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F．健康危険情報

本研究を通じて健康に危険をもたらす事項を認めていない。

G．知的財産権の出願・登録状況

出願・登録ともなし。

研究成果の刊行に関する一覧表

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1．特許取得

特許出願

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特許申請中

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「骨又は歯周組織形成用の組成物」

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Inventors: Sugimura Hiroyuki, Takai Osamu, Gomez-Vega, Jose Manual

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発明者　三浦佳子、上坊史子、小林一清

出願人　小林一清

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発明者　三浦佳子、池田高康、小林一清

出願人　小林一清

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Bone regeneration following injection of mesenchymal stem cells and fibrin glue with a biodegradable scaffold

Yoichi Yamada[1], Jae Seong Boo[1], Ryotaro Ozawa[1], Tetsuro Nagasaka[2], Yasuhiro Okazaki[1], Ken-ichiro Hata[3], Minoru Ueda[1]

[1] *Department of Oral and Maxillofacial Surgery (Head: Prof. Dr. Ueda) Japan;* [2] *Laboratory Medicine (Head: Prof. Dr. Nakashima), Nagoya University, Graduate School of Medicine, Nagoya, Japan;* [3] *Department of Tissue Engineering (Head: Assistant. Prof. Dr. Hata), Nagoya University School of Medicine, Nagoya, Japan*

SUMMARY. Aim: **The purpose of this study was to determine whether a combination of fibrin glue, β-tricalcium phosphate as a biodegradable (β-TCP) and mesenchymal stem cells would provide three-dimensional templates for bone growth resulting in new bone formation at heterotopic sites in the rat with plasticity.** Material and Methods: **Growing stem cells and developing matrices, explanted from the rat femur, were fragmented and mixed with fibrin glue in a syringe. The cells/β-TCP fibrin glue admixtures were injected into the subcutaneous space on the dorsum of the rat.** Results: **Eight weeks after implantation, gross morphology revealed a pearly opalescence and firm consistency. Histological inspections showed newly formed bone structures in all admixtures, but none in the control groups when only fibrin glue and β-TCP were injected. Osteopontin, a protein important in bone development, was identified by using antibodies in all cells/β-TCP fibrin glue admixtures.** Conclusion: **Mesenchymal stem cells/β-TCP fibrin glue admixtures can result in successful bone formation. This technique holds the promise of a minimally invasive means of generating autogenous bone to correct or reconstruct bony defects. © 2003 European Association for Cranio-Maxillofacial Surgery.**

INTRODUCTION

Tissue engineering of bone regeneration by autogenous cell transplantation is one of the most promising treatment concepts being developed, as it eliminates the problems of donor site morbidity for autologous grafts, the immunogenicity of allogenic grafts, and loosening of alloplastic implants (*Vacanti* et al., 1988, 1998). *Langer* and *Vacanti* (1993) have called it *tissue engineering*, involving the morphogenesis of new tissues formed from isolated cells and biocompatible polymers. In *tissue engineering*, man-made polymers are utilized such as hydroxyapatite (HA), polyglycolide (PGA), polylactides (PLLA, PDLA), and polycaprolactone (PCL) as scaffolds because they had enough biocompatibility and biodegradability to fabricate artifical cartilage or bone. On the other hand, mesenchymal stem cells are thought to be multipotent cells that can replicate as undifferentiated cells, and have the potential to differentiate and produce mesenchymal tissues like bone, cartilage, fat, tendon, muscle, and marrow stroma (*Pittenger* et al., 1999). They have received widespread attention because of their potential in tissue engineering.

Fibrin glue, a composite of fibrinogen and thrombin, is a physiologically relevant matrix whose principal component, fibrin, has fundamental roles in the process of blood clotting and wound healing. It is a potentially suitable biological vehicle for cell transplantation since it has proven biocompatibility, biodegradability, and binding capacity to cells (*Keller* et al., 1985). Fibrin-stabilizing factor XIII, contained in fibrin glue, favours migration of undifferentiated mesenchymal stem cells (MSCs) on the highly cross-linked structure of the glue, and it enhances proliferation of these cells (*Marktl* and *Rudas*, 1974; *Kasai* et al., 1983).

In conventional procedures, the transplantation of bone requires invasive procedures such as skin or mucosal incisions and reflection of the periosteum. An injection, leading to additions of autogenous cartilage or bone to the craniofacial skeleton would minimize surgical trauma and eliminate the need for allografts or alloplastic prostheses. If one could transplant by means of injection and onlay of large numbers of isolated cells by adapting the techniques described by *Vacanti* and coworkers (*Vacanti* et al., 1988, 1990; *Langer* and *Vacanti*, 1993), one could augment the craniofacial osseocartilaginous skeleton with autogenous tissue without extensive surgery. Furthermore, successfully implanting isolated cells would create the potential for tissue culture augmentation of osteoblasts. Unlike the porous ceramics currently used to create a cell–ceramics construct, a liquid support matrix that polymerizes to a gel would be shaped more easily and moulded for custom reconstruction or augmentation. In addition, an injection would be much less invasive than open implantation. For all these reasons, fibrin glue plus

β-tricalcium phosphate (β-TCP) were investigated as a means of delivering large numbers of isolated stem cells to promote bone formation.

The aim of this study was to determine whether a combination of fibrin glue plus β-TCP and MSCs could act as an injectable osteogenic bone substitute leading to new bone formation.

MATERIAL AND METHODS

Material

The β-TCP was supplied by NGK SPARK PLUG CO., LTD., Nagoya, Japan. The solid and porous components of the microstructure were completely interconnected. The average pore diameter was 200–400 μm the interconnection average was 60 μm and the average void volume was 90%. These ceramics were disc-shaped blocks, 5 mm in diameter and 4 mm thick.

Cell isolation, cultivation and matrix loading

MSCs' isolation and culture expansion was performed according to previously published methods (*Maniatopoulos* et al., 1988; *Dennis* et al., 1992; *Yoshikawa* et al., 1996). Briefly, two male 7-week-old Fisher F344 rats were sacrificed by pentobarbital overdose. Both ends of the femurs of each rat were removed at the level of the epiphysis, and marrow plugs were flushed out from the epiphysis using 10 ml of a culture medium expelled from a syringe through a 23-gauge needle (Fig. 1). The culture medium consisted of a minimum essential medium (MEM) containing 15% fetal bovine serum and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B, Sigma-Aldrich, Poole, UK). The released cells were collected in two tissue culture flasks ($7.5 \times 10\,cm^2$) containing 15 ml medium. The medium was changed after the first 24 h to remove non-adherent cells. Subsequently, the medium was removed 3 times a week. Cultures were maintained in a humidified atmosphere of 95% air with 5% CO_2 at 37°C.

After 10 days in primary culture, bone-marrow-derived MSCs were released from their culture substratum using 0.05% trypsin–EDTA. The cells were concentrated by a centrifuge at 900 rpm for 5 min at room temperature, resulting in 10×10^6 cells/ml. The β-TCP blocks were soaked in a cell suspension (10×10^6 cells/ml) for 2 h in a CO_2 incubator. After 2 h of incubation, each block was transferred into a 24-well plate for subcultures. One block was subcultured in one well with 1 ml of the

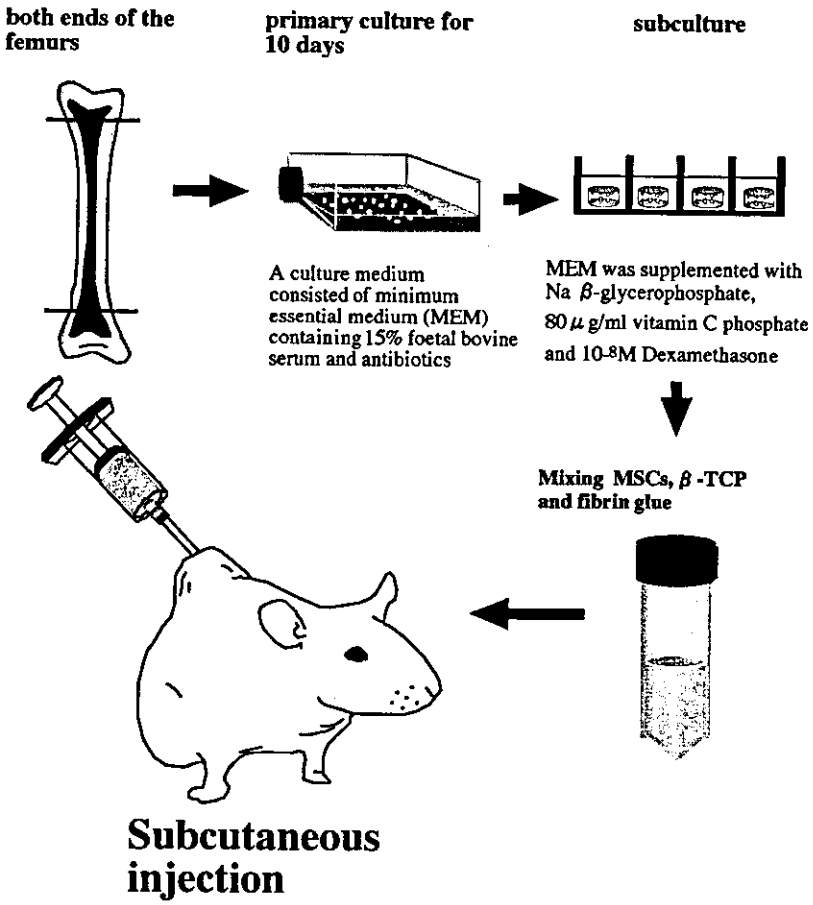

Fig. 1 – Schema of experimental protocol using MSCs/β-TCP-fibrin glue admixtures.

culture medium supplemented with 10 m mol Na β-glycerophosphate, 80 μg/ml vitamin C phosphate (L-ascorbic acid phosphate magnesium salt *n*-hydrate, $C_6H_6O_6$) and 10^{-8} mol dexamethason (Sigma-Aldrich, Poole, UK). The subcultures were maintained for 20 days.

Preparation of fibrin glue and coagulation time

Pasteurized fibrin glue (Bolheal, the ChemoSero-Therapeutic Reseach Institute, Kumamoto, Japan) was formed by mixing two separate solutions, A and B. Solution A consisted of fibrinogen (80 mg/ml) and fibrin-stabilizing factor XIII (75 units/ml) dissolved in 1 ml of plasmin inhibitor aprotinin (1000 kIE/ml). Solution B contained thrombin (250 units) dissolved in 1 ml of 40 μM $CaCl_2$. Solutions A and B were mixed in a 1:1 (vol/vol) ratio. The clotting reaction between A and B produced a semirigid three-dimensional gel at room temparature. We also investigated the correlation between coagulation time and thrombin dilution by checking the time required to confirm 'white muddiness' by mixing fibrinogen with thrombin on a glass plate (Table 1).

Injection of MSCs/β-TCP fibrin glue admixtures and harvesting

The β-TCP loaded with MSCs after 20 days of subculture (MSCs/β-TCP composite) was reduced to small lumps in a sterilized 5 ml syringe and resuspended in solution A. Solution A containing MSCs/β-TCP composite and solution B [1:1 (vol/vol) ratio] were placed in the barrel of the 5 ml sterilized syringe and mixed by inverting the syringe repeatedly.

Syngeneic 7-week-old male Fischer rats were anaesthetized by intramuscular injection of pentobarbital (nembutal 3.5 mg/100 g body weight) following light ether inhalation. The skin was prepared with povidone–iodine. An 18 gauge needle was inserted into the loose connective space between the panniculus carnosus and the muscle fascia. A total of 0.5 ml of the MSCs/β-TCP fibrin glue admixture was injected into the subcutaneous space (Fig. 1).

From preliminary studies (*Boo* et al., 2002), injection of $\sim 10 \times 10^6$ pure cells/ml in culture medium, or MSCs plus β-TCP (not subculture) produced no response over 8 weeks. These experimental groups were excluded from further study. On this basis, the matrix alone was supposed to fail to produce tissue development and this protocol was likewise excluded from additional investigations. The animals were assigned into one experimental group (injected with MSCs/β-TCP fibrin glue admixtures) and one control group (injected with the fibrin glue β-TCP admixture). All animals received humane care in compliance with the 'Guidelines for the Care and Use of Laboratory Animals' published by the National Institutes of Health (NIH Publication no. 85–23, revised 1985). They were analysed 2 ($n=5$), 4 ($n=5$), and 8 ($n=5$) weeks after injection. Sacrifice was by an overdose of ether inhalation.

Table 1 – Correlation between coagulation time and thrombine dilution

Dilution multiple	(activity (U/ml))	Coagulation time
1	(250)	10
2	(125)	15
5	(50)	25
10	(25)	40
20	(12.5)	60
50	(5.0)	120
100	(2.5)	210

Histologic analysis and mineral detection

Each implant was excised 2, 4 or 8 weeks after injection, photographed and assessed for opacity and formation of any bone-like tissues radiographically. The specimens were fixed in 10% buffered formalin, decalcified (K-CX, Falma Co., Tokyo), and stained with hematoxylin and eosin. These specimens were examined under a light microscope and analysed by a pathologist blinded to the identity of each specimen. He was asked to determine the presence or absence of bone formation. All sections were reviewed by one of the authors (YY), who agreed with the pathologist in all cases.

Immunohistochemistry was performed on 4 μm sections of formalin-fixed, paraffin-embedded specimens using a streptavidin technique (HISTOFINE SAB-PO (M) kit, Nichirei, Tokyo, Japan). The primary antibody was a murine monoclonal antibody raised against rat osteopontin.

Biochemical analysis

The implants were harvested and used for biochemical analysis after 2, 4 or 8 weeks. The osteocalcin content was determined as reported previously (*Yoshikawa* et al., 1992). Briefly, each composite was immediately crushed, homogenized with 0.5 ml of 0.2% Nonident P40 containing 1 mM $MgCl_2$, and centrifuged at 13 000 rpm for 10 min at 4°C. Osteocalcin was extracted from the sediment after extraction of 0.2% Nonidet P40 in 10 ml of 20% formic acid for 2 weeks at 4°C. An aliquot (2 ml) of the formic acid extract was then applied to an Ampure™ SA colum (Amersham, Japan) and eluted with 10% formic acid. Protein fractions were collected, lyophilized, and prepared for an osteocalcin radioimmunoassay as previously described (*Yoshikawa* et al., 1992; *Dohi* et al., 1992).

Statistical analysis

Statistical analysis was performed by paired Student's *t*-test. Significantly different findings were indicated at $p<0.05$.

RESULTS

Macroscopic observations of the injected implants

After injection of MSCs/β-TCP fibrin glue admixtures into rats, subcutaneous nodules formed by 8 weeks and these were hard and resisted compression. The admixture nodules had well-defined margins upon dissection from subcutaneous tissue (Fig. 2). Injection of only fibrin glue β-TCP failed to form nodules. Implants after 8 weeks were well vascularized, more so than at earlier times (4 weeks). At no time was evidence of malignant growth found in any of the specimens. On the other hand, control implants of injected fibrin glue β-TCP admixtures had only a shiny appearance and an elastic consistency at 2 weeks, which continued until 8 weeks. The implant margin was not detectable at 8 weeks and the nodules remained flat (Fig. 2).

Mineral detection of the implants

Determination of mineral formation was made by using radiographs of the implant and an assay for osteocalcin content. Radiographic surveys of the implants showed that calcification occurred in the MSCs/β-TCP fibrin glue admixtures 8 weeks after implantation. Only a few of the control implants of injected fibrin glue β-TCP admixtures demonstrated mineral deposition at any time (Fig. 3).

The radiographic appearance of the implants was consistent with the osteocalcin content findings. The mean calcium content in vivo increased with the duration of implantation up to 8 weeks. The differences between these specimens when compared with only fibrin glue β-TCP admixture controls were statistically significant (Fig. 4).

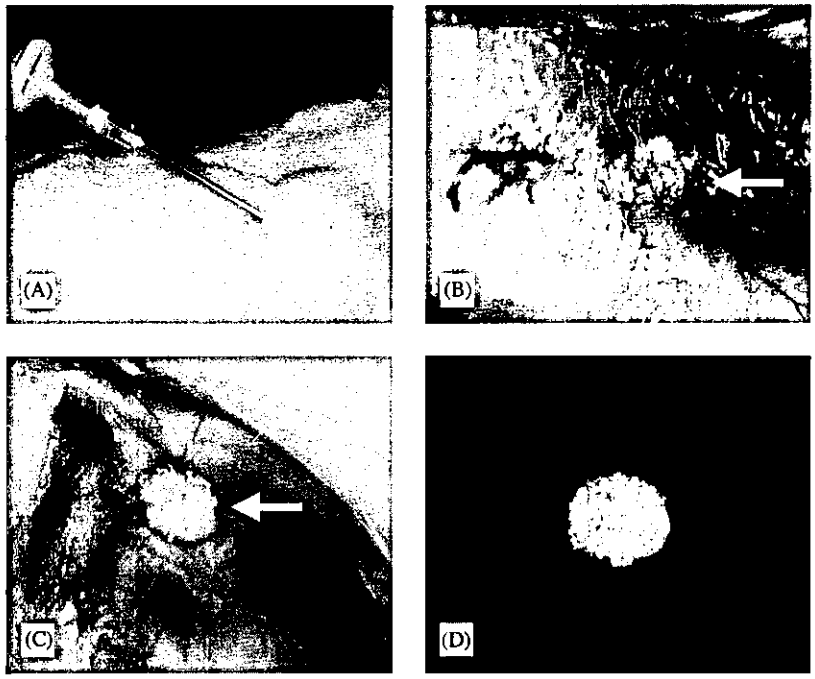

Fig. 2 – Macroscopic observations of injected material. (A) MSCs/β-TCP fibrin glue admixture injected from an 18 gauge needle. (B) Site of injection of only fibrin glue β-TCP after 8 weeks. Note some residual material that could not be distinguished from the subcutaneous layer. (C) Implant 8 weeks after injection of the MSCs/β-TCP fibrin glue admixture. A well-defined nodule is present. (D) Specimen after removal of a thin soft-tissue capsule.

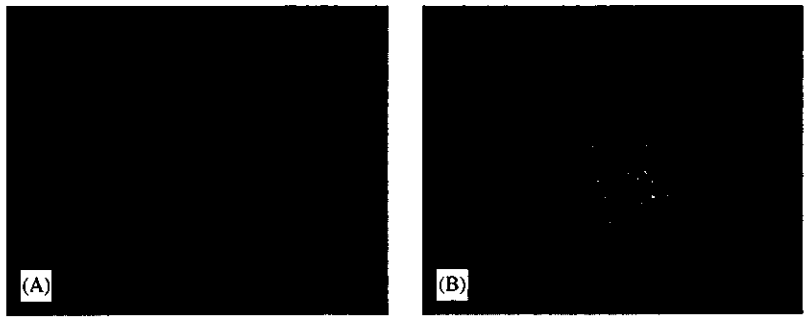

Fig. 3 – Radiography of representative implants at 8 weeks. (A) Little evidence of the presence of minerals in fibrin glue β-TCP admixture in 8 weeks control animal. (B) Radiograph of an experimental sample with mineralized material.

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

YOICHI YAMADA, D.D.S., Ph.D.,[1] MINORU UEDA, D.D.S., Ph.D.,[2]
TAKAHITO NAIKI, D.D.S.,[2] MAKOTO TAKAHASHI, D.D.S.,[2]
KEN-ICHIRO HATA, D.D.S., Ph.D.,[1] and TETSURO NAGASAKA, M.D., Ph.D.[3]

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

[1]Center for Genetic and Regenerative Medicine, [2]Department of Oral and Maxillofacial Surgery, and [3]Department of Laboratory Medicine, Nagoya University Graduate School of Medicine, Nagoya, Japan.

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.

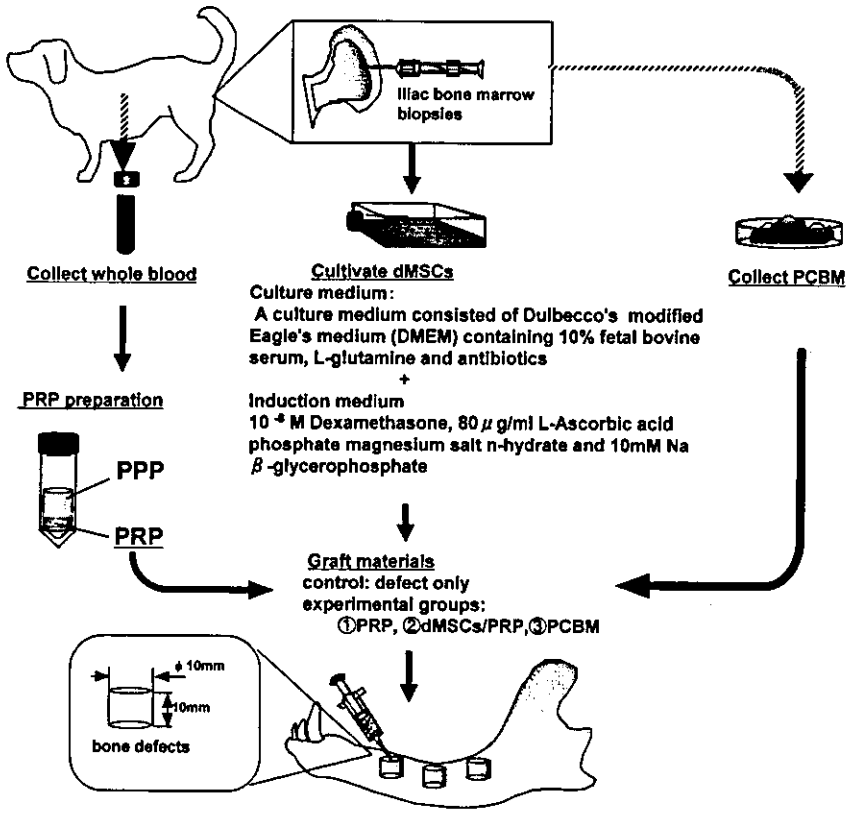

FIG. 1. Scheme of experimental protocol.

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.

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.

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).

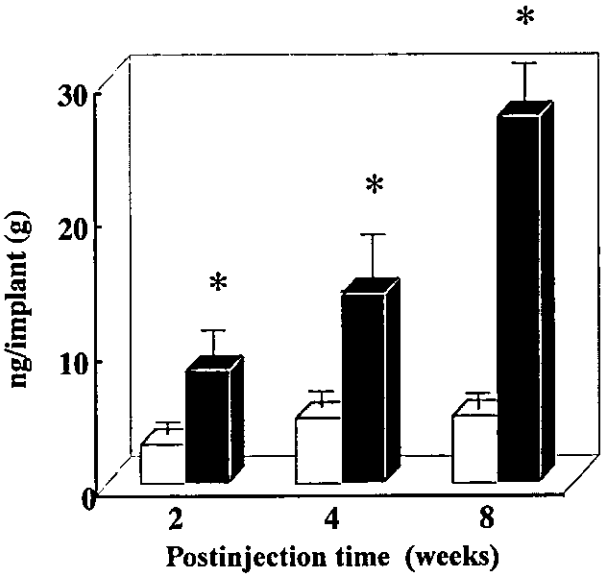

Fig. 4 – Osteocalcin content for mineral detection. Experimental samples (MSCs/β-TCP fibrin glue admixture ■) and control (fibrin glue β-TCP admixture □) harvested at 2, 4, and 8 weeks after injection. Each point represents the mean value of osteocalcin content ± SED ($n = 5$ at each point). Asterisks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

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

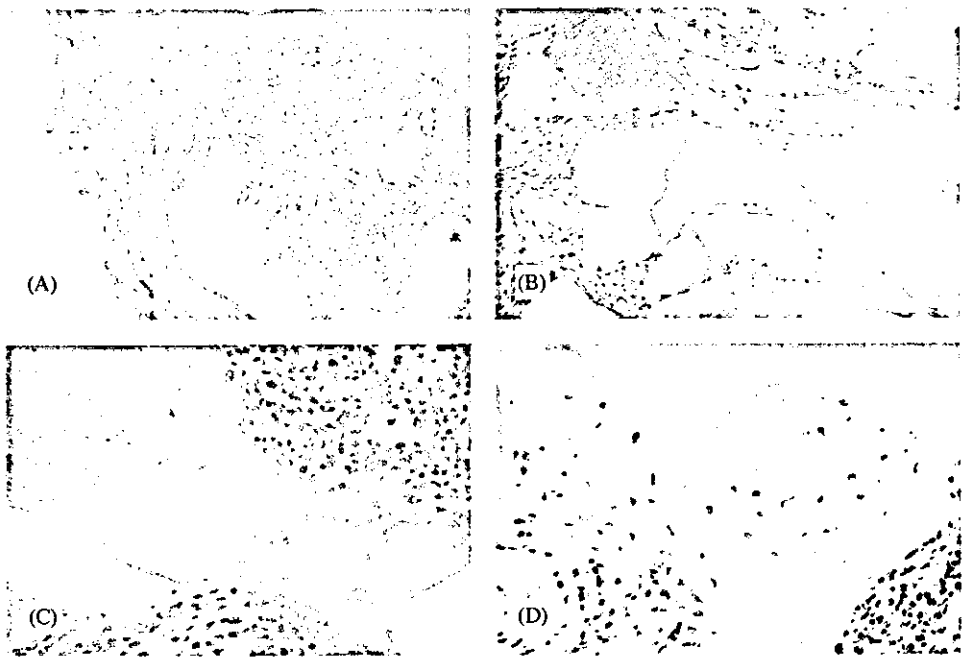

Fig. 5 – Histological comparison of the implants developed from MSCs/β-TCP fibrin glue admixtures or fibrin glue β-TCP admixtures. Sections of representative implants from the control group (fibrin glue β-TCP admixture, A), and MSCs/β-TCP fibrin glue admixture harvested at 2 (B), 4 (C), and 8 (D) weeks.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