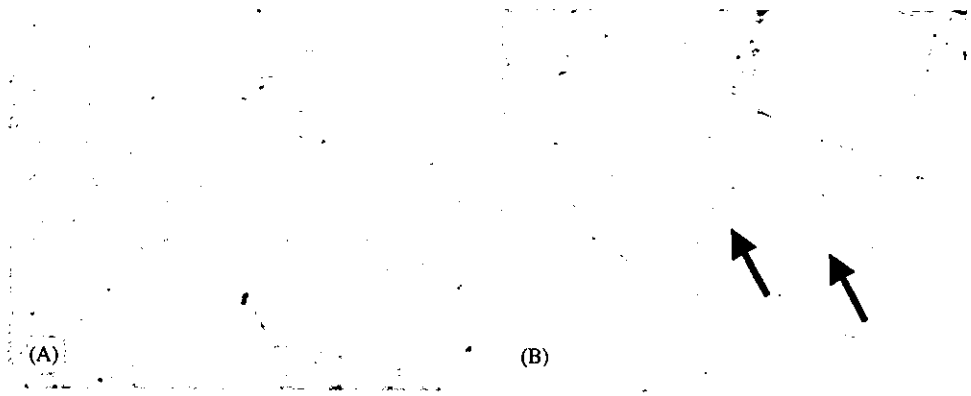

Fig. 6 – Osteopontin antibody-stained sections of the implants. Representative implants from the control group (fibrin glue β-TCP admixture, A), and MSCs/β-TCP fibrin glue admixture (B) harvested at 8 weeks. Arrows point to osteocytes. Original magnification, × 200.

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

DISCUSSION

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

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

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

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

CONCLUSION

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

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Making bone: implant insertion into tissue-engineered bone for maxillary sinus floor augmentation—a preliminary report

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SUMMARY. **Autologous, allogenic and alloplastic materials for bony reconstruction in the cranio-maxillofacial area have many drawbacks thus stimulating the on-going search for new (bio-)materials. Whereas cultivated skin and mucosa are already in clinical routine use in head and neck reconstruction, so far there has been no successful clinical application to the best of our knowledge of periosteum-derived, tissue-engineered bone for augmentation of the edentulous posterior maxilla. In a pilot study, augmentation of the posterior maxilla was carried out using a bone matrix derived from mandibular periosteal cells on a polymer fleece. This paper demonstrates fabrication of the matrix, clinical application, and the histological results in two patients. The results suggest that periosteum-derived osteoblasts on a suitable matrix form lamellar bone within 4 months which allows reliable implant insertion.**

INTRODUCTION

Augmentation procedures in oral and cranio-maxillofacial surgery prior to implant insertion are most frequently carried out with auto- or allografts or composite material (*Jensen* and *Sennerby*, 1998; *Lorenzetti* et al., 1998; *Valentini* et al., 1998; *Yildirim* et al., 2001. Donor site morbidity must be considered when using autologous grafts. However, there is a limited amount of available intraoral bone suitable for harvesting and grafting. Some other autologous graft sources are unsuitable for reconstruction of the alveolus when implants are planned owing to poor tissue quality and/or quantity and, possibly, the difficult sculpting necessary.

Alloplastic materials also have drawbacks, particularly in ischaemic areas.

Tissue-engineering procedures for bony augmentations of the maxilla offer significant advantages when compared with conventional grafts, as there is minimal or no donor site morbidity. Ideally, these procedures are undertaken in out-patient conditions under local anaesthesia, using exclusively autologous material with bone-forming capacity.

In the field of bony tissue-engineering, naturally derived and synthetic polymers, composites, ceramics, bone morphogenetic proteins (BMP), as well as cellular systems are all being studied (*Sittinger* et al., 1996; *Burg* et al., 2000). In addition to in vitro investigations, in vivo tissue-engineering approaches for bone repair are currently limited to animal research. (*Yoshikawa* and *Ohgushi*, 1999; *Tamura* et al., 2001; *Ueno* et al., 2001; *Yamanouchi* et al., 2001; *Yamagiwa* et al., 2001). Preconditions for the clinical applications described below have been developed by *Sittinger* et al. (1996).

MATERIAL AND METHODS

In this clinical pilot study two patients have been included for augmentation of the edentulous atrophic posterior maxillary alveolus with tissue-engineered bone prior to implant insertion. The study was approved by the local Ethics Committee of the University of Freiburg (ZERM). The patients showed Cawood class 4 and 5 atrophy of the posterior maxilla (*Cawood* and *Howell*, 1988).

Periosteal tissue from the lateral cortex of the mandibular angle was used to isolate periosteal cells. The periosteum was digested with collagenase CLSII (*Clostridium histolyticum*) (333 U/ml) (Biochrom, Berlin, Germany) in a 1 : 1 mixture of DMEM/Ham's F-12 (Dulbecco's modified Eagle's medium; Invitrogen, Karlsruhe, Germany). The resulting cell suspension was washed three times with phosphate-buffered saline (PBS; Invitrogen GmbH, Karlsruhe, Germany). Cell number and viability were determined by cell counts using a haemocytometer and trypan blue dye exclusion. Cell viability was 90% before seeding. The cells were re-suspended in DMEM/Ham's F-12 (1 : 1) supplemented with 10% autologous serum, placed into cell culture flasks and cultured at 37°C with 3.5% CO_2 and 95% humidified air. The medium was replaced every 2 days. Reaching 70% confluence, cells were trypsinized (0.02% trypsin, 0.02% EDTA in PBS) for 5 min, and

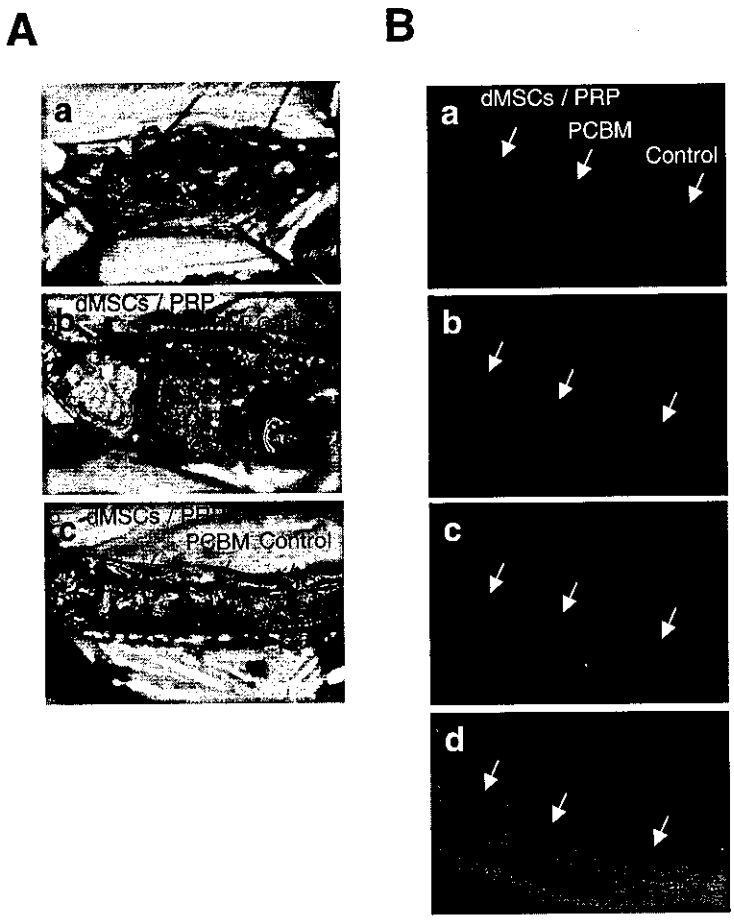

FIG. 2. (**A**) Macroscopic observations for bone regeneration: (a) The experimental design in dog mandible, prepared with a trephine bar 10 mm in diameter. (b) Implanted materials in bone defects. (c) Bone regeneration with dMSCs/PRP, PCBM, and control groups at 8 weeks. Bone regeneration by dMSCs/PRP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

DISCUSSION

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

TABLE 1. HISTOMORPHOLOGY DATA[a]

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

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

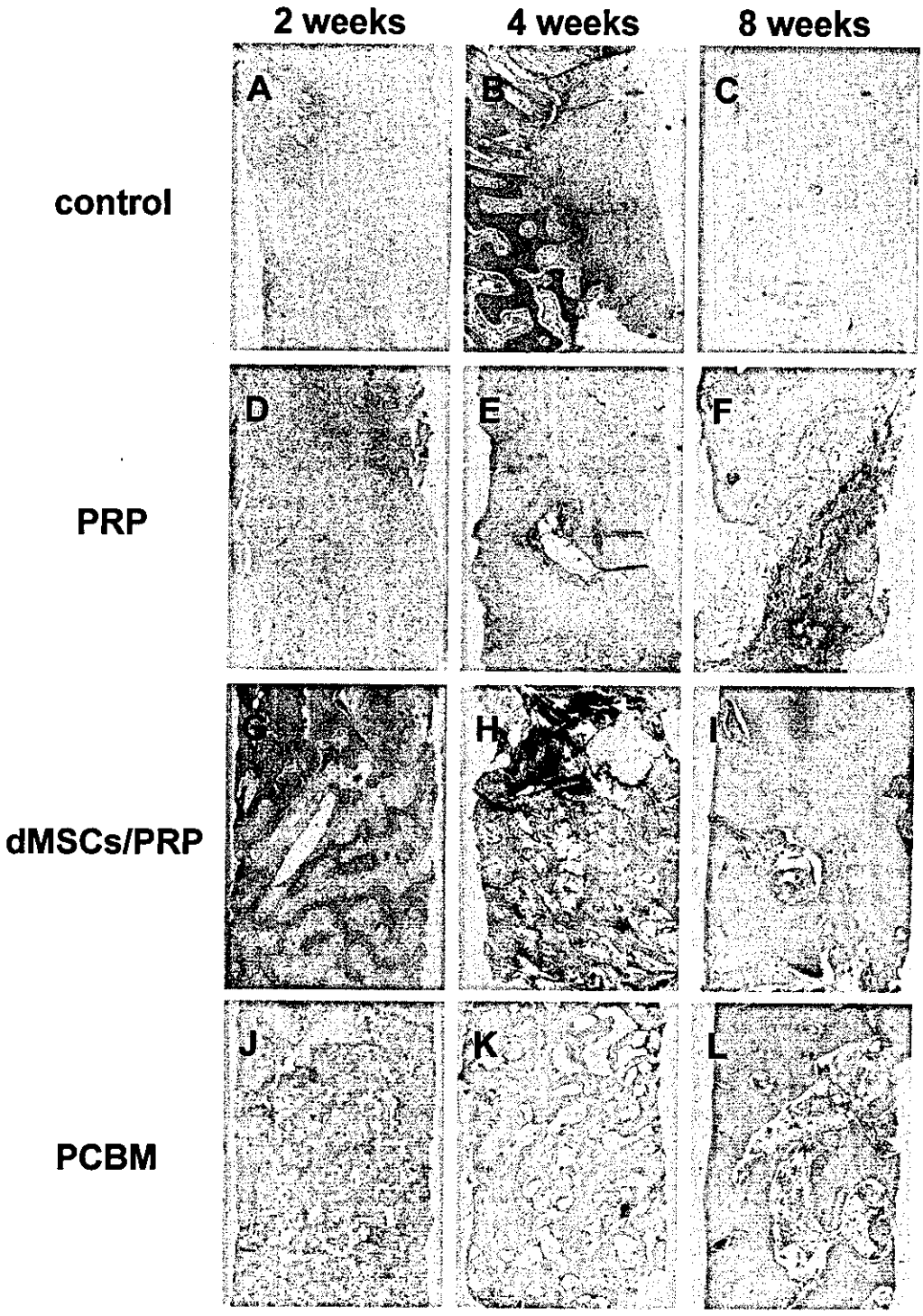

FIG. 3. (A–L) Histologic evaluation of control, PRP, PCBM, and dMSCs/PRP implantations at 2, 4, and 8 weeks: lower magnification. Sections of representative implants are shown from the respective groups. The sections were stained with hematoxylin and eosin. Original magnification: (A–L) ×40. (A) Two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

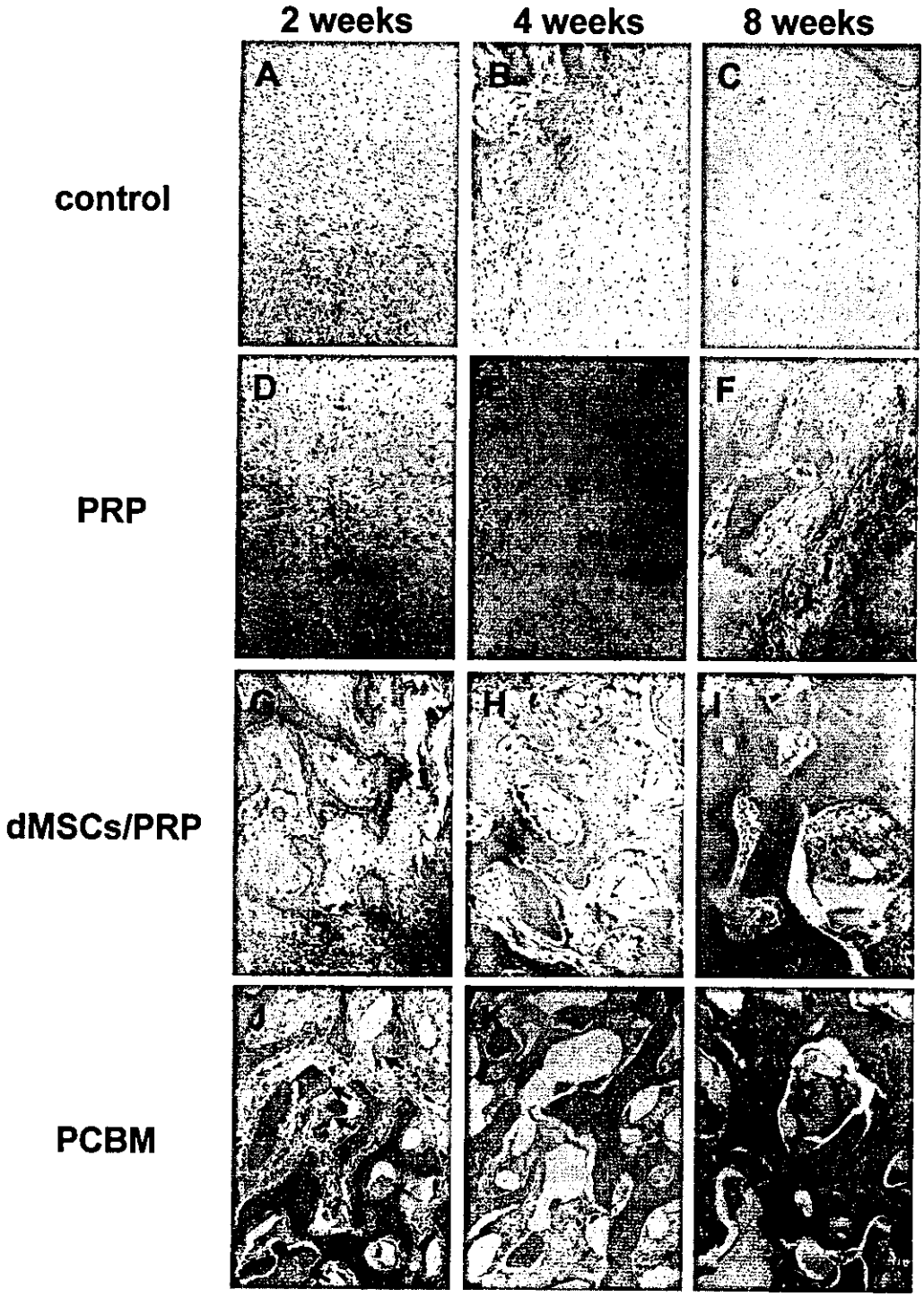

FIG. 4. Histologic evaluation of control, PRP, PCBM, and dMSCs/PRP implantations at 2, 4, and 8 weeks: higher magnification. Sections of representative implants are shown from the respective groups. The sections were stained with hematoxylin and eosin. Original magnification: (**A–L**) ×200. (**A**)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

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

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

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

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

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

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

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

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Translational Research for Injectable Tissue-Engineered Bone Regeneration Using Mesenchymal Stem Cells and Platelet-Rich Plasma: From Basic Research to Clinical Case Study

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Translational research involves application of basic scientific discoveries into clinically germane findings and, simultaneously, the generation of scientific questions based on clinical observations. At first, as basic research we investigated tissue-engineered bone regeneration using mesenchymal stem cells (MSCs) and platelet-rich plasma (PRP) in a dog mandible model. We also confirmed the correlation between osseointegration in dental implants and the injectable bone. Bone defects made with a trephine bar 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, dental implants were installed. According to the histological and histomorphometric observations at 2 months after implants, the amount of bone–implant contact at the bone–implant interface was significantly different between the PRP, PCBM, dMSCs/PRP, native bone, and control groups. Significant differences were also found between the dMSCs/PRP, native bone, and control groups in bone density. These findings indicate that the use of a mixture of dMSCs/PRP will provide good results in implant treatment compared with that achieved by autogenous PCBM. We then applied this injectable tissue-engineered bone to onlay plasty in the posterior maxilla or mandible in three human patients. Injectable tissue-engineered bone was grafted and, simultaneously, 2–3 threaded titanium implants were inserted into the defect area. The results of this investigation indicated that injectable tissue-engineered bone used for the plasty area with simultaneous implant placement provided stable and predictable results in terms of implant success. We regenerated bone with minimal invasiveness and good plasticity, which could provide a clinical alternative to autogenous bone grafts. This might be a good case of translational research from basic research to clinical application.

Key words: Translational research; Tissue engineering; Injectable bone; Mesenchymal stem cells; Platelet-rich plasma; Dental implant

INTRODUCTION

The notion of translational research has gained considerable interest over the past few years. Although there is no uniformly accepted definition, the term translational research generally "involves the application of basic scientific discoveries into clinically germane findings and, stimultaneously, the generation of scientific questions based on clinical observations" (4,5,24). The translational research studies often reflect a "bench to bedside" or a "bedside to bench and back to bedside" approach that begins with a challenging clinical problem or observation, involves rigorous investigation with application of basic science techniques and discoveries, and brings new insights about important clinical problems back to the clinical interface, along with potential directions for the next steps in future research (4).

Clinically, 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, especially for maxillofacial surgery. 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 (3), but this method is associated with substantial morbidity that includes infection, malformation, pain, and loss of function (11,25,31). A previous approach to this problem focused on the development of various

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graft materials, and bone allografts, xenografts, and alloplasts (substitutes) are being extensively studied in order to avoid the harvesting procedure of autogenous bone (7,17,18). 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 reasons most frequently cited for using alternative grafting materials are donor site morbidity and insufficient volume of harvested autogenous bone (32). These apparent shortcomings of autografts are outweighed by their safety in terms of disease transmission and immunological aspects.

We have attempted to regenerate bone in a significant osseous defect with minimal invasiveness, good plasticity, and nonimmunoreactivity, which could provide a clinical alternative to autogenous bone grafts (27–31). The new method we applied was tissue engineering (10), which 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 are thought to be multipotent cells that can replicate as undifferentiated cells and that have the potential to differentiate into lineages of mesenchymal tissue, including bone, cartilage, fat, tendon, muscle, and marrow stroma (20,21). They 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 (14). Moreover, it has been suggested that PRP may promote a 15–30% increase in the trabecular bone density (14). 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 (PDGF) and 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 (16). TGF-β has been shown to stimulate chemotaxis and mitogenesis of osteoblast precursors, to stimulate the deposition of a collagen matrix for connective tissue healing and bone formation, and to inhibit osteoclast formation and bone resorption (18,24). Furthermore, other studies have shown that PDGF and IGF-I may enhance osseous healing around endosseous dental implants (13).

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 next investigated regenerating bone with a tissue engineering method for dental implants. At present, few experimental studies have examined the behavior of tissue-engineered regeneration of bone around implants, so we investigated the correlation between the tissue-engineered bone with osseointegrated dental implants as basic research for clinical application. 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 to implants placed in nonregenerated, PRP-regenerated, PCBM-regenerated, and native bone. In addition, whether the PRP scaffold combined with MSCs improved bone formation in the bone defect with a relevant volume and whether it was able to function in dental implants was also determined.

Successful osseointegration in dental implants on the tissue-engineered bone regeneration was obtained using a combination of MSCs and PRP with minimal invasiveness. Based on this series of experimental studies, we performed a human study with the tissue-engineered bone for alveolar bone augmentation and simultaneous implant installation. The new tissue-engineered technology we developed is termed "injectable bone" (27,29, 30), which had been established by the tissue engineering concept (10), to provide a procedure with minimal invasiveness and good plasticity as a clinical alternative to autogenous bone grafts (27,29,30). The human application was successful, and these cases will be observed and monitored. Any future problems will be addressed and used to improve treatment and outcome following the translational research concept to improve patient health.

MATERIALS AND METHODS

Basic Research

Canine Animal Models. After a period of housing, five 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 dMSCs, PCBM, and control (defect only), and investigated for osteogenesis. Without any differences between the various sites in terms of bone healing, we created three defects and im-

planted the four 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.

MSC Isolation and Cultivation, 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 (9). Briefly, the basal medium, low-glucose DMEM, and growth

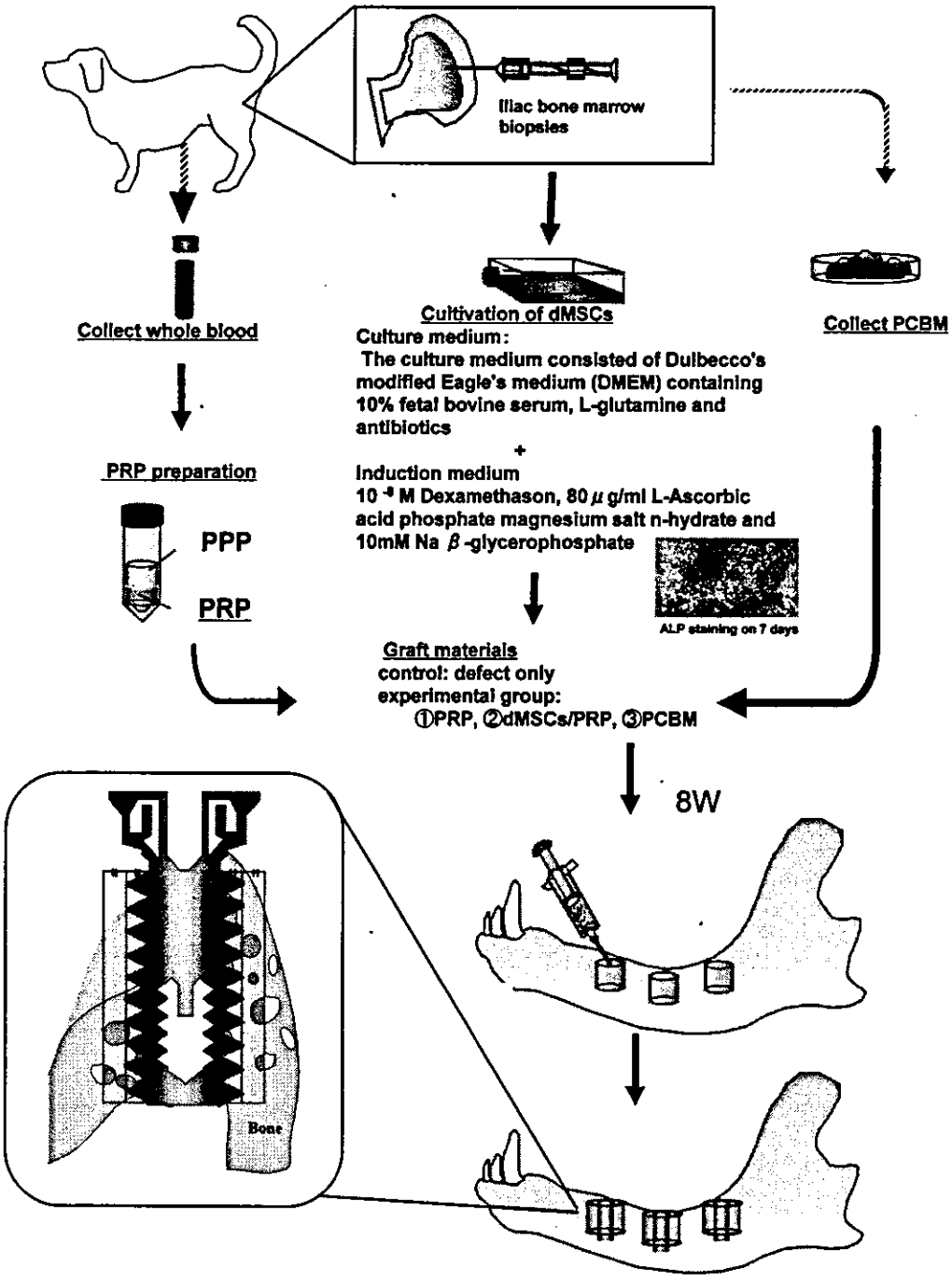


Figure 1. Schema of experimental protocol.

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 Inc. (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 Chemical Co. (St. Louis, MO). 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 control medium. The differentiated dMSCs were confirmed by detecting alkaline phosphatase activity using *p*-nitrophenylphosphatase as a substrate and alkaline phosphatase staining (30). In culture, dMSCs were trypsinized and used for implanting.

The PRP gel preparation was done according to the same method (30). 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), 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; 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., Japan). The PRP was stored at room temperature in a conventional shaker until its use. Bovine thrombin in a powder form (10,000 units) was dissolved in 10 ml 10% calcium chloride in a separate sterile cup. Next, 3.5 ml 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 were 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 transverse the two 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-gauge needle attached to a 5-ml syringe. Dental implants were installed at 8 weeks after injection ($n = 5$).

Histological and Histomorphometric Analysis. Following the manufacturer's recommended dental implant installation method, the Φ3.75 × 7 mm Brañemark implants (Nobel Biocare Norden AB, Gothenburg, Sweden) were installed into the bone defect that had been made. The dogs were sacrificed at 8 weeks after the dental implant insertion. The mandible were 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: a) the bone–implant contact (BIC) (%) = (total length of bone contact/total length of implant surfaces) × 100; (b) 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); (c) the bone density (%) = (total surface of bone in the reference area/total reference area) × 100.

Statistical Analysis. Group means and SDs were calculated for each measured parameter. The data were compared using the paired, two-tailed Student's *t*-test

Table 1. Patient Data in Clinical Cases

	Age	Sex	Location	Operation	No. of Implants	Volume of TEB (g)	Increase in Mineralized Tissue (mm)
1	74	M	7654	onlay graft	4	3.9	3.8
2	53	F	67	onlay graft	3	2.7	3.5
3	54	F	76	onlay graft	3	2.8	3.1

TEB, tissue-engineered bone.

between the control, and the PRP, dMSCs/PRP, PCBM, and native bone groups. A value of $p < 0.05$ indicated statistical significance.

Clinical Application

Patient Selection. Three partially edentulous patients were scheduled for vertical ridge augmentation. All patients had conventional denture retention problems because of severe anterior and posterior maxillary alveolar ridge atrophy. In three patients, a large part of the residual alveolar arch was atrophied in the horizontal and sagittal directions (Table 1). After routine oral and physical examinations, a patient was selected and injectable tissue-engineered bone grafting was planned, as the patient preferred not to undergo any surgery for harvesting of the autogenous bone. In the first case (No. 1 in Table 1), the reconstruction included onlay plasty in the part of the posterior maxilla with simultaneous implant placement. All patients were healthy and free from any disease that may have influenced the treatment outcome (such as diabetes, immunosuppressive chemotherapy, chronic sinus inflammation, and rheumatoid arthritis). The patients were informed extensively about the procedures, including the surgery, the graft materials, the implants, and the uncertainties of using a new bone regenerative method. They were asked for their cooperation during treatment, and the research protocol was approved by the University Ethics Committee.

Cell Preparation. One month before the operation, MSCs were isolated from the patient's iliac crest marrow aspirates (10 ml) (Fig. 2A, B), according to the reported method (22). Briefly, the basal medium, low-glucose DMEM, and growth supplements (50 ml of fetal bovine serum, 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 Bio Whittaker Inc. (Walkersville, MD). Three supplements for inducing osteogenesis (Dex, β-GP, and AsAP) were purchased from Sigma Chemical Co. The process was followed the same as for the basic research method. The differentiated MSCs were confirmed by detecting alkaline phosphatase activity using *p*-nitrophenylphosphatase as a substrate.

Osteoblasts differentiated from dMSCs showed high ALP activity (Fig. 2C). In culture, MSCs were trypsinized and used for implanting.

PRP Preparation. Preoperative hematological assessments included a complete blood count (CBC) with platelet levels. PRP was extracted 1 day prior to surgery. The PRP was isolated in a 200-ml collection bag containing the anticoagulant citrate under a sterilized condition at the blood transfusion service department. Briefly, the blood was first centrifuged for 10 min at 1100 rpm.

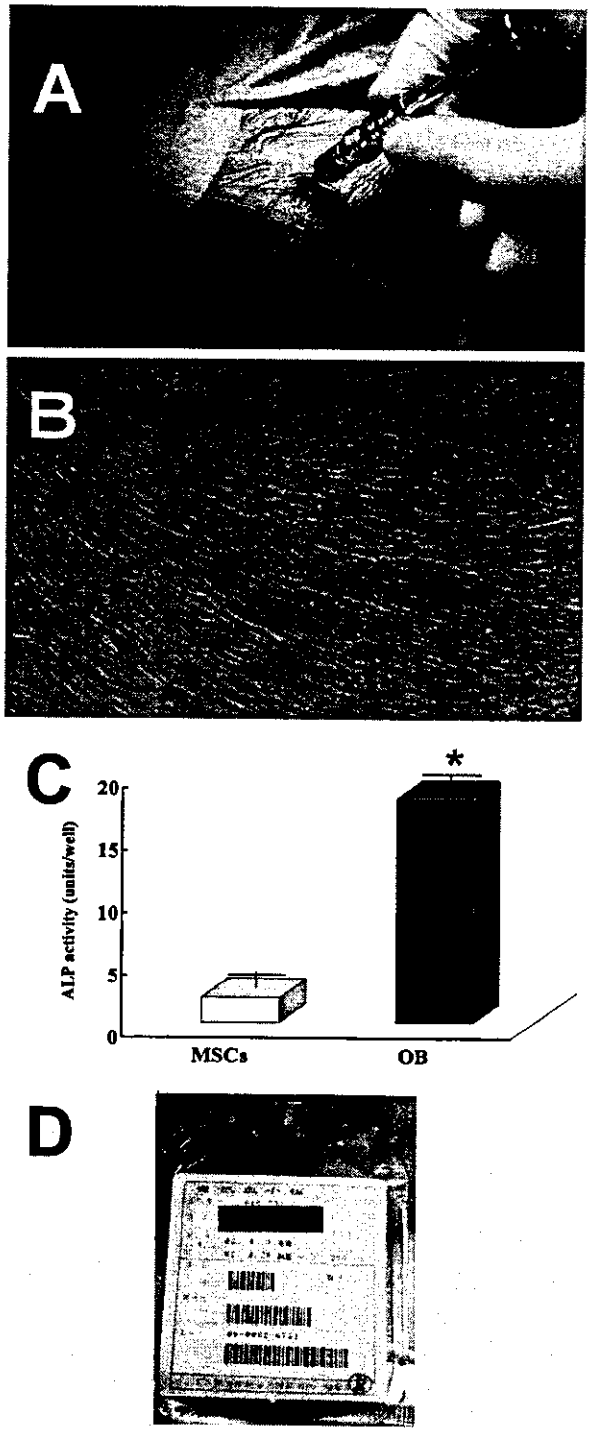


Figure 2. Graft materials and MSCs. (A) MSCs isolation from the patient's iliac crest marrow aspirates. (B) Morphologic observation of the cell of MSCs at day 7. (C) Alkaline phosphatase activity (ALP) in MSCs: open column, osteoblasts differentiated from mesenchymal stem cells; filled column, osteoblasts differentiated from MSCs showed with high ALP activity. Bar: SD. Statistically significant differences between MSCs and differentiated MSCs after 7 days were observed. $*p < 0.01$. (D) PRP preparation: 200-ml collection bag containing the anticoagulant citrate.

Subsequently, the yellow plasma (containing the buffy coat, which contained the platelets and leukocytes) was taken up. A second centrifugation at 2500 rpm for 10 min was performed to combine the platelets into a single pellet; the plasma supernatant, which was PPP and contained relatively few cells, was removed. The resulting pellet of platelets, the buffy coat/plasma fraction (PRP), was resuspended in the residual 20 ml of plasma and used in the platelet gel (Fig. 2D). The PRP was stored at 22°C in a conventional shaker until used. Human thrombin in a powder form (10,000 units) was dissolved in 10 ml 10% calcium chloride in a separate sterile cup. Next, 3.5 ml PRP, MSCs (1.0×10^7 cell/ml), and 0.5 ml of air were aspirated into a 5-ml sterile syringe. In a second 2.5-ml syringe, 500 µl of the thrombin/calcium chloride mixture was aspirated. The cells were resuspended directly into the PRP. The two syringes were connected and the injectable bone was mixed with our developed syringe (Fig. 6C). The contents assumed a gel-like consistency as the thrombin affected the polymerization of the fibrin to produce an insoluble gel.

Surgical Technique: Alveolar Ride Augmentation. Standard titanium implants were placed into the atrophied maxilla or mandible at a depth of at least 5 mm with most of the threads of the fixture exposed. The injectable tissue-engineered bone was applied around the implant to cover the exposed threads completely. After coagulation of the tissue-engineered bone, the grafted area was covered by the titanium membrane to protect the mucosal flap compression. The membrane was fixed with coverscrews and microscrews. Finally, the buccal and labial periosteum was extended in the customary way, and the wound was closed in a tension-free manner. The patients were instructed not to wear any removable prosthesis for 30 days and not to blow their noses for 7 days.

RESULTS

In Vivo Macro Findings, and Histological Evaluation of the Implants

The dMSCs were trypsinized at day 7 and were used for the implants at a concentration of 1.0×10^7 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 PRP and the control (defect only) was not complete. The dMSCs/PRP scaffold had almost completely disappeared without infection after implantation (Fig. 3A–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. 3D).

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 bone regenerated by dMSCs/PRP showed newly formed woven and lamellar bone (Fig. 4G–H). In the native bone sites, normal dense, compact bone was found at both the buccal and lingual implant aspects. This bone showed characteristic remodeling, with newly formed osteons in the area adjacent to the implant surface (Fig. 4I–J).

Bone density was $63.2 \pm 7.6\%$ for the control group, $68.2 \pm 10.3\%$ for the PRP group, $70.3 \pm 8.2\%$ for the PCBM group, $79.4 \pm 3.3\%$ for the dMSCs/PRP group, and $80.6 \pm 4.8\%$ for the native bone. There were significant differences in bone density between the dMSCs/PRP, native bone group, and the control group ($p < 0.05$), but no significant difference was seen between the PRP, PCBM, and the control groups. The implants exhibited a varying degree of bone–implant contact (BIC). The BIC was $26.4 \pm 9.5\%$ for the control group, $44.2 \pm 10.8\%$ for the PRP group, $49.9 \pm 8.2\%$ for the PCBM group, $58.6 \pm 9.7\%$ for the dMSCs/PRP group, and $65.0 \pm 12\%$ for the native bone. The BIC of the PRP and PCBM ($p < 0.05$), dMSCs/PRP, and native bone ($p < 0.005$) groups showed a significant increase in the implant surface compared with the control (Fig. 5).

Clinical Observation

The three patients in this study included two women and one man, ranging in age from 53 to 74 years, with a mean of 60.3 years. A total of 10 implants were inserted simultaneously with onlay plasty. None of the patients had postoperative complications besides normal swelling and inflammation at the surgical sites. At the second surgery, which was performed after a mean healing period of 5.3 months, the mucosal flap was elevated relatively widely to observe the grafted site. All 10 implants were clinically successful as defined by complete coverage of the entire implant up to the cover screw, and the absence of mobility. In the three cases of vertical ridge augmentation, the spaces underneath the titanium membranes were filled with newly formed tissue, which appeared to be calcified tissue (Table 1). All implants maintained stability at 6 months after loading, as tested

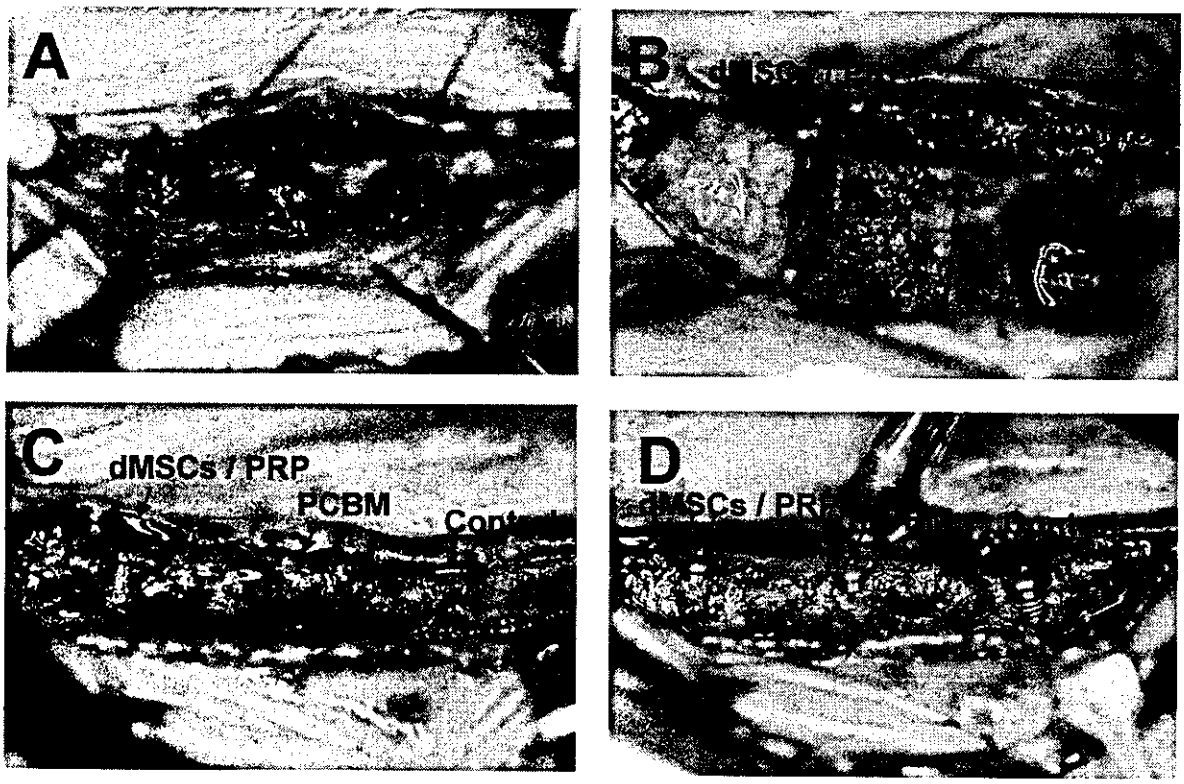
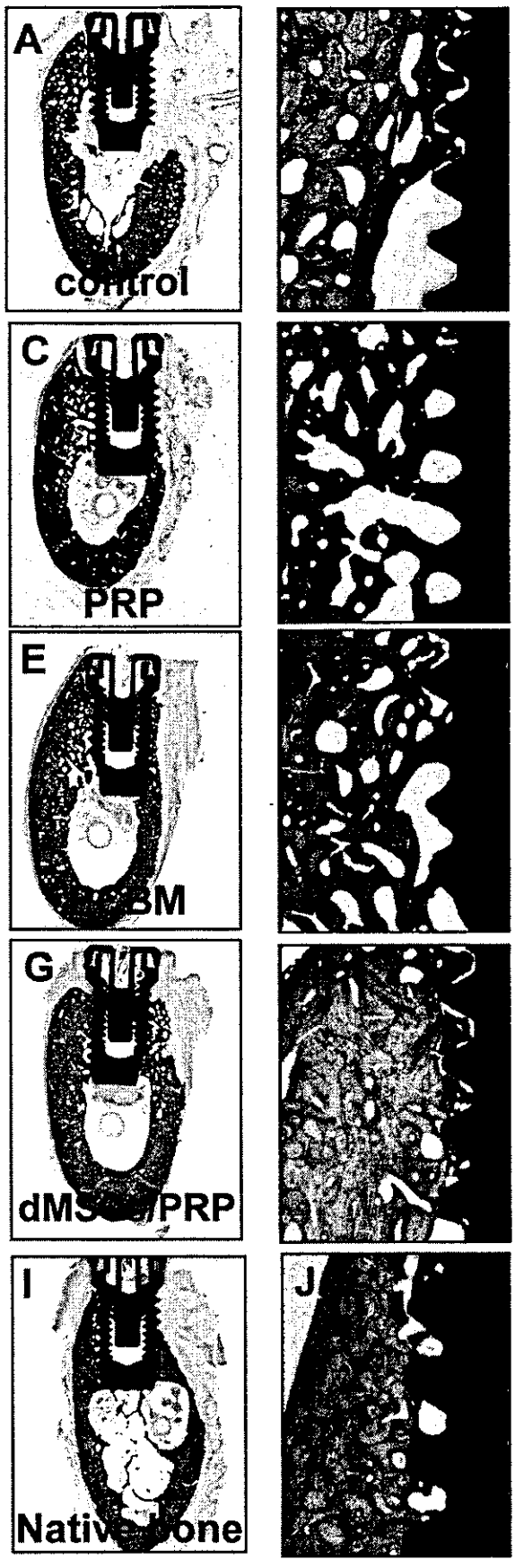


Figure 3. Macroscopic observations for bone regeneration. (A) The experimental design in the dog mandibular prepared with a trephine bar with a 10-mm diameter. (B) Implanted materials in bone defects. (C) New bone regeneration in the dMSCs/PRP, PCBM, and control groups at 8 weeks (PRP group data not 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. (D) The osseointegrated dental implants in the bone regeneration areas at 8 weeks. The dental implant thread was exposed in the control group.

after removal of the prosthetic reconstruction. Marginal bone resorption at 6 months after loading did not exceed 1.5 mm.

In the representative first case, a 74-year-old man presented with severe bone resorption of the alveolar arrests in the right maxilla (Fig. 6A). A crestal incision within the keratinized tissue, circumscribing the cervical aspects, was extended intrasulcularly to the mesial line-angle of the first premolar buccally. A buccal mucoperiosteal full-thickness flap was raised. Inflammatory granulomatous tissue was removed from the inner aspects of both mucoperiosteal flaps and from the bony defects using hand curettes. Abundant sterile saline rinses were delivered to the defects. All four standard implants of 15 mm in length presented bone resorption that was morphologically differentiated in horizontal and vertical components. The implants presented mainly a moat-type infrabony lesion of approximately 5 mm in depth, with 10 exposed threads (Fig. 6B). The injectable tissue-engineered bone was positioned around the exposed threads to completely cover them (Fig. 6C–D). A titanium membrane was bent to obtain close adaptation to the underlying bone and to the implants. The lateral portions overlapped the edge of the bone beyond the defect margins by approximately 4 mm. The titanium membrane was stabilized to the bone with a fixation screw. Horizontal mattress sutures with U stitches were used to create two contact surfaces at least 3 mm thick (first line of closure). No pressure was applied to the surgical area. Healing was uneventful. Sutures were removed after 14 days and the patient was examined monthly. Despite a prolonged healing period, the titanium membrane remained completely submerged and the surrounding tissue was completely healthy, without any sign of inflammation. After the fixation screw was removed, the membrane was raised with small surgical pliers from its most apical portion. All space underneath the membrane was completely filled with regenerated, hard, bone-like tissue (Fig. 6E). Clinically, this regenerated tissue was hard and appeared to consist of bone tissue. The newly formed tissue reached the uppermost part of the implant system, partially covering the cover screws. After the abutment, the implant-supported bridge connecting prosthesis, the flaps were sutured back to their original positions. Nine months after membrane removal, clinical probing depth measurements were made (Fig. 6F). These



did not exceed 2 mm, and a healthy and firm peri-implant mucosa had been established. After a 12-month loading period, a periapical radiograph showed a radiographic bone fill within the infrabony defects and around the previously exposed threads, reaching the neck of the implants (Fig. 6G–H). Routine panoramic radiographs also clearly showed the positions of both types of graft material and the height of the new alveolar ridge. Radiographic findings were consistent with integration between the implant and the regenerated bone (no bone loss or peri-implant radiolucency). Decreased graft height was not observed in any radiographs.

DISCUSSION

Translational research involves the application of basic scientific discoveries into clinically germane findings and, simultaneously, the generation of scientific questions based on clinical observations (4,5,24). Translational research studies involve rigorous investigation with application of basic science techniques and discoveries, and they bring new insights about important clinical problems back to the clinical interface, along with potential directions for the next steps in future research (4).

At present, there are some problems that 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, autografts have become a predictable treatment and are unequivocally accepted as the standard of care (3), but this method is associated with substantial morbidity that includes infection, malformation, pain, and loss of function (11,25,32) for patients. Therefore,

Figure 4. Photographs of the histology sections, as seen with light microscopy. Nondecalcified ground sections, surface stained with toluidine blue. Original magnification: 12.5× (A, C, E, G, I) and 250× (B, D, F, H, J). (A, B) In the control group, the buccal wall was not sufficiently regenerated for dental implants. (C, D) In the PRP group, most of the threads on the buccal aspect were covered by soft tissue. (E, F) In the PCBM group, the buccal wall was thin but reached the smooth/rough implant border. Extensive bone–implant contact was present. After the absorbance by PCBM, it underwent recalcification. The dead space underwent grafted bone absorption. (G, H) In the dMSCs/PRP group, the fully regenerated buccal bone plate was as wide as the lingual cortex. This group showed good reconstruction of the former alveolar width. Good bone remodeling, as well as extensive bone–implant contact, was seen on the sides of the implant. (I, J) In the native bone, bone remodeling, as well as bone–implant contact, were identical on the sides of the implant. The bone consisted of compact bone with comparable remodeling activity.

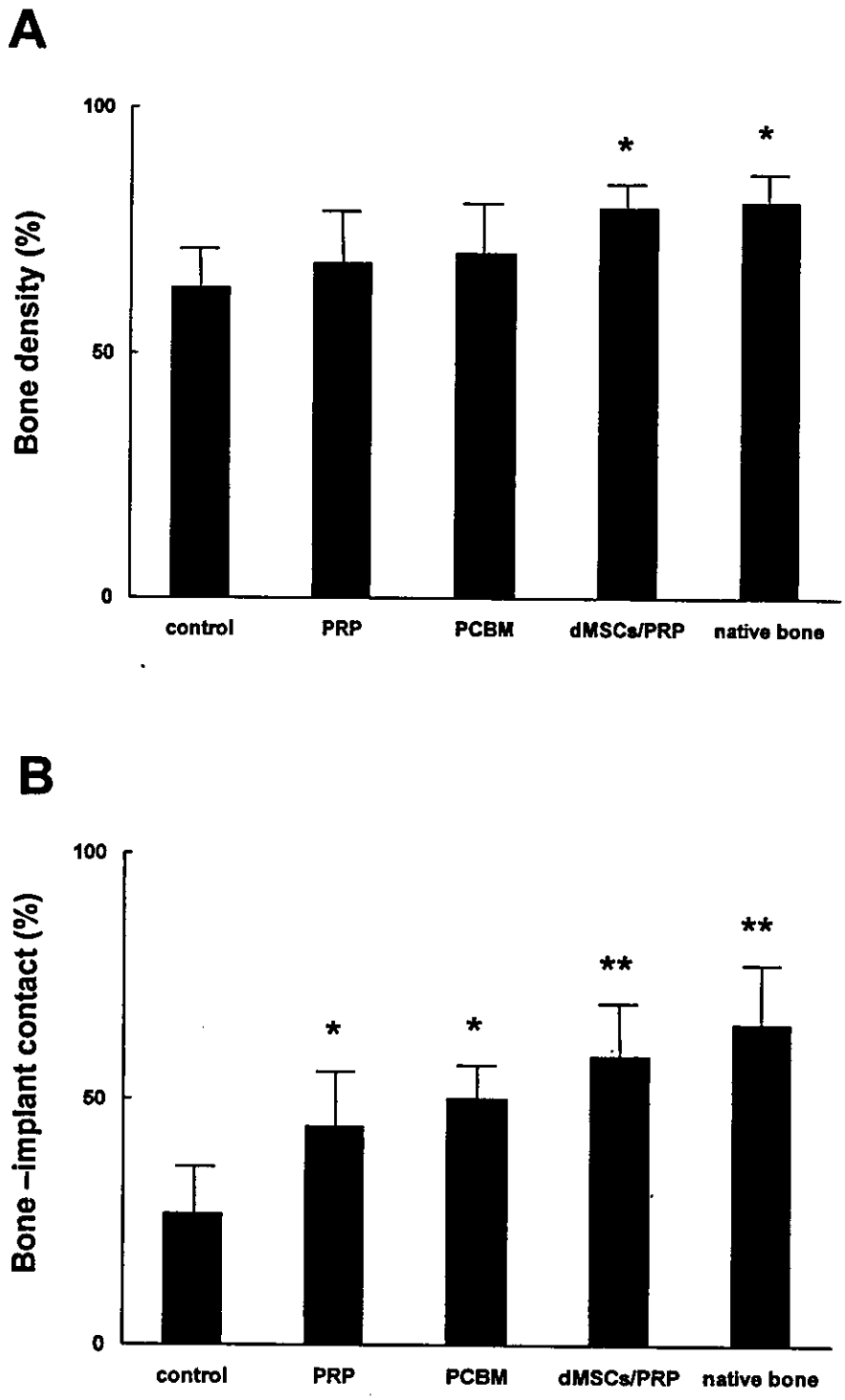


Figure 5. Histomorphometrical evaluation. (A) Comparison of the mean percentage of bone density among the graft materials. A statistically significant difference was seen between the dMSCs/PRP, native bone, and control groups. *Significant difference at $p < 0.05$. (B) Comparison of the mean percentage 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, PCBM, dMSCs/PRP, native bone, and control groups. *Significant difference at $p < 0.05$, **significant difference at $p < 0.005$.

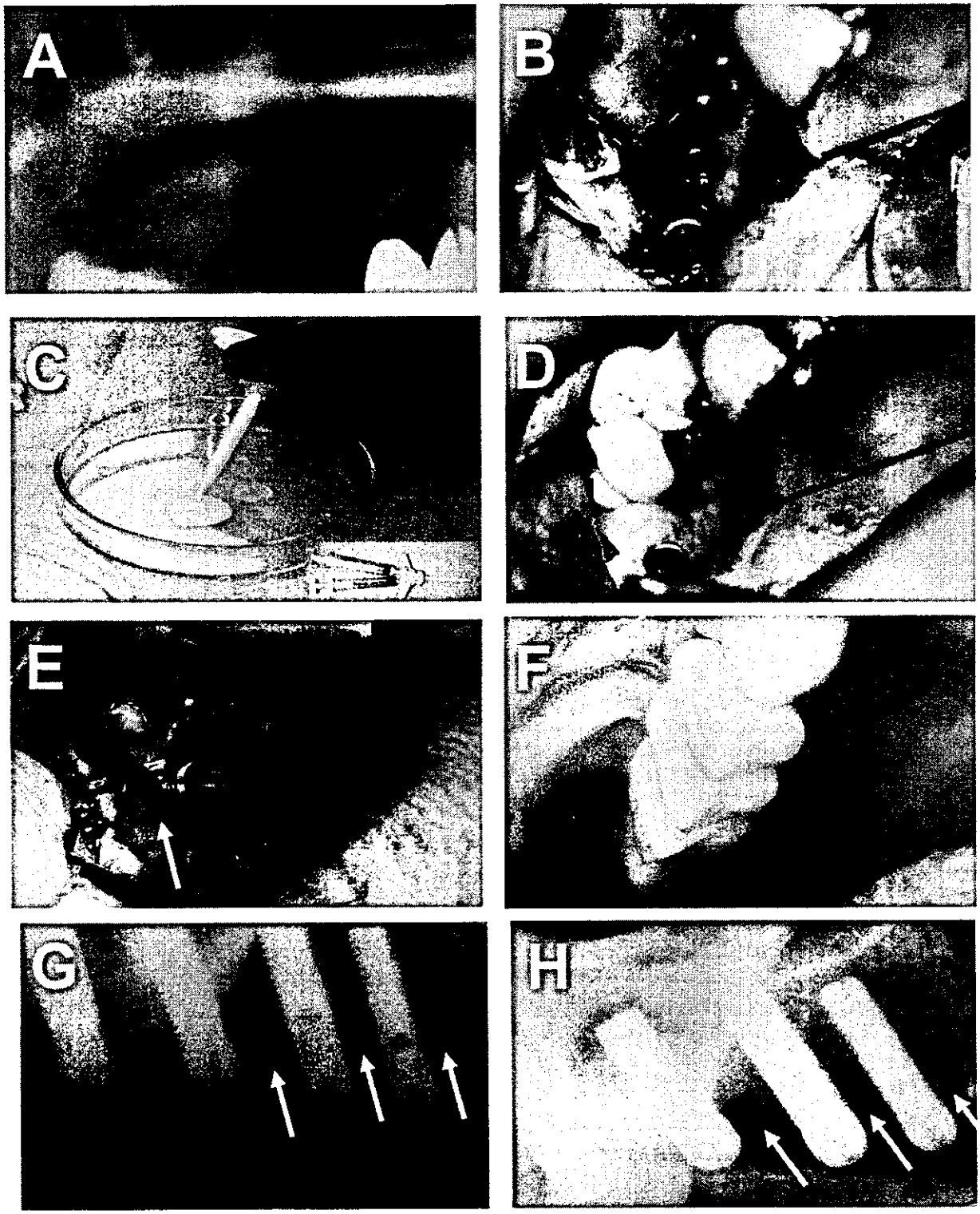


Figure 6. (A) Panoramic radiograph of the patient, preoperative. (B) Macro view of a 15-mm Tio-blast titanium dental implant insertion into a prepared implant site. (C) Preparation of injectable bone by injectable instrument and two syringes. (D) Macro view of a tissue-engineered bone insertion. (E) Observation of second-stage surgery 6 months after the implant installation. The exposed thread was surrounded by newly formed bone (see the arrow) and confirmed successful osseointegration. (F) Last prosthesis observation by porcelain fused to a metal crown. (G) A periapical radiograph after a postpoperative time at 1 week; the radiolucent area around the installed dental can be found. Arrow shows the radiolucent area. (H) A periapical radiograph after a 6-month period; the radiograph shows a bone fill within the infrabony defects and around the previously exposed threads, reaching the neck of the implants (see the arrows).

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 by applying the concept of tissue engineering.

Accordingly, the recent tissue-engineering approaches had attempted to create new bone based on MSCs seeded onto porous ceramic scaffolds. These attempts have given suboptimal results due to the slow resorption rate of the hydroxyapatite-based ceramics. In our previous study (2), we used a biodegradable material, a β-TCP block loaded with MSC, which had excellent osteogenic characteristics. However, these delivery substances did not have good plasticity and the cellular implantation procedure was complicated by problems associated with the delivery vehicles. Optimally, these should combine with an appropriate rate of biodegradability, with the capacity for the respective cells to multiply. Presumably, the disappearance of the osteogenic cells left in place induced bone tissue formation, which then self-organized according to the surrounding environment. In this study, we used a combination of MSCs with PRP and found a progressive, complete resorption of the scaffold, leaving relatively mature remodeled bone. So we were able to explore the potential ability of MSCs and PRP to increase the rate of bone formation and to enhance the bone regeneration, compared with autogenous bone grafts (PCBM). And we also investigated the correlation between injectable tissue-engineered bone with osseointegrated dental implants as basic research for clinical application.

Due to experimental design of Berglundh and Lindhe's study (1), the defects made in extraction 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 (15). And the use of PRP might provide conditions to obtain more rapid and effective bone regeneration for dMSCs. PRP contains 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 (15). In addition to these growth factors, other proteins carried within platelets (26) 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 bone implant contact and, thus, PRP in the defect did not result in improved osseous healing well.

The successful result of this basic research was then applied to clinical cases following the concept of translational research. Various clinical investigations (12,19) and case reports (28) have indicated that, although sinus augmentation or onlay graft can be clinically successful with various grafting materials, autogenous bone still provides the best osteogenic potential and biomechanical properties of regenerated bone. However, the quantitative limitations of autogenous bone harvested from intraoral sites often constrain the clinician to combine the autograft with other types of grafts in order to obtain an adequate amount of grafting material. Autogenous bone, when used as a graft, has an osteogenic potential related to the number of surviving osteoblasts, and a potential osteoinductive effect brought about by the release of bone morphogenic proteins and other growth factors.

This study evaluated the performance of MSCs, PRP, and MSCs/PRP admixture (injectable tissue-engineered bone) in one-stage sinus or mandible onlay plasty, with simultaneous implant placement. While numerous studies (8,14,23) have recommended the two-step procedure in patients with less than 5 mm of alveolar bone height in the posterior maxilla or alveolar ridge, the results of this investigation suggest that injectable tissue-engineered bone graft yields adequate bone quality and volume for predictable simultaneous implant placement in such patients. The one-step procedure offers the advantages of reducing the number of surgical procedures and the time needed to complete the implant-supported prosthesis.

The results of the clinical and radiological examinations, relating to the use of injectable tissue-engineered bone, permit conclusions concerning the successful healing and regeneration of bone. Osteointegration between implants and regenerated bone can be seen clinically, and can be followed in the same way for injectable tissue-engineered bone as for autogenous bone. The use of injectable tissue-engineered bone provides conditions for obtaining more rapid and effective bone regeneration. Also, this tissue-engineered bone, which is a coagulated mass, is easy to manipulate. And our clinical findings also demonstrated that injectable tissue-engineered bone implants can elicit bone regeneration, as well as autogenous bone grafts, with a complete disappearance of the biomaterial and formation of new tissue in a bone