and **I**) are shown. Original magnification: (**A–C**) ×40; (**D–I**) ×100. Representative patterns of four independent experiments are shown.

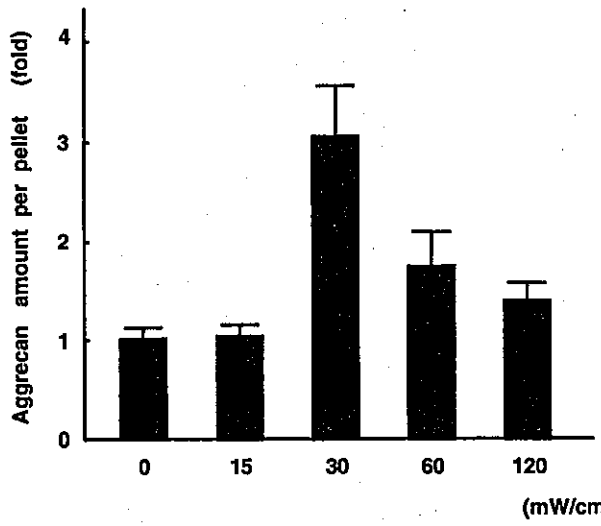

FIG. 4. Effects of US at different intensities on aggrecan synthesis of cells in pellets in the presence of TGF-β. Aggrecan amount at 30 mW/cm^2 is greater than for any other group ($p <$ 0.01). The same results were obtained from two independent experiments.

whereas nontreated pellets only slightly stained (Fig. 3A–F). The TGF-β-treated pellets contained spindle-shaped, oval, and round cells and mature extracellular matrix stained with alcian blue, indicating that these pellets formed cartilage-like tissue. In contrast, nontreated pellets contained mainly large plump cells like adipocytes. The pellets treated with both TGF-β and US showed stronger staining with alcian blue. Interestingly, the bottom half was better stained.

We confirmed the formation of cartilage matrix by immunostaining for aggrecan (Fig. 3G–I). Aggrecan is one of the major structural macromolecules of cartilage,[1] and therefore is widely used as a marker for cartilage tissue. The staining patterns were similar to those with alcian blue. These results indicate that TGF-β induces chondrocyte differentiation of MSCs in the pellets, and that US treatment enhances the differentiation.

As US treatment enhanced aggrecan deposition, we optimized its intensity (Fig. 4). Treatment at an intensity of 30 mW/cm^2 showed a 3.06-fold enhancement, and those with 60 and 120 mW/cm^2 were enhanced 1.75-fold and 1.38-fold, respectively. Treatment at 15 mW/cm^2 did not appear to enhance aggrecan deposition. Thus, we applied US at 30 mW/cm^2 in all the following experiments.

As better cartilage matrix formation may result in larger pellet size, we measured the sizes of pellets on day 10 (Fig. 5A). Pellet size was 0.83 $\pm$ 0.12, 0.87 $\pm$ 0.04, and 0.66 $\pm$ 0.02, and 0.69 $\pm$ 0.05 mm^2 for pellets without treatment, with US treatment, with TGF-β treatment, and both TGF-β and US treatment, respectively. These data indicate that TGF-β-treatment reduces pellet size but that US has few effects.

During culture for 10 days, cells may grow at different rates. We examined the total DNA content on day 10. Because more than 99% of DNA remained in the insoluble fraction after extraction (data not shown), the insoluble fraction was used for the analysis. The total amount of DNA was approximately 10 mg in all four groups (data not shown), indicating little effect of both TGF-β and US on cell proliferation.

We also measured the amount of protein in the pellets

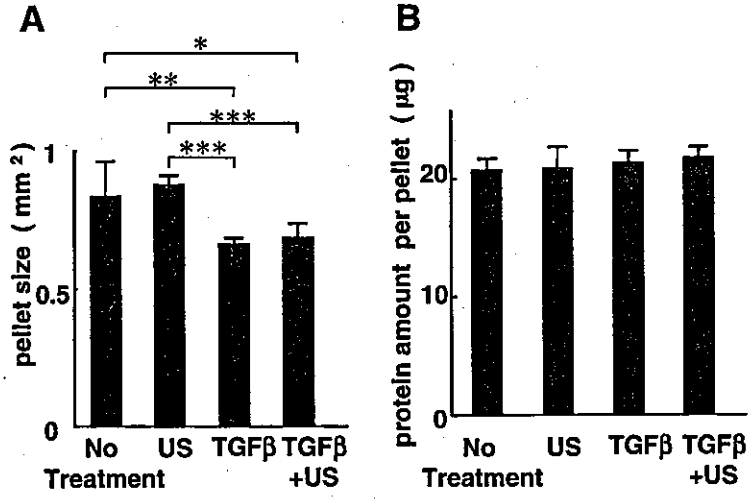

FIG. 5. The size of pellets (A) and total protein amount (B). (A) TGF-β treatment reduces pellet sizes but application of US has little effect (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). (B) There is no significant difference in protein amount among all groups. (No treatment, nontreated; US, ultrasound treated; TGF-β, TGF-β treated; and TGF-β + US, treated with both TGF-β and US). Essentially the same results were obtained from three independent experiments.

(Fig. 5B). The protein content was 20.85 ± 1.05, 21.00 ± 2.10, 21.15 ± 1.05, and 21.45 ± 1.50 μg in pellets without treatment, with US treatment, with TGF-β treatment, and with both TGF-β and US treatment, respectively (Fig. 5B). There was no significant difference among them.

Finally, we compared aggrecan deposition among four groups. The aggrecan amount was 0.30 ± 0.02, 0.32 ± 0.02, 0.86 ± 0.13, and 2.63 ± 0.25 ng in pellets without treatment, with US treatment, with TGF-β treatment, and with both TGF-β and US treatment, respectively (Fig. 6). These results indicate that TGF-β actually accelerated cartilage matrix formation and that US further enhanced the matrix formation of TGF-β-treated pellets, whereas application of US alone showed little enhancement.

DISCUSSION

In this study, we demonstrate for the first time the enhancing effect of low-intensity US on TGF-β-mediated chondrocyte differentiation of human MSCs. Although MSCs were not able to differentiate when cultured on plates, they were able to do so in pellets, especially in the presence of TGF-β. US significantly enhanced cartilage matrix formation of TGF-β-treated cells in pellets, whereas it had little effect on nontreated cells as assessed by aggrecan deposition. These results indicate that pellet culture of hMSCs is essential for the induction of chondrocyte differentiation, and that TGF-β both accelerates differentiation and facilitates acquisition of cell machinery to respond to the US signal. Interestingly, US treatment had little effect on cell proliferation. Although US treatment upregulated aggrecan synthesis, the total protein levels were not significantly increased, suggesting little effect of US on proteins not involved in chondrocyte differentiation.

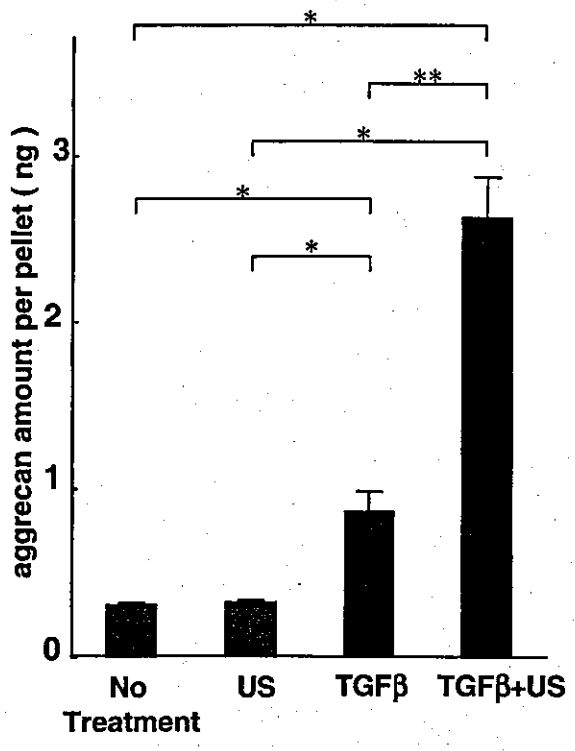

FIG. 6. Effects of TGF-β and US on aggrecan synthesis. The amount of aggrecan in induced groups (TGF-β and TGF-β + US) is significantly larger than that of non-TGF-β-treated groups (no treatment and US). The amount of aggrecan in the TGF-β + US group is increased by 3.06-fold compared with the TGF-β group (*$p < 0.001$, **$p < 0.01$). Essentially the same results were obtained from three independent experiments.

The fact that US enhanced the differentiation of TGF-β-treated cells but not of nontreated cells leads to two interpretations. One is that chondrocytes normally respond to US signals and treatment of MSCs with TGF-β was required for differentiation. In fact, mature chondrocytes synthesize larger amounts of aggrecan when treated with US.[21] The other interpretation is that TGF-β is required for the acquisition of signal transduction pathways mediated by US, which is independent of chondrocyte differentiation. As mature chondrocytes do not appear to require TGF-β-mediated signals and still respond to US, US-mediated signal transduction pathways may not be associated with TGF-β-mediated pathways.

Although several studies have shown that US stimulation of chondrocytes enhances cartilage matrix formation,[21–23] its precise mechanism has not been determined. Parvizi *et al.*[28] demonstrated that calcium signaling was required for US-stimulated aggrecan synthesis. Similar effects of tensile strain are observed on osteoblastic differentiation of hMSCs,[29] where NF-κB is implicated in this process. Integrins and stretch-activated cation channels have been known as candidates for converting mechanical signals to chemical signals on the cell surface.[30–32] Molecules such as mitogen-activated protein kinase (MAPK) and NF-κB, and the calcium concentration, may mediate their signal transduction pathways. Further study is required to determine which signal transduction pathways are mediated by US in chondrocytes and chondrogenic MSCs.

We have shown that the US effect had a range of optimal intensity at 30 mW/cm^2, which gave rise to ~3-fold aggrecan synthesis. US had little effect below 30 mW/cm^2 and the effect was only 1.4-fold at 120 mW/cm^2. These results suggest that the machinery consisting of the receptors and downstream signaling molecules is quite sensitive to the intensity of US. As the sensitivity to mechanical stress depends on the tissue density and structure, the optimal intensity may differ during formation of the cartilage matrix. We observed a polarity of

cartilage matrix formation in the pellets. The cells close to the US probe might have formed a matrix better than did the cells inside the pellet, and the matrix formed on the surface might have attenuated transmission of the US.

MSCs had to be cultured in pellets for differentiation into chondrocytes.[10,11] Even in the presence of TGF-β, they did not differentiate when cultured on plates. Induction of chondrocyte differentiation in MSCs in pellets may imply a requirement for cell–cell interaction different from that in plate culture, as confluent cells show little differentiation, and the pellet culture may provide a microenvironment similar to mesenchymal condensation,[33] which normally takes place on initiation of chondrogenesis. It has been demonstrated that chondrocytes maintain differentiation in pellets[12] or in three-dimensional culture coupled with scaffolds such as alginate beads,[34] collagens,[35] and polyglycolic acid.[36] The cells in the pellet may have an appropriate microenvironment for differentiation. Studies on the expression patterns of MSCs cultured in pellets during chondrocyte differentiation demonstrate that these cells exhibit sequential expression of molecules involved in chondrocyte differentiation.[35] The pellet culture system of MSCs will enable us to study US effects at different stages of chondrocyte differentiation.

BMPs have been described as the most potent factors of chondrocyte differentiation.[37] However, MSCs in pellets did not show chondrocyte differentiation when treated with BMP-6 at 10 ng/mL, which is an adequate concentration for osteoblast differentiation. MSCs in pellets have been shown to differentiate into chondrocytes when treated with BMP-6 at 500 ng/mL.[38] Shukunami *et al.*[39] also demonstrated that chondrogenic ATDC5 cells attain differentiation in the presence of BMP-2 at 1,000 ng/mL. These data suggest that MSCs and chondrogenic cells are less responsive to BMPs and that the concentration we employed was far from saturation. In contrast, TGF-β at 10 ng/mL is adequate for the induction of differentiation, indicating that MSCs have TGF-β-mediated signal transduction pathways. Signal transduction mediated by TGF-β in the initial step of differentiation may be indispensable for establishment of a response to BMPs, which dramatically accelerate further differentiation.

Compared with other bioreactors, the US system has several advantages, and is therefore widely used. The system is simple, easy to handle, and relatively inexpensive. It is not only available for plate culture and three-dimensional culture *in vitro*, but is also applicable to *in vivo* study and treatment. In fact, US has been widely used for treatment of bone fracture,[18] and its application was approved by the Food and Drug Administration (FDA). Accumulating data on the effects of US on differentiating MSCs and chondrocytes will help establish treatments for cartilage defects.

For clinical application of differentiating MSCs in pellets, there remain at least two issues to be resolved. The pellet grew to 1.5 mm even after 3 weeks. A large pellet with 1×10^6 cells exhibited central necrosis. To obtain a larger size of cartilage, collection of small pellets followed by their fusion or embedding them in scaffolds may be needed. The other issue is the mechanical strength of the pellets. In this study, the size of the pellet was too small to evaluate its mechanical strength. Provided that these issues are resolved, treatment with US of differentiating MSCs in pellets will facilitate its clinical application toward cartilage tissue engineering.

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