defect of clinically relevant volume. The ability to inject tissue-engineered bone 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 tissue-engineered bone grafts. Theoretically, one could obtain a host's marrow tissue by biopsy with minimal invasiveness, proliferate the cells to osteoblasts in vitro, and then reimplant them in a controlled manner to produce direct contour augmentation, reconstruction, periodontosis, or dental implants. And the results of the present investigation indicate that injectable tissue-engineered bone used for maxillary or mandible onlay graft, with simultaneous implant placement in patients, provided stable and predictable results in terms of implant success.

We regard this translational research as a method in accelerating therapy development. Based on the success of the approach, we also would develop additional new strategies in bone regeneration. Just as translational research must be fluid and interactive to succeed, we will continually modify our efforts on behalf of the patients that we ultimately serve. More important, most translational research studies require close collaboration between basic scientists and clinical researchers, with synergistic effects resulting from shared expertise and dedicated efforts to solve challenging problems. Although the clinical research enterprise currently is facing several important challenges, breakthrough discoveries in basic biomedical science would continue to be translated effectively to human studies to advance clinical science, expand medical knowledge, and ultimately improve patient care and health.

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Tissue-engineered injectable bone regeneration for osseointegrated dental implants

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Key words: dental implant, injectable bone, mesenchymal stem cells, platelet-rich plasma, tissue engineering

Abstract: The present study investigated a correlation between osseointegration in dental implants and an injectable tissue-engineered bone, using mesenchymal stem cells (MSCs) and platelet-rich plasma (PRP). Initially, the teeth in the mandible region were extracted and the healing period was 1 month. Bone defects on both sides of the mandible were prepared with a trephine bar. The defects were implanted with graft materials as follows: PRP, dog MSCs (dMSCs), and PRP, autogenous particulate cancellous bone and marrow (PCBM), and control (defect only). Two months later, the animals were evaluated by histology, and at the same time dental implants were installed. Two months later, the animals were sacrificed and nondecalcified sections were evaluated histologically and histometrically. According to the histological observations, the dMSCs/PRP group had well-formed mature bone and neovascularization, compared with the control (defect only) and PRP groups, as was the same for the PCBM group. A higher marginal bone level was observed around implants with PRP, PCBM, and dMSCs/PRP compared with the control. Furthermore, the values describing the amount of bone–implant contact (BIC) at the bone/implant interface were significantly different between the PRP, PCBM, dMSCs/PRP, and control groups. Significant differences were also found between the dMSCs/PRP and control groups in bone density. The findings of this experimental study indicate that the use of a mixture of dMSCs/PRP results in good results such as the amount of BIC and bone density comparable with that achieved by PCBM.

Predictable bone regeneration of large alveolar defects with complex morphology can pose a significant clinical challenge, particularly when there is a significant vertical component involved and a large tooth socket. Among the various techniques to reconstruct or enlarge a deficient alveolar bone, autogenous bone grafting (autografts) has become a predictable and well-documented surgical approach and is unequivocally accepted as the standard of care (Buser et al. 1999), but this method is associated with substantial morbidity that includes infection, malformation, pain, and loss of function (Laurie et al. 1984; Sommers & Eisenstein 1984; Younger & Chapman 1989). A previous approach to this problem focused on the development of various graft materials, and the bone allografts, xenografts, and alloplasts (substitutes) are being extensively studied in order to avoid the harvesting procedure of autogenous bone (Misch & Dietsh 1993; Gross 1997). Allografts are also in limited supply because of a scarcity of tissue donors. Synthetic materials suffer from increased susceptibility to infection, incidences of extrusion, and an uncertain long-term interaction with the host's physiology. The most reasons frequently cited

for using alternative grafting materials are donor site morbidity and insufficient volume of harvested autogenous bone (Younger & Chapman 1989). These apparent shortcomings of autografts are outweighted by their safety in terms of disease transmission and immunological aspects. So 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 (Yamada et al. 2003, 2004). The new technology we applied was called as *tissue engineering* (Langer & Vacanti 1993), and involves the morphogenesis of new tissue using constructs formed from isolated cells with biocompatible scaffolds and growth factors. In this study, we used mesenchymal stem cells (MSCs) as the isolated cells, and platelet-rich plasma (PRP) as the growth factors and scaffold.

MSCs have been thought to be multipotent cells that can replicate as undifferentiated cells and that have the potential to differentiate into lineages of mesenchymal tissue including the bone, cartilage, fat, tendon, muscle, and marrow stroma (Owen & Friedenstein 1998; Pittenger et al. 1999), and have received widespread attention because of their potential utility in tissue engineering applications. On the other hand, PRP, which is a mixture of growth factors and an autologous modification of the fibrin glue, is believed to result in early consolidation and graft mineralization in approximately half the time that it would take using an autogenous graft alone (Marx & Gard 1999). Moreover, it has been suggested that PRP may promote a 15–30% increase in the trabecular bone density (Marx et al. 1998). The use of PRP is based on the premise that the large numbers of platelets found in PRP release significant quantities of mitogenic polypeptides, such as platelet-derived growth factors (PDGFs), transforming growth factor-β (TGF-β), as well as insulin-like growth factor-I (IGF-I). The potential effects of PDGF include the stimulation of mitogenesis of marrow stem cells, and the stimulation of angiogenesis (Marx & Gard 1999). TGF-β has been shown to stimulate chemotaxis and mitogenesis of osteoblast precursors, stimulate the deposition of a collagen matrix for connective tissue healing and bone formation, as well as inhibit osteoclast formation and bone resorption (Mohan & Baylink 1991; Pierce et al. 1992). Furthermore, other studies have shown that PDGF and IGF-I may enhance osseous healing around endosseous dental implants (Lynch et al. 1991).

Implant–bone tooth restorations have become a standard of care in modern dentistry for occulusion restoration. However, the presence of sufficient bone volume is an important prerequisite for dental implant placement. Therefore, we tried to regenerate bone with a tissue engineering method for dental implants. At present, no experimental studies have examined the behavior of tissue-engineered regeneration of bone around implants, so we investigated the correlation between tissue-engineered bone with osseointegrated dental implants.

We designed the present experimental study to evaluate the osseointegration of dental implants placed in bone regenerated with different grafting materials. Implants placed in injectable tissue-engineered bone regeneration areas were compared with implants placed in nonregenerated, PRP regenerated, and particulate cancellous bone and marrow (PCBM) regenerated. In this paper we explored the potential ability of MSCs and PRP to increase the rate of bone formation and to enhance the bone

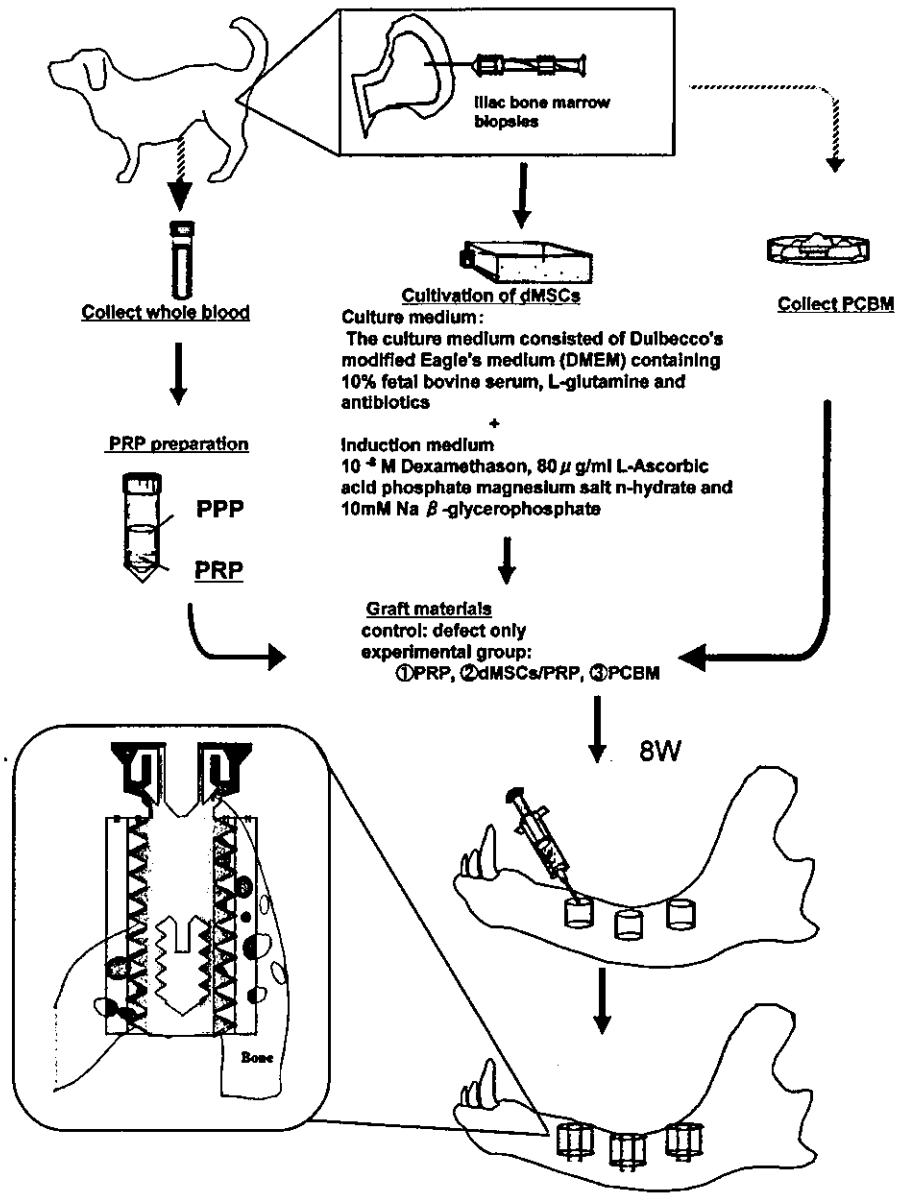

Fig. 1. (a) Schema of experimental protocol and the measurement around the dental implant. Red line, total length of bone contact; blue line, total length of implant surfaces. dMSCs, dog mesenchymal stem cells; PCBM, particulate cancellous bone and marrow; PPP, platelet-poor plasma; PRP, platelet-rich plasma.

regeneration, compared with autogenous bone grafts, PCBM. Secondly, to determine whether the combination of the PRP scaffold combined with MSCs improved bone formation in the bone defect with a clinically relevant volume and whether it is able to function in dental implants. Successful osseointegration in dental implants on tissue-engineered bone regeneration was obtained using a combination of MSCs and PRP with minimal invasiveness.

Material and methods

Canine animal models

After a period of housing, 5 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 1 month. Bone defects on both sides of the mandible were prepared with a trephine bar with a diameter of 10 mm. 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 between the various sites in terms of bone healing, we created 3 defects and implanted the 4 materials randomly without being specific to the sites. PCBM was also harvested from the iliac crest (Fig. 1). After 8 weeks, the osseointegrated dental implant was inserted into the bone regeneration areas.

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

The dMSCs were isolated from the dog's iliac crest marrow aspirates (10 ml) according to the reported method (Kadiyala et al. 1997). Briefly, the basal medium, low-glucose Dulbecco's modified Eagle's medium (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 U of penicillin and 25 μg of streptomycin) were purchased from Bio-Whittaker Inc. (Walkersville, MD, USA). Three supplements for inducing osteogenesis, dexamethasone (Dex), sodium β-glycerophosphate (β-GP) and L-ascorbic acid 2-phosphate (AsAP), were purchased from Sigma Chemical Co. (St Louis, MO, USA). The cells were incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. We replated the dMSCs at densities of 3.1×10^3 cells/cm^2 in 0.2 ml/cm^2 of the control medium. The differentiated dMSCs were confirmed by detecting alkaline phosphatase activity using *p*-nitrophenylphosphatase as a substrated and alkaline phosphatase staining (Yamada et al. 2003, in press). In culture, dMSCs were trypsinized and used for implanting.

The PRP gel preparation was done according to the same method (Yamada et al. 2003, in press). In short, approximately 50 ml whole blood was drawn from the canine into centrifuge tubes containing 10 ml of the culture medium with 250 U/ml of preservative-free heparin. The blood was first centrifuged in a standard laboratory centrifuge machine, Himac CT (Hitachi koki, 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 Sysmex XE-2100 (SYSMEX Co., Kobe, Japan). The PRP was stored at room temperature in a conventional shaker until its use. Bovine thrombin in a powder form (10,000 U) was dissolved in 10 ml 10% calcium chloride in a separate sterile cup. Next, 3.5 ml PRP, dMSCs (1×10^7 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 2 syringes were connected with a 'T' connector and the plungers of the syringes were pushed and pulled alternatively, allowing the air bubble to transverse the 2 syringes. Within 5–30 s, the contents assumed a gel-like consistency as the thrombin affected the polymerization of the fibrin to produce an insoluble gel. The gel was injected into the bone defect field using a 16-G needle attached to a 5 ml syringe. Samples were analyzed at 8 weeks after injection ($n = 5$).

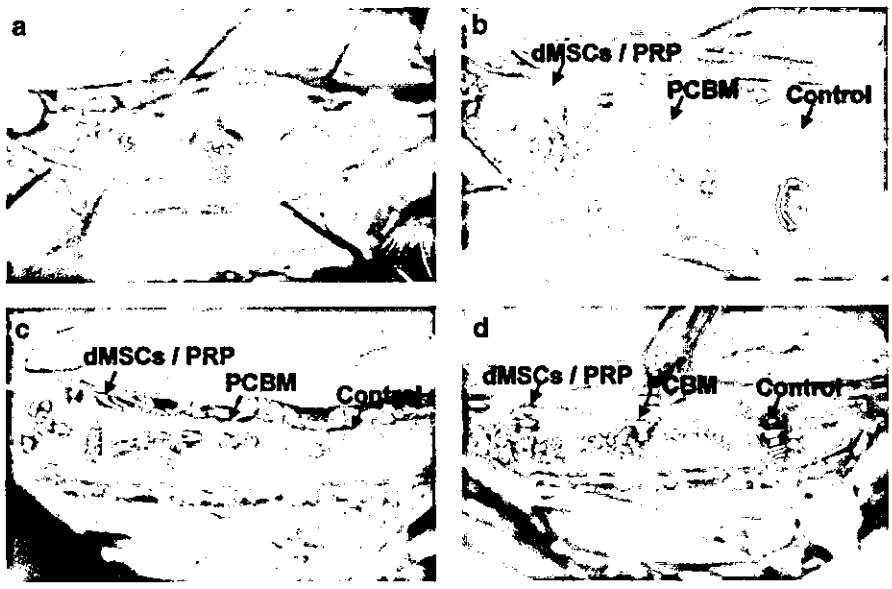

Fig. 2. Macroscopic observations for bone regeneration. (a) The experimental design in the dog mandibular prepared with a trephine bar with a 10 mm diameter. (b) Implanted materials in bone defects. (c) New bone regeneration in the dog mesenchymal stem cells/platelet-rich plasma (dMSCs/PRP), particulate cancellous bone and marrow (PCBM), and control groups at 8 weeks (PRP group data not shown). Bone regeneration by dMSCs/PRP and PCBM was regenerated to a natural level, but regeneration by PRP and the control (defect only) was not complete (PRP group data not shown). (d) The osseointegrated dental implants in the bone regeneration areas at 8 weeks. The dental implant thread was exposed in the control group.

Histological and histomorphometric analysis

Each implantation site was excised with a trephine bar with a diameter of 2 mm at 8 weeks after implantation, was assessed histologically. The specimens were fixed in 10% buffered formalin, decalcified (K-CX, Falma Co., Tokyo, Japan), and stained with hematoxylin and eosin. Then the Ø3.75 × 7 mm Brånemark implants Nobel Biocare Norden AB, Gothenburg, Sweden were installed into the bone defect, which had been made by bone sampling. The dogs were sacrificed at 8 weeks after the dental implant insertion. The mandible was dissected and cut into smaller blocks. Block sections were fixed in 10% formaldehyde. The sections were embedded in methylmethacrylate (Technovit 7200VLC, Kulzer GmbH, Germany) and polymerized. The specimens were sectioned and ground to about 10 μm thick using the Exact Cutting-Grinding System (Exact Apparatebau, Norderstedt, Germany), and stained with toluidine blue. A histological analysis was performed to obtain a general description of the tissue surrounding the implants. The histomorphometrical analysis was done by means of a light microscope (Hitachi Tablet Digitizer HDG-1212D, Hitachi Seiko Ltd., Tokyo, Japan) connected to a PC, equipped with a video and an image analysis system (System Supply Co. Ltd., Ina, Japan). The following histomorphometrical analyses were carried out:

$$\text{The bone – implant contact (BIC)(\%)} = \frac{\text{total length of bone contact}}{\text{total length of implant surfaces}} \times 100$$

The bone density was measured in a reference area defined between the lowest part of the shoulder and the screw thread bottom, and its mirror image (Fig. 1).

$$\text{The bone density(\%)} = \frac{\text{total surface of bone in the reference area}}{\text{total reference area}} \times 100$$

Statistical analysis

Group means and standard deviations were calculated for each measured parameter. The data were compared using the paired, 2-tailed Student's *t*-test between the control, and the PRP, dMSCs/PRP, and PCBM groups. A *P*-value of <0.05 indicated statistical significance.

Results

In vivo macrofindings and histological evaluation of the implants (PRP, dMSCs/PRP, PCBM) compared with the control

The dMSCs were trypsinized at day 7 and were used for the implants at a concentration of 1×10^7 cells/ml. The PRP mean platelet count was 1,293,400, with a range of 935,000–1,840,000. These values confirmed the platelet sequestration ability of the process, which showed that the concentration was 438% above the baseline platelet counts. Macroscopic findings showed that the bone regeneration by dMSCs/PRP and PCBM was to a natural level, but the regeneration by the PRP and control (defect only) was not complete. The dMSCs/PRP scaffold had almost completely disappeared without infection after implantation (Fig. 2a–c). When the osseointegrated dental implant was installed into the bone regeneration areas, it was found that the dental implant thread was exposed in the PRP and control groups, but not in the dMSCs/PRP and PCBM groups (PRP group data not shown) (Fig. 2d).

In the histological observation, in the controls and defects filled with PRP, the cortical continuity was never restored, the cavities were invaded by a vascular,

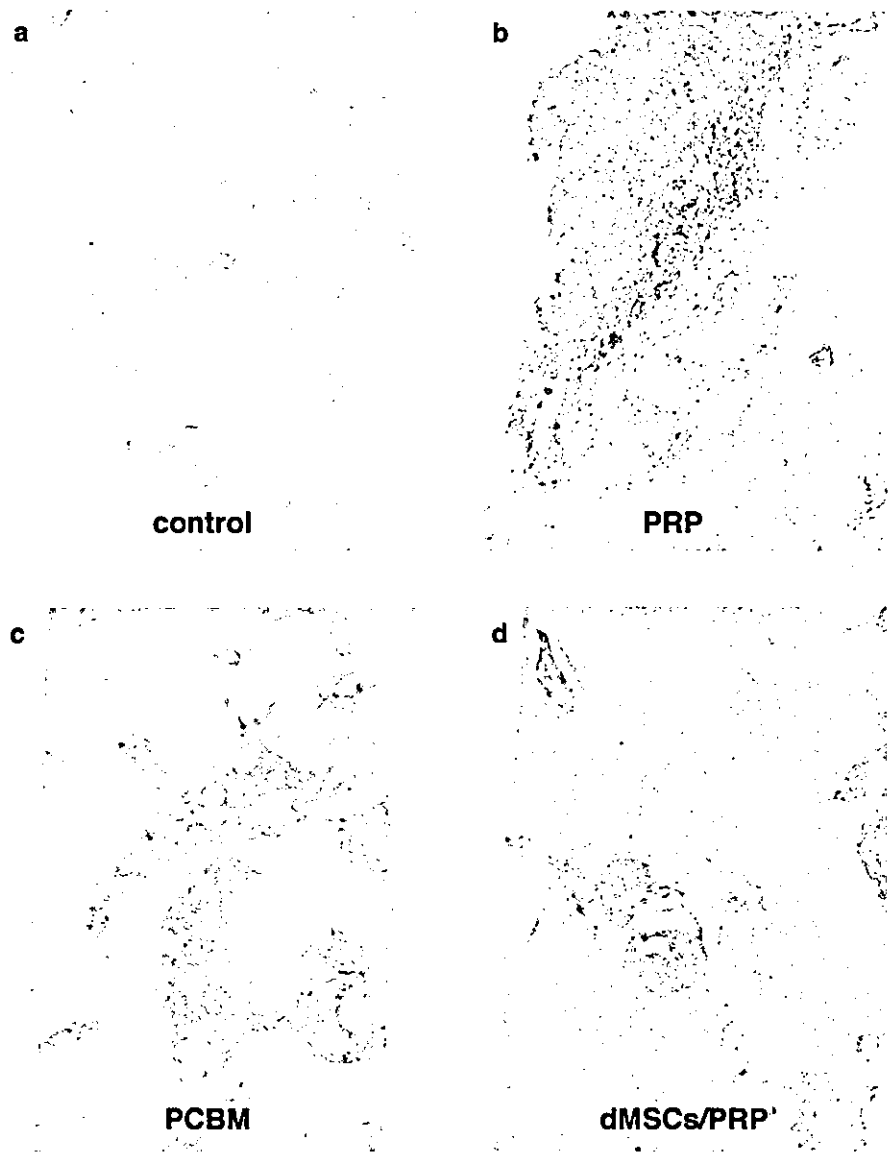

Fig. 3. Histologic evaluation of control, platelet-rich plasma (PRP), particulate cancellous bone and marrow (PCBM), and dog mesenchymal stem cells (dMSCs)/PRP implantations at 8 weeks. Sections of representative implants are shown from the respective group. The sections were stained by hematoxylin and eosin. Original magnification, × 40 for all prints. (a) Section from the control group, (b) section from the PRP group, (c) section from the PCBM group, and (d) section from the dMSCs/PRP group.

fibrous tissue, and few new bone formations (Fig. 3) were seen. On the other hand, cavities filled with dMSCs/PRP resulted in new bone formation with a tubular pattern at 8 weeks and abundant vascularization. This pattern reflected normal bone macrostructure, with well-differentiated marrow cavity and cortices, compared with cavities filled with PCBM, which showed dead space by grafted PCBM resorption (Fig. 3).

Histological findings and histomorphometric analysis around dental implants

All implants healed uneventfully and remained stable throughout the experimental period. In the control and PRP sites, the bone regeneration was not sufficiently regenerated for dental implant (Fig. 4a–d). In the PCBM grafted sites, the grafted bone exhibited good remodeling in spite of PCBM resorption (Fig. 4e,f). On the other hand, the regenerated bone by dMSCs/PRP showed newly formed woven and lamellar bone (Fig. 4g,h).

Bone density was 63.2 ± 7.6% for the control group, 68.2 ± 10.3% for the PRP group, 70.3 ± 8.2% for the PCBM group, and 79.4 ± 3.3% for the dMSCs/PRP group. There were significant differences in bone density between the dMSCs/PRP and control groups ($P<0.05$), but no significant difference was seen between the PRP, PCBM, and control groups. The implants exhibited a varying degree of bone–implant contact (BIC). The BIC was 26.4 ± 9.5% for the control group, 44.2 ± 10.8% for the PRP group, 49.9 ± 8.2% for the PCBM group, and 58.6 ± 9.7% for the dMSCs/PRP group, respectively. The BIC of the PRP and PCBM ($P<0.05$), and dMSCs/PRP ($P<0.005$) groups showed a significant increase in the implant surface compared with the control (Fig. 5).

Discussion

Berglundh & Lindhe (1997) reported a lower BIC for implants placed in a bone formation stage approach. After extraction in that study, the defects in the test sites were filled with a demineralized deproteinized bovine bone allograft (DFDBA) material without a barrier membrane. The control sites were not filled, and were left to heal spontaneously with a blood clot, as in our study. Three months later in their study, nonsubmerged implants were placed. Following a healing period of 4 months, the BIC measured along the entire implant surface was 44.1% for the test implants and 45.8% for the control implants. The BIC percentage was similar to the PRP and PCBM groups in our study. On the other hand, our dMSCs/PRP groups showed a higher percentage in comparison, irrespective of the short healing time. The results may be due to a bone promoting effect by PRP, which is known to enhance the formation of new bone and accelerate existing wound healing (Marx et al. 1998). And the use of PRP might provide conditions to obtain more rapid and effective bone regeneration for dMSCs. PRP contains

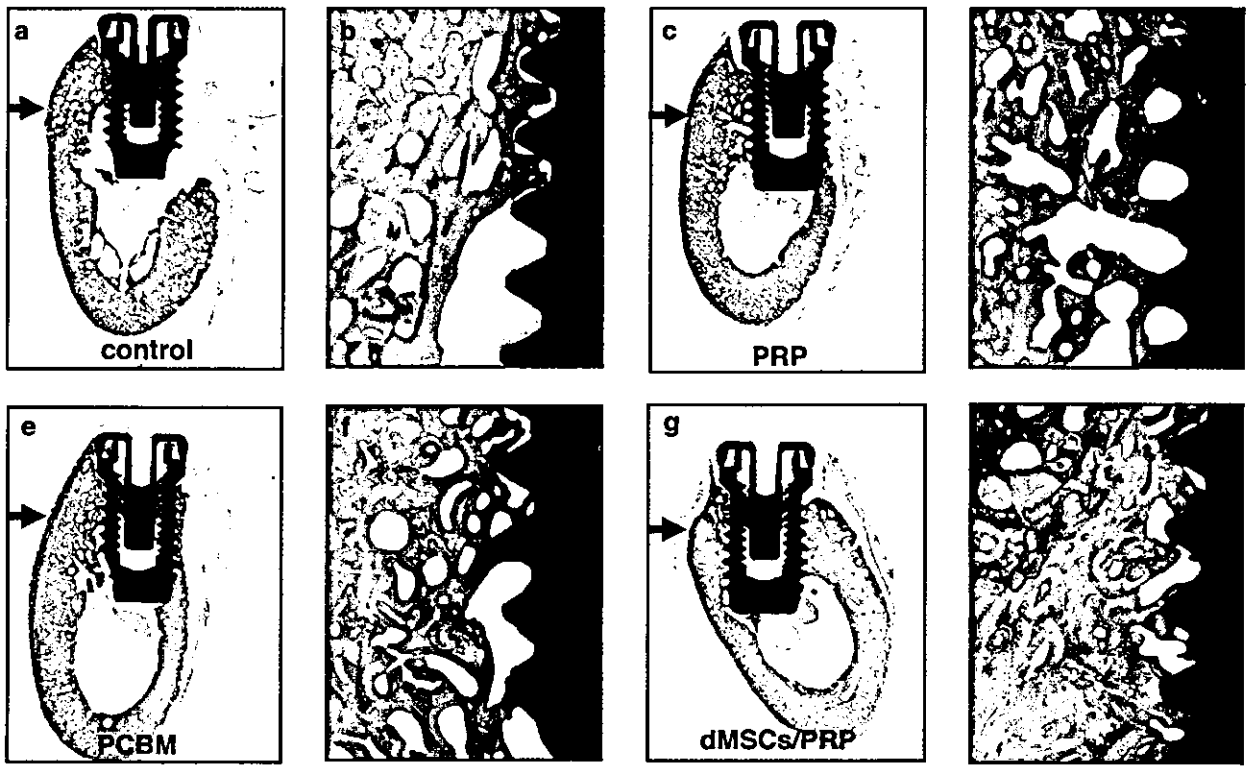

Fig. 4. Photographs of the histology sections, as seen with light microscopy. Nondecalcified ground sections surface stained with toluidine blue. Original magnification, × 12.5 for a, c, e, g, and × 250 for b, d, f, h. Arrows indicated the lingual side. (a) In the control group, the buccal wall was not sufficiently regenerated for dental implants (lower magnification). (b) In the control group (higher magnification). (c) In the platelet-rich plasma (PRP) group (lower magnification). Most of the threads on the buccal aspect were covered by soft tissue. (d) In PRP the group (higher magnification). (e) In the particulate cancellous bone and marrow (PCBM) group (lower magnification). The buccal wall was thin but reached the smooth/rough implant border. Extensive bone–implant contact was present. (f) In the PCBM group (higher magnification). After the absorbance by PCBM, it underwent recalcification. The dead space underwent grafted bone absorption. (g) In the dog mesenchymal stem cells (dMSCs)/PRP group (lower magnification). The fully regenerated buccal bone plate was as wide as the lingual cortex. This group showed good reconstruction of the former alveolar width. (h) In the dMSCs/PRP group (higher magnification). Good bone remodeling, as well as extensive bone–implant contact, was seen on the sides of the implant.

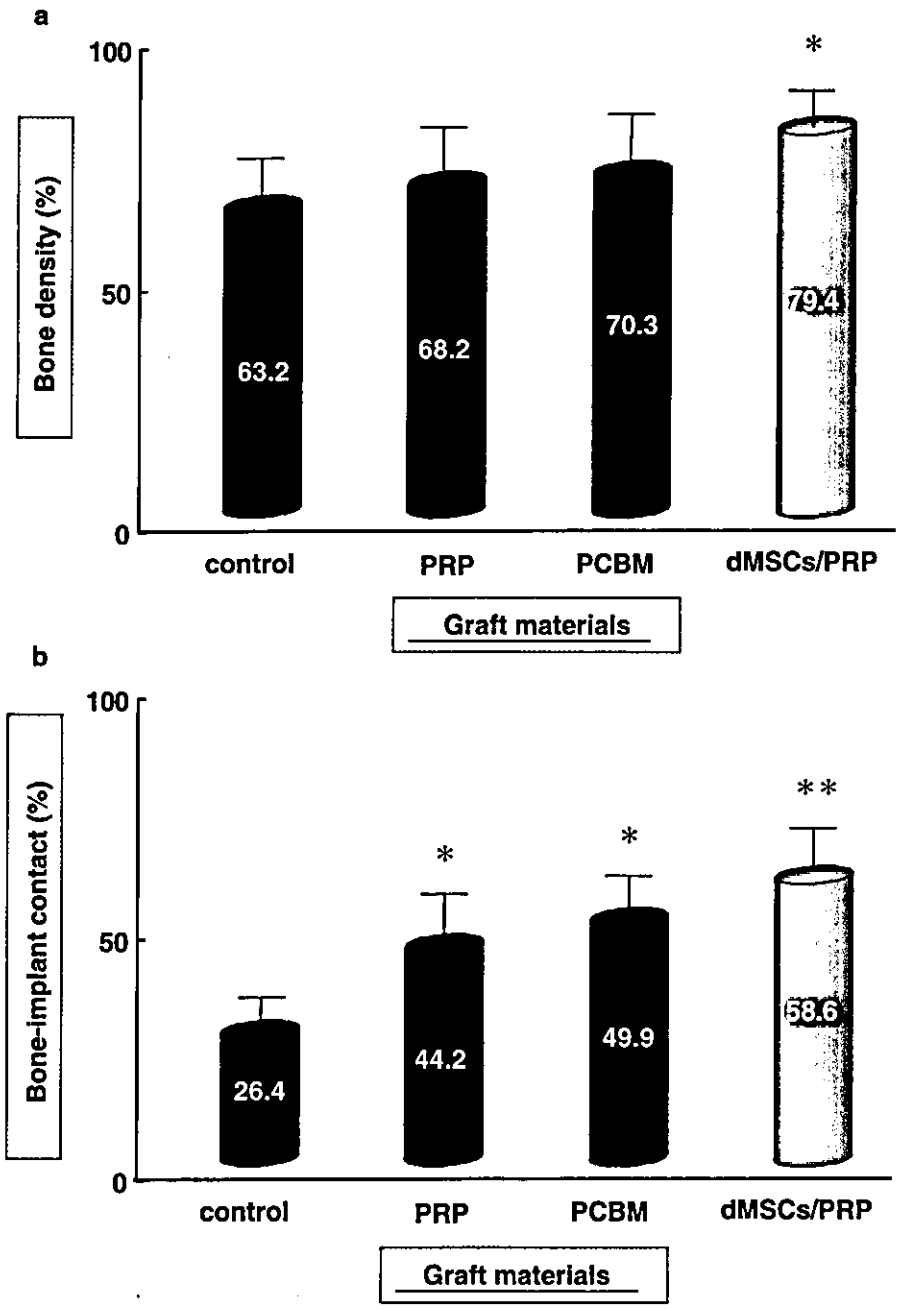

Fig. 5. Histomorphometrical evaluation. (a) Comparison of the mean percentage of bone density among the graft materials. A statistically significant difference was seen between the dog mesenchymal stem cells/platelet-rich plasma (dMSCs/PRP), and control groups. Asterisks indicate significant differences ($^{*}P<0.05$). (b) Comparison of the mean percentage of bone–implant contact among the graft materials. The measurements were made on all threads on both the buccal and lingual aspects of the implants (see Materials and Methods and Fig. 1). A statistically significant difference was seen between the PRP, particulate cancellous bone and marrow (PCBM), dMSCs/PRP, and control groups. Asterisks indicate significant differences ($^{*}P<0.05$, $^{**}P<0.005$).

an autologous source of PDGF and TGF-β. This dMSCs/PRP gel, which is a coagulated mass, is easy to manipulate, but it must be applied without delay to preserve growth factor activity (Marx et al. 1998). In addition to these growth factors, other proteins carried within platelets (Soslau et al. 1997) may act in concert with cytokines released from other cellular sources, thus modulating hemostasis. These results would suggest that reinforcing growth factor concentration through the application of PRP, by applying to it with dMSCs, improved bone regeneration, and osseointegration of dental implants. But the PRP alone was least effective in bone density and BIC and thus, PRP in the defect did not result in improved osseous healing.

Implant position and angulation in the bone may also affect the level of BIC, as well as the buccal and lingual width of the crestal bone around the implants (Von Arx et al. 2001). It must be emphasized that no attempt was made to standardize the angulation and position of the implants, or the original osseous defect, upon placement in the present study. It was rather a post-experimental observation that the long axis of the placed implants seldom matched the long axis of the alveolar ridge. The inadvertent buccal inclination of many of the evaluated implants may be explained by 2 reasons: (1) placement of implants in intubated dogs lying on their side makes the tilting of the drills toward the surgeon more likely; and (2) augmentation regions, particularly those with a bone grafting material, may demonstrate low resistance on the buccal aspect, facilitating the swerving of the drills. The control group was surrounded by soft tissue, which never healed, thereby confirming the critical size of this defect, and was not sufficient for dental implantation. We 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 implant areas. Recent advances in the culturing of multipotent 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 and osseointegration may be possible.

Recent bone augmentation materials have been used with autogenous bone, β-tricalcium phosphate particles (β-TCP), DFDBA, coral-derived hydroxyapatite (HA) granules and so on, and barrier membrane (Misch & Dietsh 1993; Gross 1997; Groeneveld et al. 1999; Al Ruhaimi 2001; Boo et al. 2002). Excised autogenous tissue, including fat, fascia, cartilage, and bone chips, have also frequently been used (Burchardt 1983; Wood & Moore 1988; Jensen et al. 1990). However, each of these alternative treatments causes specific problems (Younger & Chapman 1989). The preferred autogenous material causes specific problems: it is limited in its supply,

has an 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 (Laurie et al. 1984; Sommers & Eisenstein 1984; Younger & Chapman 1989). Autogeneous bone grafts from the ileum and tibia also must use general anesthesia, and it involves not only more invasiveness but also donor-site morbidity (Laurie et al. 1984; Sommers & Eisenstein 1984). On the other hand, this bone marrow biopsy with tissue engineering concept in compensation for autogenous bone grafts, can perform under local anesthesia, at least in our hospital, clinically and would pose few invasiveness than autogenous bone grafts taken from the ileum and tibia for this purpose when using implant placements. Synthetic materials of HA, β-TCP ceramics (Kadiyara et al. 1997; Bruder et al. 1998), DFDBA, or coral scaffolds (Petite et al. 2000), suffer from increased susceptibility to infection, incidences of extrusion, and an uncertain long-term interaction with the host's physiology, and that they virtually show no signs of resorption during some months after implantation (Schlegel & Donath 1998) or slight resorption (Piatelli et al. 1999). However, the scaffold, the autogenous dMSCs/PRP using PRP, which is nontoxic, nonimmunoreactive, would be resorbed at a rate commensurate with new bone formation within a few weeks (Yamada et al. in press). This makes it very different from most other grafting materials. Presumably, the dMSCs/PRP left in place might induce bone tissue formation, which then self-organized according to the surrounding environment. In this study, we used a combination of PRP with MSCs and found that the scaffold was regenerated as good mature bone as autogenous bone (PCBM) at 8 weeks histologically. To our knowledge, we were not able to find the biomaterial unlike synthetic materials when used in conjunction with MSCs, and it seemed to be mature bone with the appropriate architecture. Also, guided bone regeneration (GBR) has become not only the most extensively studied technique, but also probably the most popular bone reconstructive procedure in implant dentistry (Von Arx et al. 2001). Although it was demonstrated in several experimental studies that the bone-promoting effect of this membrane technique and indication was reproducible, complications such as membrane exposure or membrane collapse have also been reported (Becker et al. 1990; Arora et al. 1992). With these contaminations, osseointegrated implants are too unsuccessful in terms of bone regeneration in some cases (Haas et al. 2000; Lorenzoni et al. 2002).

In summary, the dMSCs/PRP group had well-formed mature bone and neovascularization, compared with the control (defect only) and PRP groups, as was the same for the PCBM group. Furthermore, the values describing the amount of BIC were significantly different between the PRP, PCBM, dMSCs/PRP, and control groups. Significant differences were also found between the dMSCs/PRP and control groups in bone density. The findings of this experimental study indicate that the use of a mixture of dMSCs/PRP results in good results such as the amount of BIC and bone density comparable with that achieved by autogenous bone, PCBM.

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Résumé

L'étude présente a analysé une corrélation entre l'ostéïntégration d'implants dentaires et un os injectable traité en utilisant des cellules souches mésenchymateuses (MSC) et du plasma riche en plaquette (PRP). Initialement, les dents de la région mandibulaire ont été avulsées et la période de guérison a été d'un mois. Les lésions osseuses des deux côtés de la mandibule ont été préparées avec un trépan. Les lésions ont été traitées avec les matériaux de greffe suivants : PRP, MSC canin (dMSC) et PRP, moelle l'os spongieux en petites particules autogènes (PCBM) et contrôle (lésion seulement). Deux mois plus tard, les animaux ont été évalués et en même temps les implants dentaires ont été placés. Deux mois plus tard, les animaux ont été euthanasiés et des coupes non-décalcifiées ont été évaluées histologiquement et histométriquement. Suivant les observations histologiques, le groupe dMSC/PRP avait de l'os mûr bien formé et une néovascularisation comparé au contrôle et aux groupes PRP, comme pour le groupe PCBM. Un niveau osseux marginal plus important a été observé autour des implants avec PRP, PCBM et dMSC/PRP comparé au contrôle. De plus les valeurs décrivant la quantité de contact os/implant à l'interface os/implant étaient significativement différentes entre les groupes PRP, PCBM, dMSC/PRP et contrôles. Des différences significatives étaient aussi trouvées entre les groupes dMSC/PRP et contrôle en ce qui concernait la densité osseuse. Ces découvertes indiquent que l'utilisation d'un mélange dMSC/PRP résulte en de bons résultats tels que la quantité de contact os-implant et la densité osseuse comparé à ce qui se passe après l'utilisation du PCBM.

Zusammenfassung

Gewebemanipulierte injizierbare Knochenregenerate für osseointegrierte dentale Implantate

Die vorliegende Studie untersuchte eine Korrelation zwischen der Osseointegration bei dentalen Implantaten und einem injizierbaren gewebemanipulierten Knochenregenerat aus mesenchymalen Stammzellen (MSCs) und plättchenreichem Plasma (PRP). Zuerst wurden die Zähne im Unterkiefer extrahiert. Darauf folgte eine Heilungsperiode von 1 Monat. Auf beiden Seiten der Unterkiefer wurden mit einer Hohlfräse Knochendefekte präpariert. Die Defekte wurden mit folgenden Transplantatmaterialien aufgefüllt: PRP, Hunde MSCs (dMSCs) und PRP, autologer zerkleinerter Knochen und Knochenmark (PCBM) und kein Füllmaterial (Kontrolle). Zwei Monate später wurden die Tiere histologisch untersucht und es wurden dentale Implantate eingesetzt. Nach weiteren zwei Monaten wurden die Tiere geopfert und nicht entkalkte Schnitte wurden histologisch und histometrisch ausgewertet. Gemäss der histologischen Beobachtungen zeigten die dMSCs/PRP Gruppe im Vergleich zur Kontrollgruppe und zur PRP Gruppe gut ausgebildeten reifen Knochen und neue Gefässe. Das Selbe galt für die PCBM Gruppe. Bei den Implantaten mit PRP, PCBM und dMSCs/PRP konnte im Vergleich zur Kontrollgruppe ein höheres marginales Knocheniveau beobachtet werden. Zudem waren die Werte, welche das Ausmass an Knochen-Implantat-Kontakt an der Kochen/Implantat Berührungsfläche beschreiben, zwischen den PRP, PCBM, dMSCs/PRP und der Kontrollgruppe signifikant verschieden. Signifikante Unterschiede wurden auch bezüglich Knochendichte zwischen der dMSCs/PRP und der Kontrollgruppe gefunden. Die Ergebnisse dieser experimentellen Studie zeigen, dass die Anwendung eines Gemisches aus dMSCs/PRP zu guten Resultaten bezüglich Ausmass an Knochen-Implantat-Kontakt und Knochendichte führt. Die erzielten Resultate sind mit den mit PCBM erreichten vergleichbar.

Resumen

El presente estudio investigó la correlación entre osteointegración en implantes dentales y un tejido-elaborado óseo inyectable, usando células madre mesenquimales (MSCs) y plasma rico en plaquetas (PRP). Inicialmente, se extrajeron los dientes de la mandíbula y el periodo de cicatrización fue de un mes. Se prepararon defectos óseos en ambos lados de la mandíbula con una fresa trépano. Los defectos se implantaron con material de injerto de la siguiente manera: PRP, MSCs de perro (dMSCs) y PRP, partículas de hueso esponjoso y médula autógenos (PCBM), y control (defecto solo). Dos meses mas tarde, los animales se evaluaron por histología, al mismo tiempo se instalaron implantes dentales. Dos meses mas tarde se sacrificaron los animales y se evaluaron secciones no descalcificadas histológica e histometricamente. De acuerdo con las observaciones histológicas, el grupo dMSCs/PRP había formado bien un hueso maduro y una neovascularización, comparado con el control (defecto solo) y grupos PRP, también fue igual para el grupo PCBM. Se observó un nivel óseo marginal mas alto alrededor de los implantes con PRP, PCBM, y dMSCs/PRP comparados con el control. Mas aun, los valores que describen la cantidad de contacto hueso-implante en la interfase hueso/implante fueron significativamente diferentes entre los grupos PRP, PCBM, dMSCs/PRP, y de control en la densidad ósea. Estos hallazgos de este estudio experimental indican que el uso de una mezcla de dMSCs/PRP resulta en buenos resultados tales como la cantidad de contacto hueso-implante y densidad ósea comparable a aquella lograda por PCBM.

要旨

本研究は、間葉系幹細胞（ＭＳＣｓ）と多血小板血漿（ＰＲＰ）を用いて、組織工学的手法による注入型培養骨と歯牙インプラントとの骨性結合の相関性を調べた。まず下顎の歯牙を抜去し、１ヶ月の治癒期間をおいた後に、トレファン・バーで下顎両側に骨欠損を形成した。欠損部に次の移植材料を充填した：ＰＲＰ、犬のＭＳＣｓ（ｄＭＳＣｓ）とＰＲＰ、粒子状の自家海綿骨と骨髄（ＰＣＢＭ）及び対照（欠損のまま）。２ヵ月後に動物の組織学的評価を行うと同時に、歯牙インプラントを埋入した。さらに２ヵ月後に動物を屠殺し、非脱灰組織切片を組織学及び組織形態測定法によって評価した。組織学的観察によると、対照群（欠損のまま）とＰＲＰ群に比べ、ｄＭＳＣｓ/ＰＲＰ群では成熟骨と新生血管がよく形成されており、ＰＣＢＭ群と同様であった。ＰＲＰ群、ＰＣＢＭ群及びｄＭＳＣｓ/ＰＲＰ群では、インプラント周囲の辺縁骨レベルは対照群より高かった。さらに骨/インプラントの界面における骨ーインプラントの接触率には、ＰＲＰ、ＰＣＢＭ、ｄＭＳＣｓ/ＰＲＰと対照群の間に有意差があった。さらに骨密度にもＰＲＰ、ＰＣＢＭ、ｄＭＳＣｓ/ＰＲＰと対照群の間に有意差がみとめられた。本実験研究の所見は、ｄＭＳＣｓ/ＰＲＰの混合物の使用は、骨ーインプラントの接触率、骨密度などの観点からしても、ＰＣＢＭに匹敵する良い結果をもたらすことを示唆している。

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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

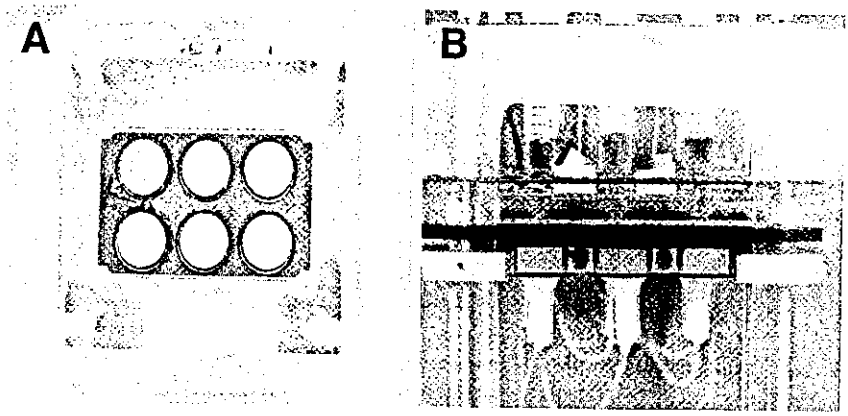

FIG. 1. The apparatus for low-intensity US. **(A)** Upper view; **(B)** side view. In **(B)** polypropylene tubes containing cell pellets are placed on a transducer.

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

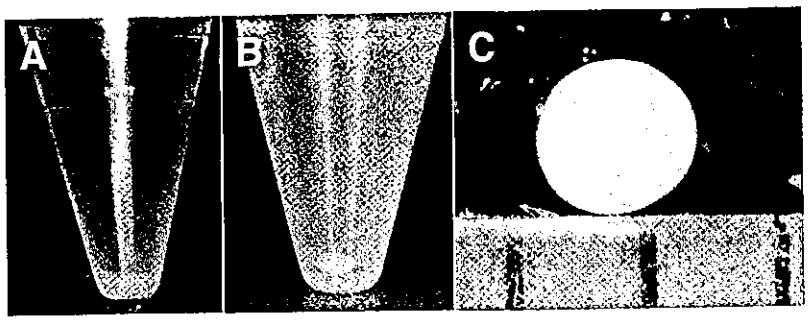

FIG. 2. Pellet culture. (**A**) Cell pellet of hMSCs immediately after centrifugation appears quadrilateral. (**B**). The cell pellet after 24 h of incubation appears oval. (**C**) Stereomacroscopic view of a pellet on day 10. The pellet appears spherical. Scale indicates 1 mm.

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,

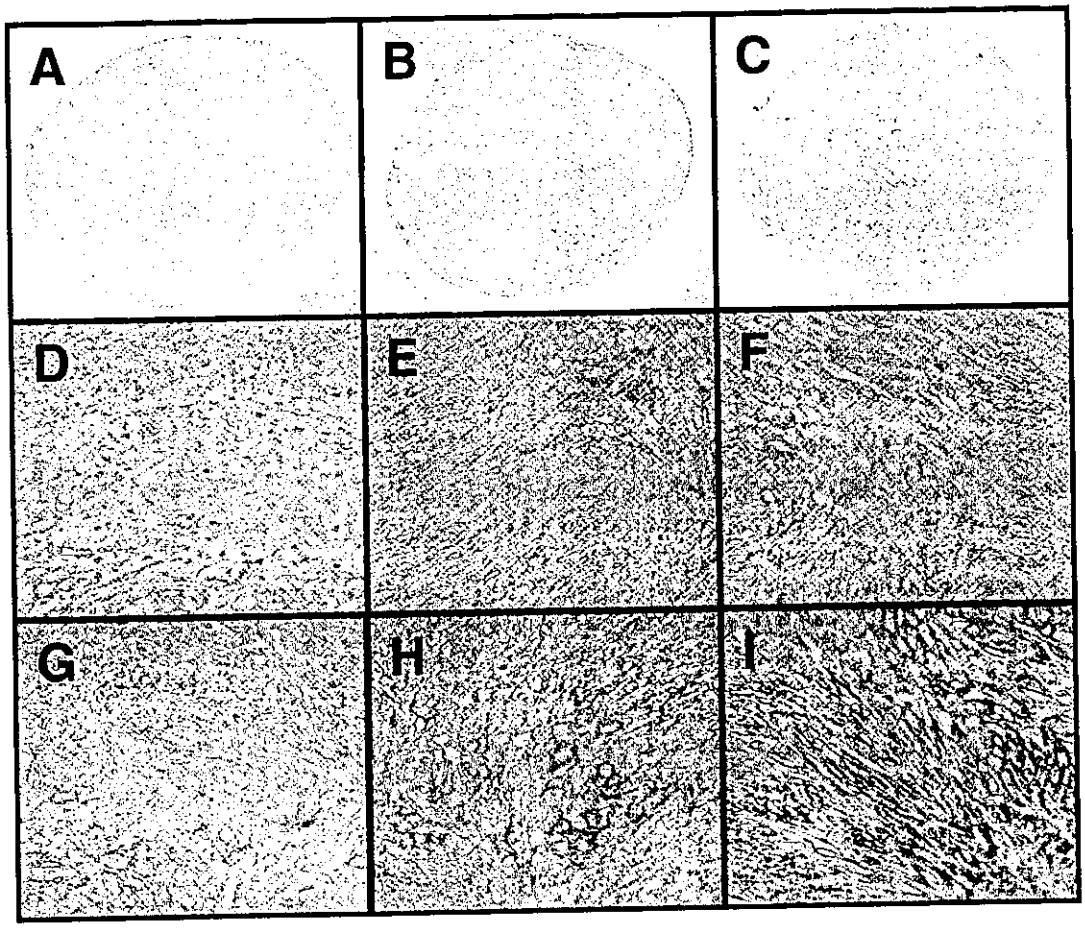

FIG. 3. Patterns of alcian blue staining (**A–F**) and immunostaining for aggrecan (**G–I**). Cell pellets without treatment (**A**, **D**, and **G**), treated with TGF-β (**B**, **E**, and **H**), those treated with both TGF-β and US (**C**, **F**, and **I**) are shown. Original magnification: (**A–C**)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

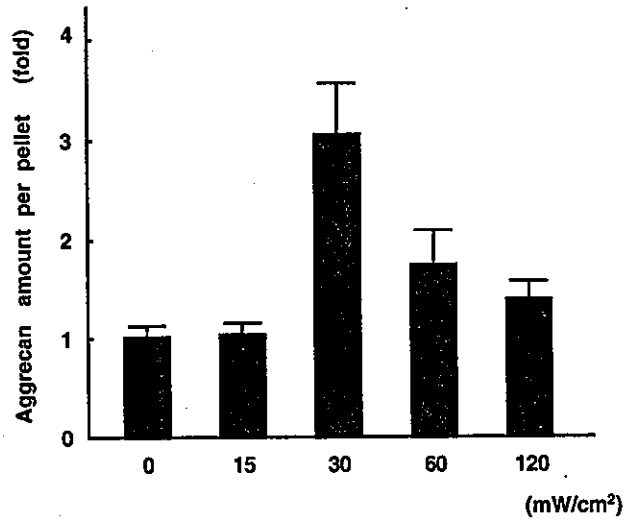

FIG. 4. Effects of US at different intensities on aggrecan synthesis of cells in pellets in the presence of TGF-β. Aggrecan amount at 30 mW/cm^2 is greater than for any other group ($p < 0.01$). The same results were obtained from two independent experiments.

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

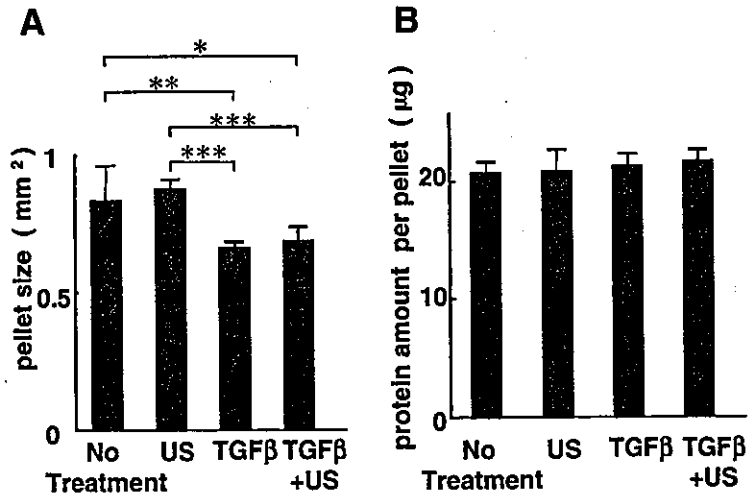

FIG. 5. The size of pellets (A) and total protein amount (B). (A) TGF-β treatment reduces pellet sizes but application of US has little effect (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). (B) There is no significant difference in protein amount among all groups. (No treatment, nontreated; US, ultrasound 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.

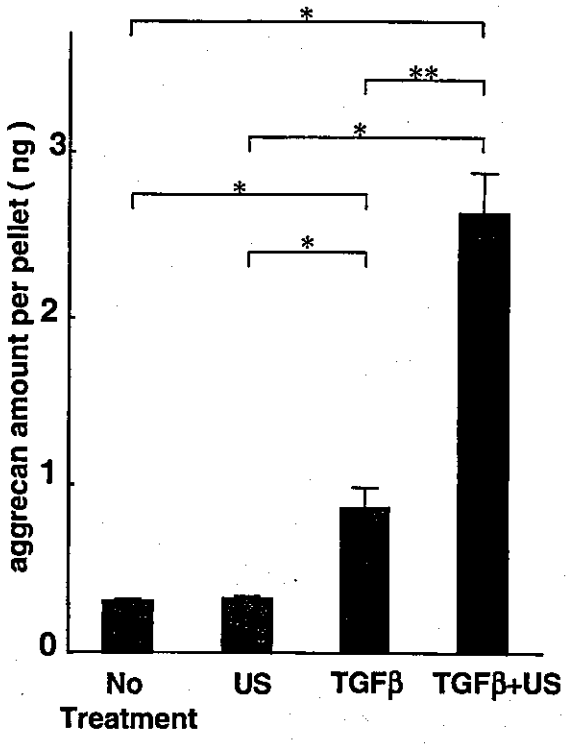

FIG. 6. Effects of TGF-β and US on aggrecan synthesis. The amount of aggrecan in induced groups (TGF-β and TGF-β + US) is significantly larger than that of non-TGF-β-treated groups (no treatment and US). The amount of aggrecan in the TGF-β + US group is increased by 3.06-fold compared with the TGF-β 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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