Chapter 3

Bone

The use of dental implants in oral rehabilitation is becoming a standard method of care in dentistry. In the case of insufficient bone volume, a procedure for augmentation is needed. The ability to augment the alveolar ridge has gradually expanded the scope of implant dentistry. During the past 10 years, alveolar augmentation techniques have become established treatment modalities. Dahlin et al. reported an experimental study on rabbits involving the formation of new bone around titanium implants using the membrane technique [1]. In addition, various bone-grafting materials have been used for augmentation, including autologous grafts, freeze-dried bone grafts, hydroxyapatite and xenografts [2, 3]. Although the results of these investigations indicate that augmentation is clinically successful for various graft materials, it is questionable whether these materials, except for autologous bone, have adequate osteogenic potential and biomechanical properties [4, 5]. On the other hand, autologous bone, which currently remains the material of choice, is available for bone reconstructive procedures [6]. However, its use is limited due to donor site morbidity and limited amounts of graft material available for harvesting.

To avoid these problems, we attempted to regenerate bone in a significant osseous defect with minimal invasiveness, and to provide a clinical alternative to the graft materials described above. The new technology that we developed is called "injectable tissue-engineered bone", and involves the morphogenesis of new tissue using constructs formed from isolated cells with biocompatible scaffolds and growth factor, and was established based on tissue engineering concepts [7-9].

Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are frequently used for bone tissue engineering and increasingly applied in the clinic. Although engineering bone tissue using MSCs is feasible, the size of the regenerated bone is limited by nutrient transport. The grafted cells require an oxygen/nutrition supply to survive and early neovascularization is considered essential for successful bone tissue engineering. Development of an efficient neovascularization method to sustain the engineered implants is clinically important. The relationship between vascular endothelial factors (e.g. VEGF) and bone regeneration emphasizes the important role of vasculature not only for survival but also for the proper formation of tissue-engineered bone.

In 1997, Asahara et al. characterized endothelial progenitor cells (EPCs) in human peripheral blood using magnetic beads selection [10]. Since EPCs can give rise to endothelial cells (ECs) and are known to facilitate the neovascularization of an implanted site, EPCs may be used to facilitate collateral vessel growth into ischemic tissues through delivery of antiangiogenic or proangiogenic agents. EPCs have been implanted into various ischemic tissue models, for example, ischemic hindlimbs and areas of myocardial infarction. Recently, EPCs have also been used to engineer blood vessels. Taken together, it is conceivable that supplementing osteogenic cells with ECs or EPCs may facilitate osteogenesis in tissue-engineered bone

constructs. However, little is known about how EPCs may influence development of tissue-engineered bone.

We investigated the potential of EPCs to facilitate neovascularization in implants and evaluated their influence on bone regeneration. The influence of EPC soluble factors on osteogenic differentiation of MSCs was tested by adding EPC culture supernatant to MSC culture medium. To evaluate the influence of EPCs on MSC osteogenesis, canine MSCs-derived osteogenic cells and EPCs were seeded independently onto collagen fiber mesh scaffolds and cotransplanted to nude mice subcutaneously. Results from co-implant experiments were compared to implanted cells absent of EPCs 12 weeks after implantation. Factors from the culture supernatant of EPCs did not influence MSC differentiation. Co-implanted EPCs increased neovascularization and the capillary score was 1.6-fold higher as compared to the MSC only group (P<0.05). Bone area was also greater in the MSC + EPC group (P<0.05) and the bone thickness was 1.3-fold greater in the MSC + EPC group than the MSC only group (P<0.05). These results suggest that soluble factors generated by EPCs may not facilitate the osteogenic differentiation of MSCs; however, newly formed vasculature may enhance regeneration of tissue-engineered bone (Fig. 9).

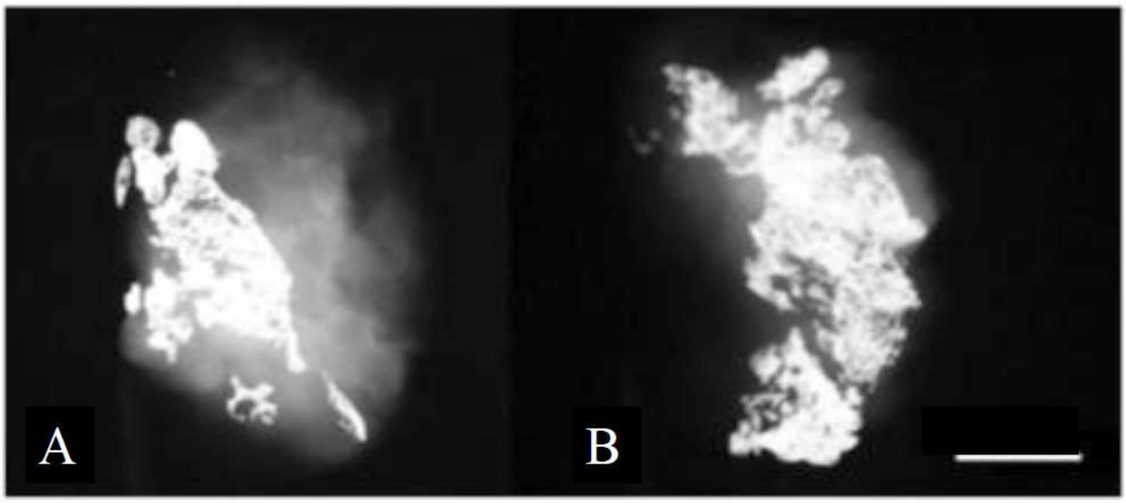

Fig. 9. Soft X-ray radiographs of implants 12 weeks after implantation. A: MSC only group: MSC-derived osteogenic cells were seeded onto collagen, which was then wrapped with the same scaffold without cells. B: MSC and EPC group: MSC-derived osteogenic cells were seeded onto one scaffold, then covered with another scaffold seeded with EPCs (From Usami et al. 2006. Reprinted with permission).

Tissue engineered bone 'injectable bone'

We previously reported that tissue-engineered bone induces excellent bone regeneration and promotes bone formation in a grafted area treated with platelet-rich plasma (PRP), which contains various growth factors [8].

After a period of housing, 12 adult hybrid dogs with a mean age of 2 years were operated dMSCs were isolated from 10 ml samples of dog iliac bone marrow aspirates. Bone marrow cell isolation and expansion was performed according to previously published methods [11]. Briefly, basal medium (condition medium), low-glucose Dulbecco's modified Eagles medium (DMEM) and growth supplements consisting of 50 ml of mesenchymal cell growth

supplement, 10 ml of 200 mM L-glutamine and 0.5 ml of a penicillin–streptomycin mixture containing 25 U of penicillin and 25 μg of streptomycin. The three supplements used for inducing osteogenesis, dexamethasone (Dex), sodium β-glycerophosphate (β-GP) and L-ascorbic acid 2-phosphate (AsAP). The cells were incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. dMSCs were replated at a density of 3.1×10^3 cells/cm^3 in 0.2 ml/cm^2 condition medium. The implants were assessed by histological and histomorphometric analysis, 2, 4 and 8 weeks after implantation. The implants exhibited varying degrees of bone-implant contact (BIC). The BIC was 17%, 19% and 29% (control), 20%, 22% and 25% (fibrin), 22%, 32% and 42% (dMSCs/fibrin) and 25%, 49% and 53% (dMSCs/PRP/fibrin) after 2, 4 and 8 weeks, respectively. This study suggests that tissue-engineered bone may be of sufficient quality for predictable enhancement of bone regeneration around dental implants when used simultaneous by with implant placement. Recent tissue engineering approaches have attempted to create new bone based on the use of MSCs seeded onto porous ceramic scaffolds with osteoconductive properties [12] (Fig. 10). These attempts have yielded sub-optimal results due to the slow resorption rate of hydroxyapatite-based ceramics. Also, these delivery substances do not exhibit good plasticity and the cellular implantation procedure is complicated by problems associated with the delivery vehicles because the block materials do not have plasticity. Isogai et al. reported that a combination of fibrin glue with delivery vehicle and cultured periosteal cells resulted in new bone formation at heterotopic sites in nude mice [13]. In numerous reports about materials, fibrin was found to have hemostatic effects and to promote wound healing. In a bone regeneration study using the rabbit tibia, Bosch et al. and Schwarz et al. reported that fibrin stimulated neovascularization of bone with accelerated healing and earlier new bone formation [14, 15]. Additionally, the use of fibrin as an osteoconductive material has been recommended [16]. Therefore, we used fibrin as a scaffold, which is one of the three key factors in the tissue engineering concept [9].

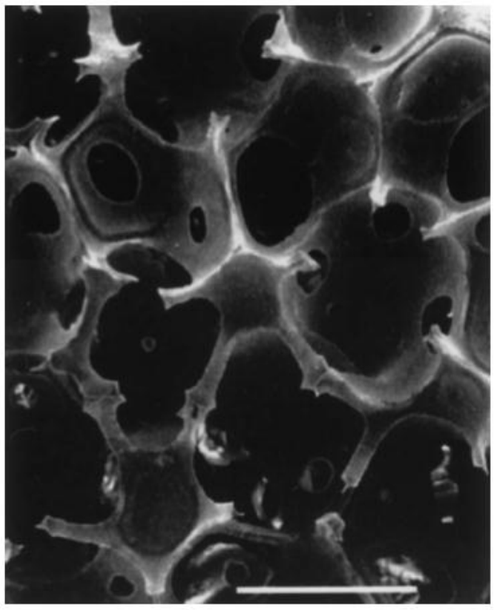

Fig. 10. Scanning electron microscopy photomicrograph of a cross-section of β-tricalcium phosphate. Bar = 500 μm (From Boo et al. 2002. Reprinted with permission).

Distraction osteogenesis using tissue engineered bone

Distraction osteogenesis has become a widely accepted technique for reconstructing bone defects in the maxillofacial region. This technique provides autologous and predictable bone formation without grafting procedures but requires long-term treatment that includes latent, lengthening, and consolidation periods. The long treatment time results in a high rate of complications such as infection, pin loosening, and fracture. The recommended rate of gradual bone lengthening as described by Ilizarov is 1 mm per day [17]. A lower rate of distraction tends to result in bony union, whereas a higher rate of distraction may delay bone union or result in fibrous union.

To promote bone formation and shorten the consolidation period, some attempts at applying hyperbaric oxygenation or electrical, ultrasonic, or chemical stimulation have been made [18]. Several recent studies have shown that injecting cells with osteogenic potential into distracted callus enhances its consolidation, but there have been few attempts at higher-rate distraction [19-22].

We previously reported on a tissue-engineered osteogenic material (TEOM) [23]. This material is an injectable gel of autologous MSCs, which are culture-expanded then induced to be osteogenic in character, and PRP activated with thrombin and calcium chloride. The injection of TEOM into the distraction gap has advantages. MSCs can be expanded and induced to osteoblastic lineage *ex vivo*. Moreover, both MSCs and PRP are autologous materials.

Bilateral maxillary distraction was performed at a higher rate in rabbits to determine whether locally applied TEOM enhances bone regeneration (Fig. 11). The material was an injectable gel composed of autologous mesenchymal stem cells, which were cultured then induced to be osteogenic in character, and PRP. After a 5-day latency period, distraction devices were activated at a rate of 2.0 mm once daily for 4 days. Twelve rabbits were divided into 2 groups. At the end of distraction, the experimental group of rabbits received an injection of TEOM into the distracted tissue on one side, whereas, saline solution was injected into the distracted tissue on the contralateral side as the internal control. An additional control group received an injection of PRP or saline solution into the distracted tissue in the same way as the experimental group. The distraction regenerates were assessed by radiological and histomorphometric analyses. The radiodensity of the distraction gap injected with TEOM was significantly higher than that injected with PRP or saline solution at 2, 3, and 4 weeks postdistraction. The histomorphometric analysis also showed that both new bone zone and bony content in the distraction gap injected with TEOM were significantly increased when compared with PRP or saline solution.

TEOM injection at the end of distraction promotes new bone formation following a higher rate of distraction. TEOM injection may be able to compensate for the insufficient distraction gap at a higher rate (Fig. 12).

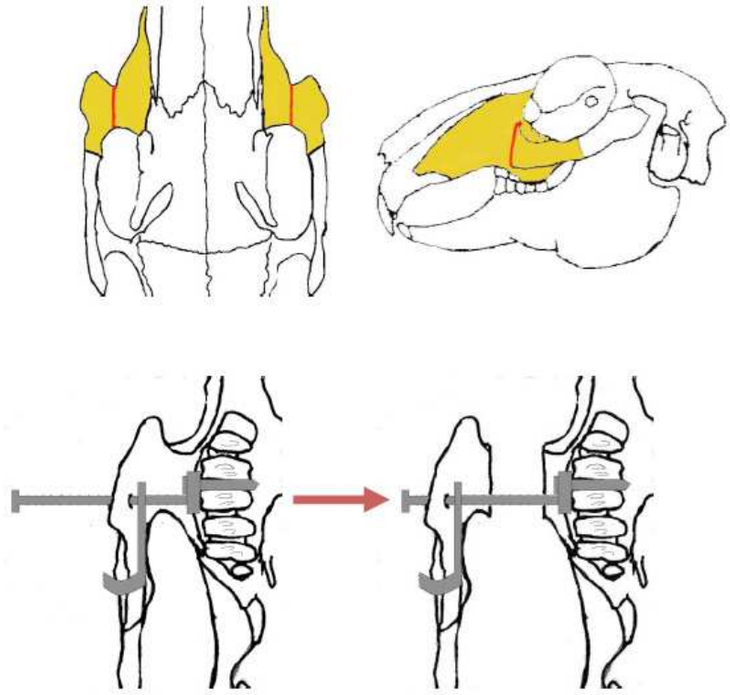

Fig. 11. A: Schematic drawing of the maxilla and maxillary distraction. Red line, osteotomized line; yellow area, maxilla.

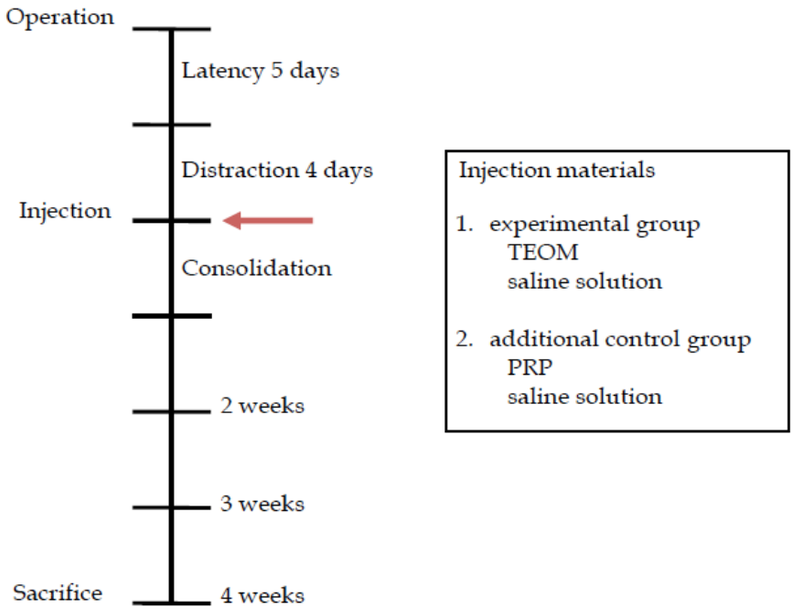

Fig. 11. B: Distraction protocol and experimental design (From Kinoshita et al. 2008. Reprinted with permission).

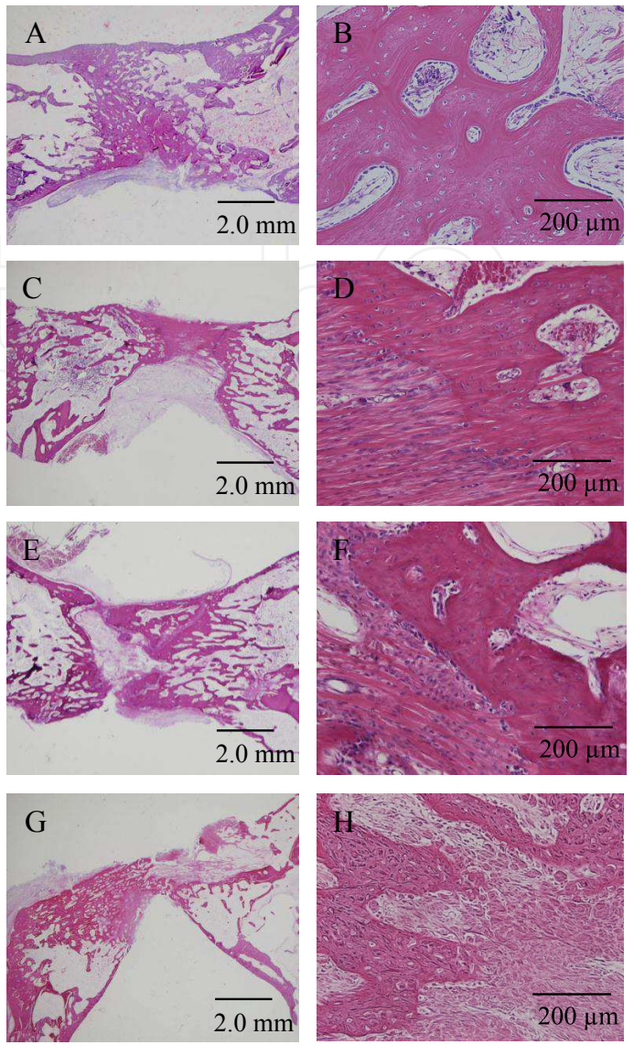

Fig. 12. Histological view of the distracted maxilla, staining H-E. Experimental group A: TEOM-injected side; B: center of the distraction zone on the TEOM-injected side; C: saline solution-injected side; D: center of the distraction zone on the saline solution-injected side. Additional control group, E: PRP-injected side; F: center of the distraction zone on the PRP-injected side; G: saline solution-injected side; H: center of the distraction zone on the saline solution-injected side (From Kinoshita et al. 2008. Reprinted with permission).

Bone regeneration using 'periosteum'

The periosteum is comprised of two tissue layers: the outer fibroblast layer that provides attachment to soft tissue, and the inner cambial region that contains a pool of undifferentiated mesenchymal cells, which support bone formation [24]. Recently, studies have reported the existence of osteogenic progenitors, similar to MSCs, in the periosteum [25, 26]. Under the appropriate culture conditions, periosteal cells secrete extracellular matrix and form a membranous structure [27]. The periosteum can be easily harvested from the patient's own oral cavity, where the resulting donor site wound is invisible. Owing to the above reasons, the periosteum offers a rich cell source for bone tissue engineering.

Our group has previously demonstrated bone regeneration using a cultured periosteum (CP) in a critical-sized rat calvaria bone defect [28] (Fig. 13). CP has also been shown to regenerate bone in a surgically created furcation bone defect using a canine model [27]. Considering the biocompatibility of CP and its capacity for alveolar bone regeneration, it should be useful to investigate the potential of CP for bone regeneration around an implant site. The purpose of this study was to investigate the potential of CP to regenerate bone to mitigate implant dehiscence defects.

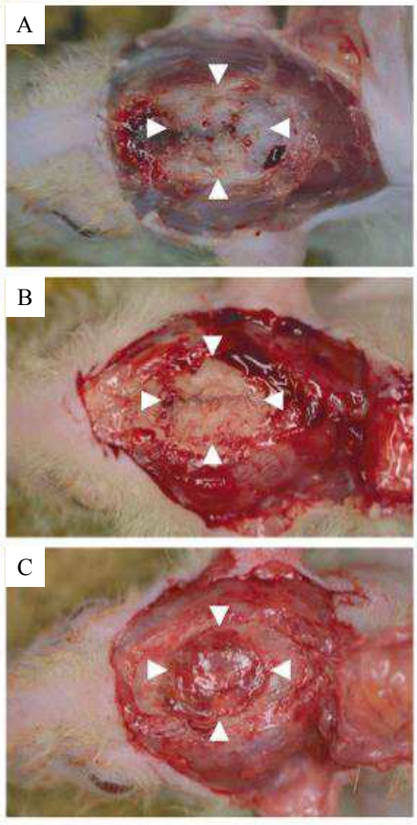

Fig. 13. A–C: Photographs showing representative calvarial bone defect of athymic rats 3 months after surgery. A: Animals with grafted fresh CP showed complete closure of calvarial defect. B: Animals, grafted with cryopreserved CP also showed complete closure of the defect, and there was no apparent difference between fresh and cryopreserved CP macroscopically. C: However, the bone defect of the control group without CP remained almost the same size as before grafting. Arrowheads indicate margin of original bone defect (From Mase et al. 2006. Reprinted with permission).

Four healthy beagle dogs were used in this study. Implant dehiscence defects (4 × 4 × 3 mm) were surgically created at mandibular premolar sites where premolars had been extracted 3 months back. Dental implants (3.75 mm in diameter and 7 mm in length) with machined surfaces were placed into the defect sites (14 implants in total). Each dehiscence defective implant was randomly assigned to one of the following two groups: (1) PRP gel without cells (control) or (2) a periosteum cultured on PRP gel (experimental). Dogs were killed 12

weeks after operation and nondecalcified histological sections were made for histomorphometric analyses including percent linear bone fill (LF) and bone-implant contact (BIC) (Fig. 14). Bone regeneration in the treatment group with a CP was significantly greater than that in the control group and was confirmed by LF analysis. LF values in the experimental and the control groups were 72.36 ± 3.14% and 37.03 ± 4.63%, respectively (P<0.05). The BIC values in both groups were not significantly different from each other. The BIC values in the experimental and the control groups were 40.76 ± 10.30% and 30.58 ± 9.69%, respectively (P = 0.25) and were similar to native bone.

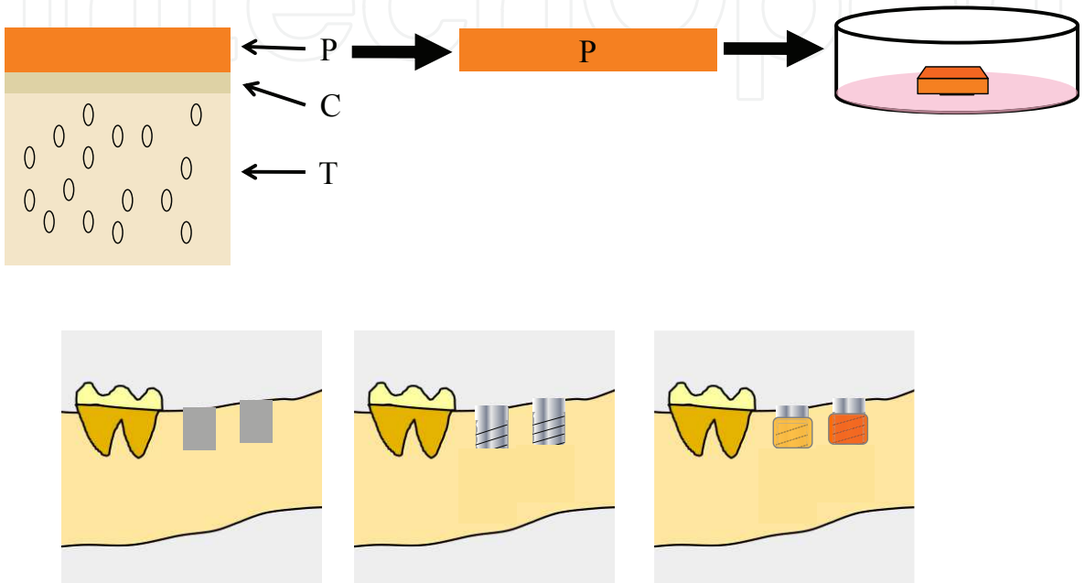

Fig. 14. Schematic drawing of the surgical procedures used in this study (P, Periosteum; C, Cortical bone; T, Trabecular bone). (a) Dog mandibular defect model (4 × 4 × 3 mm). (b) Implant placement. (c) Transplantation PRP gel (left) and cultured periosteum membrane on PRP gel (right) (From Mizuno et al. 2008).

The consensus tissue engineering paradigm includes cells, scaffolds, and bioactive molecules. For periodontal therapy, there are several reports based on this tissue engineering paradigm that incorporate various polymers such as collagen and gelatin as a scaffold material [29-31]. However, natural biodegradable materials cannot eliminate the possible risk of infection and degraded products may interfere with the regeneration process. Instead of culturing cells on natural biodegradable scaffolds, we have been able to stimulate periosteal cells to form their own matrix and generate a cell-populated membrane *in vitro* [27]. This method creates a CP durable enough to be held by forceps, making it feasible to transplant CP without the need for a biodegradable support. Furthermore, the thickness of the CP is approximately 200 µm, which may be beneficial to cells, allowing oxygen and nutrients to diffuse into the transplanted tissue (Fig. 15).

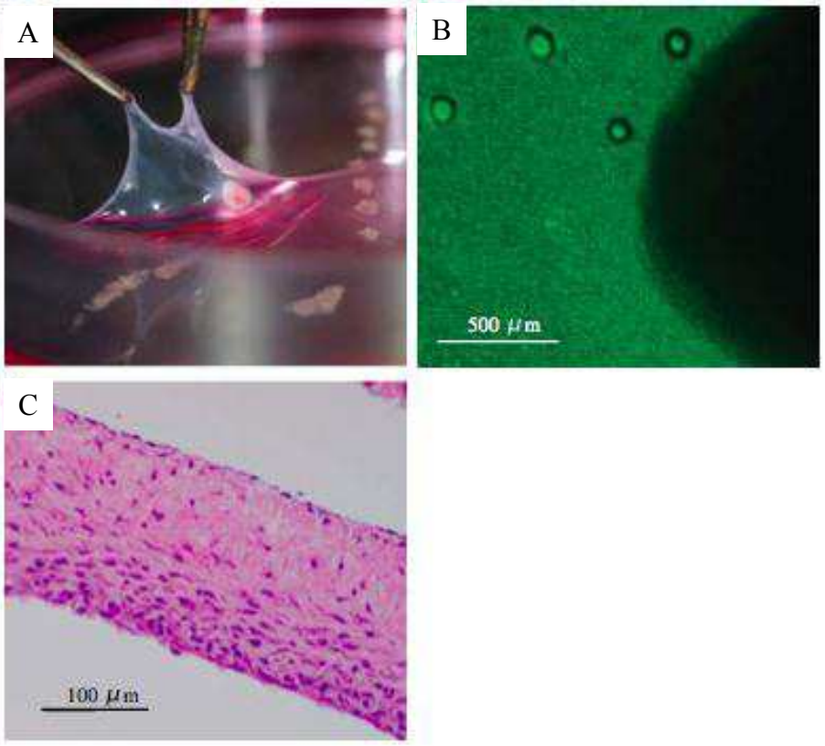

Fig. 15. A, B: CP macroscopic and microscopic findings. Bovine periosteal cells were cultured for 4 weeks. A: The cells became a membranous structure with enough mechanical strength to handle with forceps. Cells can be obtained by a conventional explant culture of periosteal fragment. B: Phase-contrast photomicrograph showing the cultured CP. C: Photomicrograph showing hematoxylin and eosin staining of CP section. The CP is approximately 100–300 μm in thickness and consists of 20–30 cellular layers (From Mase et al. 2006. Reprinted with permission).

A major disadvantage of using CP for clinical treatment might be the time period required for tissue culture. Four to 6 weeks is a typical time frame for obtaining CP with enough mechanical strength to be transplanted. Furthermore, the cultured period would likely differ for each patient and may be unpredictable at the beginning of culture. This uncertainty makes it difficult to formulate a treatment schedule in advance. To overcome this problem, we have investigated the potential of CP cryopreservation [28]. In this study, the optimal preincubation protocol for CP was investigated and it was found that CP could be successfully cryopreserved under specific conditions without loss of osteogenic potential. Cryopreservation of CP should increase the usefulness of these approaches in future clinical applications. This study demonstrated the feasibility of a CP to regenerate bone at implant dehiscence defect.

Translational Research

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 MSCs and 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, autologous 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 BIC 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 autologous 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 autologous bone grafts. This might be a good case of translational research from basic research to clinical application.

Clinical application

From these animal experiments, we adopted "injectable tissue-engineered bone" to regenerate bone in a significant osseous defect that was minimally invasive and had good plasticity, and to provide a clinical alternative to the graft materials mentioned above. One of the advantages of injectable tissue-engineered bone is the use of autologous cells for bone regeneration. Tissue engineering technology by autologous cell transplantation is one of the most promising therapeutic concepts being developed because it may solve problems, including donor site morbidity from autologous grafts, immunogenicity of allogenic grafts, and loosening of alloplastic implants. In this method, we use differentiated bone marrow derived stem cells (BMDSCs) as isolated cells for bone formation, and PRP as a growth factor and scaffold. In our hospital, we experienced many clinical cases using this method, such as maxillary sinus augmentation, periodontal treatment, and distraction [32-34]. We here report the procedures and results for these cases.

Preparation of cells

One and a half month before surgery, BMDSCs were isolated from the patient's iliac crest bone marrow aspirates (20 ml) and cultured. The control medium contained the following: basal medium, low-glucose DMEM, 10% patient serum or 10% fetal bovine serum, and growth supplements (10 ml of 200 mM L-glutamine and 0.5 ml of a penicillin-streptomycin mixture containing 25 units of penicillin and 25 μg of streptomycin). Each patient could choose the type of serum (patient serum or fetal bovine serum) for cultivation of BMDSCs. For human serum preparation, human blood was isolated in a 200 ml collection bag under sterile conditions. Subsequently, the blood collected was centrifuged at 3500 rpm for 10 minutes and the supernatant was collected. Three supplements for inducing osteogenesis—100 nM of dexamethasone, 10 mM of sodium β-GP, and 80 μg/ml of AsAP. Cells were incubated at 37°C in a humid atmosphere containing 95% air and 5% carbon

dioxide. Differentiated BMDSCs were trypsinized and used for implant placement. To verify the safety of cultured cells, the culture media were examined for contamination by bacteria, fungi, or mycoplasmas before transplantation.

Preparation of PRP

Preoperative hematology included complete blood count with platelet counts. PRP, extracted 1 day before surgery, was isolated in a 200 ml collection bag containing an anticoagulant, citrate, under sterile conditions in the Blood Transfusion Service of Nagoya University Hospital. Briefly, the blood collected was first centrifuged at 1500 rpm for 10 minutes. Subsequently, the yellow plasma (containing the buffer coat that contained platelets and leukocytes) was removed. The second centrifugation was conducted at 3500 rpm for 5 minutes to combine platelets with a single pellet. The plasma supernatant, which was platelet-poor plasma and contained a relatively small number of cells, was removed. The resulting pellet of platelets, the buffer coat/plasma fraction (PRP), was resuspended in remaining 20 ml of the plasma before use in the platelet gel.

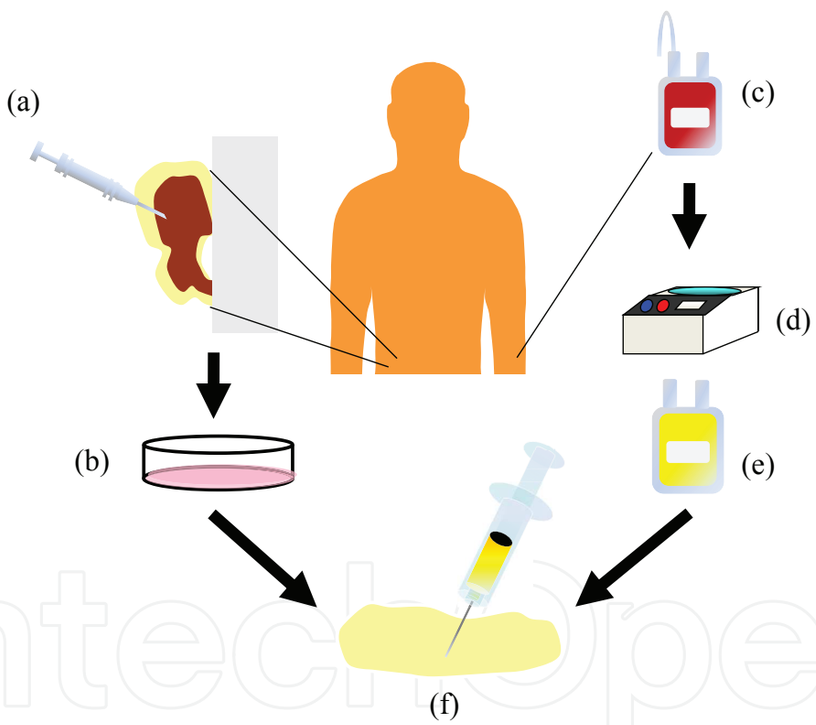

Fig. 16. Protocol of tissue engineered bone. (a) Harvest of bone marrow. (b) Cell culture and osteoinduction. (c) Collection of whole blood. (d) Centrifuge. (e) Platelet rich plasma. (f) Injection of tissue engineered bone (From Ueda et al. 2008).

Preparation of injectable tissue-engineered bone

PRP was stirred and stored at 22°C in a conventional shaker until used. Powdered human thrombin (500 units) was dissolved in 10% calcium chloride in a separate sterile cup. The PRP, BMDSCs (5.0×10^6 cells/ml), and air were aspirated into the first 2.5 ml sterile syringe. The thrombin-calcium chloride mixture (300 µl) was aspirated into the second 2.5 ml syringe. The two syringes were connected with a T connector, and the plungers of the syringe were

pushed and pulled alternatively, allowing air bubbles to flow back and forth between the two syringes. Within 5-30 seconds, the contents gained gel-like consistency because thrombin affected the polymerization of fibrin to produce an insoluble gel (Fig. 16).

Application for maxillary sinus augmentation

After tooth loss, alveolar augmentation of the extensively atrophied maxillary process may be required to restore the masticatory function of the patient by means of substitute teeth anchored on dental implants. In order to obtain adequate volume of bone enough to insert dental implants, elevation of the maxillary sinus floor has been carried out as a routine clinical procedure for more than 15 years [35-39]. Where the bone thickness between the maxillary sinus and the alveolar crest is less than 8 mm, sinus floor elevation without bone graft materials is insufficient. A bone graft-induced increase in the thickness of the alveolar sinus floor is necessary to support longer implants that are required [40, 41]. The success of the dental implants is to be evaluated over a long period of time.

Sixteen sinus augmentations in 12 patients, partially or totally edentulous patients 44-60 years of age (mean age: 54 years), were performed. All the patients had a conventional problem of denture retention due to severe posterior alveolar ridge atrophy; the average height of their residual sinus floor was <6 mm, for which sinus graft and dental implants would solve the problem.

After routine oral and physical examinations, patients who did not desire to undergo any surgery for harvesting autologous bone were selected for injectable tissue-engineered bone grafting. They were healthy and free of any disease that might affect treatment outcomes (e.g., diabetes, immunosuppressive chemotherapy, and rheumatoid arthritis). Each patient was given detailed information about the intervention, including surgical techniques, types of graft material and dental implants, and the uncertainties of conducting a new bone-regenerative procedure. Informed consent in writing was requested of each patient.

Surgical techniques

Sinus augmentation was conducted under general anesthesia. The sinus grafting procedure followed Tatum's classical description [35]. Briefly, the mucoperiosteal flap was elevated to create a trap door with a round hollow burr in the lateral wall of the maxillary sinus. After mobilization, the door was reflected inward. The space created by this procedure was filled with 1.8-5.4 g of injectable tissue-engineered bone to simultaneously place dental implants. The mucoperiosteal flap was repositioned and sutured in the usual manner. After surgery, patents received cephalosporins (300 mg/day) as antibiotics, and loxoprofen sodium (180 mg/day) as analgesics for 3 days.

Postoperative course

The incidences of grafted bone resorption and implant loss after sinus augmentation with various bone substitutes have been recorded. The complete resorption of bone substitutes, especially autologous bone, was observed in 2.7% of patients [37]. However, this regenerated bone did not show resorption and remained in the sinus floor that had been elevated by injectable tissue-engineered bone. The mineralized tissue 2 years after operation increased by 8.8 ± 1.6 mm, presumably due to the properties of tissue-engineered bone (Fig. 17).

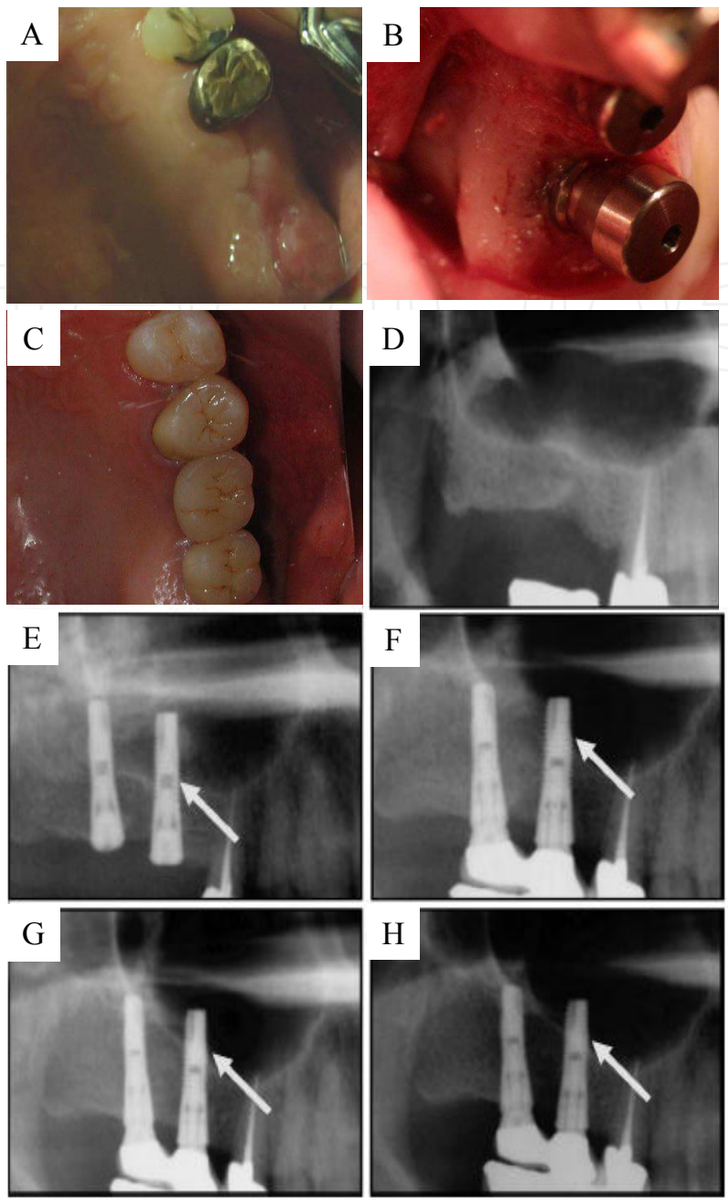

Fig. 17. A: Preoperative macro view. B: Observation of second-stage surgery 6 months after the implant installation. The exposed thread was surrounded by newly formed bone and confirmed successful osseointegration. C: Last prosthesis observation by porcelain fused to a metal crown. These did not exceed 2 mm, and a healthy and firm peri-implant mucosa had been established. D: Panoramic radiograph, preoperative. E: Panoramic radiograph, postoperative. F: Panoramic radiograph, postoperative 1 year. G: Panoramic radiograph, postoperative 2 years. H: Panoramic radiograph, postoperative 3 years (From Ueda et al. 2008. Reprinted with permission).

For long periods of time, maxillary sinus floor augmentation has constituted a surgical procedure to gain bone mass required for placing dental implants. Further, there is also consensus that some threshold of osseous deficiency, vertical, horizontal, or both, exists at a

site where a sinus bone graft is required for successful implant treatment regardless of residual bone quality. If there is vertical bone less than 8 mm in height in the posterior maxilla, sinus floor elevation without bone graft materials is insufficient. A sinus graft should be strongly recommended to provide adequate support for the placement of dental implants [40, 41]. In these cases, the average residual bone height was 5.5 ± 1.6 mm (range: 2-10 mm); therefore, we applied tissue-engineered bone as a graft material for the sinus graft. On the other hand, in our previous study [8, 23, 42-44], we found that the tissue-engineered bone was well-formed mature bone, and the bone-regenerating ability increased significantly compared to the nongrafted control of defect-only sites or PRP-only sites that had been reported not to function effectively for bone regeneration [45], confirming the radiological and histological data [8, 45]. In addition, we measured the values from a Vickers hardness test, which indicates mechanical properties for bone formation. The values of nongrafted control, PRP, autologous bone, and tissue-engineered bone were 8, 9, 13, and 17, respectively, 2 weeks after operation [44]. Moreover, in our experiment we used rabbit maxillary sinus that has well-defined ostium similar to that of humans. The augmented height and bone volume showed peaks as early as 2 weeks in tissue-engineered bone sites; on the other hand, the volume of newly formed bone reached a peak value within 4 weeks in autologous bone sites at 2, 4, and 8 weeks of experimental time [42, 46]. Thus bone regeneration may indicate early bone formation and enhanced bone quality as the main advantages of BMDSCs, since our results were consistent with the report of our previous animal experiments [8, 23, 44].

This technique might be effective for maxillary sinus augmentation. This result also might be the effect of this grafted material, tissue-engineered bone by BMDSCs and PRP, and not the effect of sinus membrane elevation alone. The BMDSCs in the bone marrow are induced in cells with osteogenic capacity, and the MSCs in BMDSCs are considered more feasible for tissue engineering because the former proliferates faster due to a lower degree of differentiation. The PRP contains not only fibrinogen, which forms a fibrin network acting as a matrix, but also cytokinetic substances such as PDGF, TGF-β, IGF, and VEGF, which can stimulate MSCs to transform into osteoblasts [47]. The growth factors are believed to have an osseous regenerative effect on the MSCs and contribute to cellular proliferation, matrix formation, collagen synthesis, osteoid production, and other processes that accelerate tissue regeneration. However, further research will be required to examine the effect.

Additionally, osseointegration is the most important condition for success in dental implant treatment. In clinical cases, implant loss occurs with no osseointegration because of infection, absorption and loss of bone volume. In our other study, we showed that the surface of implants attached to regenerated bone by tissue-engineered bone. Further, no infection was observed, and regenerated bone volume was not reduced 2 years after operation from the above-mentioned result. As a result, no implant loss was observed whatever because our procedures used autologous graft materials, autologous BMDSCs and PRP, in our cases (Fig. 18).

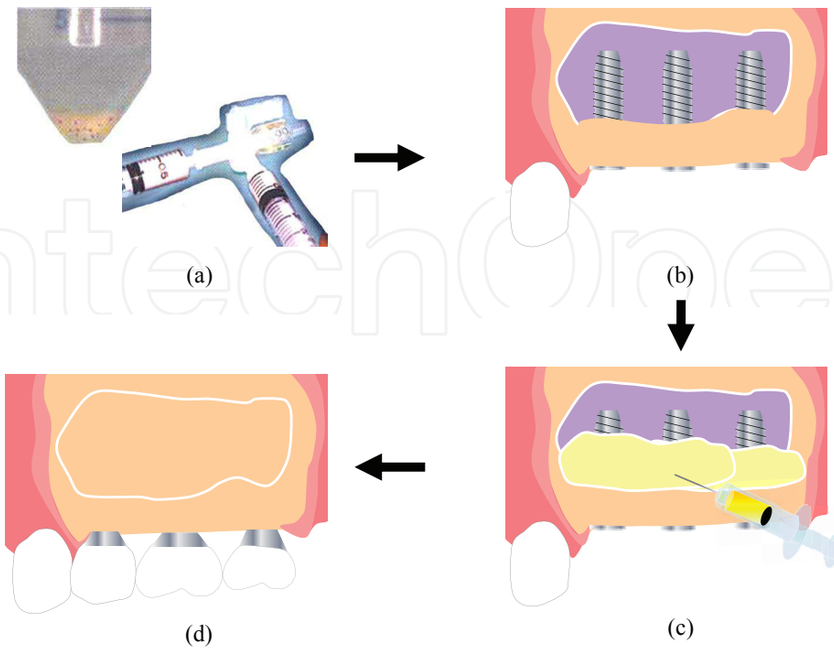

Fig. 18. Protocol of tissue engineered bone for sinus lift. (a) Mixing osteoblasts and PRP. (b) Installation of dental implants. (c) Injection of tissue engineered bone. (d) Bone regeneration (From Ueda et al. 2005).

Application for periodontal treatment

Periodontal disease is an infectious disease that affects tooth-supporting tissues. Clinically, the color changes of the gingival, periodontal pocket formation, bleeding, clinical attachment loss of alveolar bone as detected on radiolucent disease is due to bacterial plaque in the periodontal pockets. The main aim of conventional periodontal therapy is to halt and possibly reverse the attachment loss resulting from the disease. To this end, initial therapy is focused on removal of bacterial plaque from teeth and periodontal pockets and prevention of supragingival plaque accumulation. Subgingival plaque can be removed by a nonsurgical form of therapy, such as scaling and root planing, or by surgical means. The efficacy of nonsurgical methods is well documented [48].

Several studies have confirmed the efficacy of mechanical subgingival plaque control in periodontal therapy, irrespective of the approach used [49]. Adequate supragingival plaque control by patients is required for successful periodontal treatment [50]. However, subgingival bacteria in deep pockets with compared anatomy, in infrabony pockets, and in areas of furcal involvement are sometimes difficult to remove with nonsurgical therapy. In those pockets, open access with surgical therapy may be indicated to clean the root surfaces. Even when inflammation has been eliminated and healthy periodontal tissue has been established after pathogenic microorganisms are removed from periodontal pockets, the anatomy of healed defects can be a problem, particularly in areas in which esthetics is critical and maintenance is difficult, such as in dentitions with gingival recession, infrabony defects, and furcations.

In the early 1980s, a series of experimental studies was conducted on a procedure to regenerate the lost attachment apparatus. A membrane was placed under the flap to prevent epithelial downgrowth and to create space for periodontal reformation [51]. The procedure, termed guided tissue regeneration (GTR), was introduced into the clinical setting by Gottlow et al. [52]. GTR therapy has been applied to furcation and infrabony sites under certain conditions, and its efficacy has been reported [53, 54]. However, it has been claimed that the new attachment between regenerated cementum obtained by GTR procedures and root dentin may not be as strong as the attachment between the original cementum and root dentin [55]. Because cementum formed following GTR therapy is apparently different from cementum formed during tooth development (a cellular cementum), the appropriateness of the term regeneration in the context of GTR therapy has been questioned [56]. Recently, some authors have started using the term true periodontal regeneration, which has been defined as "healing after periodontal treatment that results in the regain of lost supporting tissues, including a new cellular cementum attached to the underlying dentin surface, a new periodontal ligament with functionally oriented collagen fibers inserting into the new cementum, and new alveolar bone attached to the periodontal ligament [57]." Another technique, developed recently, is the application of enamel matrix derivatives to root surfaces. However, although these treatments have been reported to be effective for periodontal tissue regeneration, the indications for such treatments are rather limited and the amounts of regenerated tissue are not predictable. This indicates that further theoretical and technical developments are needed in the field of periodontal regenerative therapies before such therapy can be widely used in daily practice.
In the previous cases, we used injectable tissue-engineered bone grafting to effectively regenerate bone for dental implant placement, and the result confirmed that tissue engineering can elicit as much bone regeneration as autologous bone grafts. So, we applied this method as periodontal regenerative therapy.

Surgical techniques
Immediately before surgery, the patient rinsed her mouth with 0.2% chlorhexidine solution for 90 seconds. The surgical area was anesthetized with lidocaine adrenaline 2%. Following pocket and releasing incisions, buccal and lingual full-thickness flaps were elevated and the epithelium was removed from the inside of the flaps. Granulation tissue residing in the defect area was carefully excised, and the root surface was scaled and planed. No bone recontouring was performed. Subsequently, MSCs-PRP gel was applied to the root surface and adjacent defect space. The flaps were replaced and closed with sutures. After 2 weeks, the sutures were removed. The patient was instructed to rinse three times daily with a 0.1% or 0.2% solution of chlorhexidine digluconate. Mechanical cleaning of the surgical site was not recommended during the first 4 postoperative weeks. Supportive care, including professional tooth cleaning, was performed every 2 months.

Postoperative course
By 1 year after this treatment, the pocket depth decreased from 5 mm to 1 mm. This clinical improvement should be predictable for teeth. Radiographic assessments revealed that the bone defect was indeed reduced in depth. But this may be a result of the short time allowed for a change in radiopacity; after a longer period, the postoperative progress would have become radiographically apparent. Whereas some regeneration may occur in humans

following a regenerative surgical approach, complete and predictable true regeneration is still difficult to attain. Based on recent clinical results, GTR therapy appears to have the most promising prospects for regeneration, although its clinical efficacy and predictability in periodontal defects have yet to be thoroughly tested in controlled clinical trials [57]. Also, when the GTR approach is used to treat periodontal defects, the risk of membrane exposure must be considered as a major complication [58-60]. The reported prevalence of membrane exposure is in the 70% to 80% range [61]. In such cases, adequate membrane fixation and soft tissue coverage can be difficult to perform. On the other hand, it is easier to apply injectable tissue-engineered bone, which is a formed gel, than to position a membrane around a defect, and since gingival recession and unesthetic outcomes occur when membranes become exposed, it seems logical to use tissue-engineered bone rather than a membrane. Also, the tissue-engineered bone assumes a firm, gel-like consistency and may have the ability not only to immobilize to implants in place but also to provide a seal around the tooth. In a preliminary animal study, tissue-engineered bone prevented downgrowth of the epithelium equally well as the GTR method. In addition, it has been claimed that the new attachment between regenerated cementum obtained from GTR procedures and root dentin may not be as strong or continuous as the attachment between the original cementum and root dentin [55]. On the other hand, in a periodontal tissue regeneration study using this treatment in dogs, MSCs played an important role in cementification, and the structure of regenerated cementum was similar to that of natural cementum on roots versus that regenerated using the GTR method. Therefore, injectable tissue-engineered bone treatment might be more useful than the GTR method for true periodontal and interdental papilla regeneration (Fig. 19).

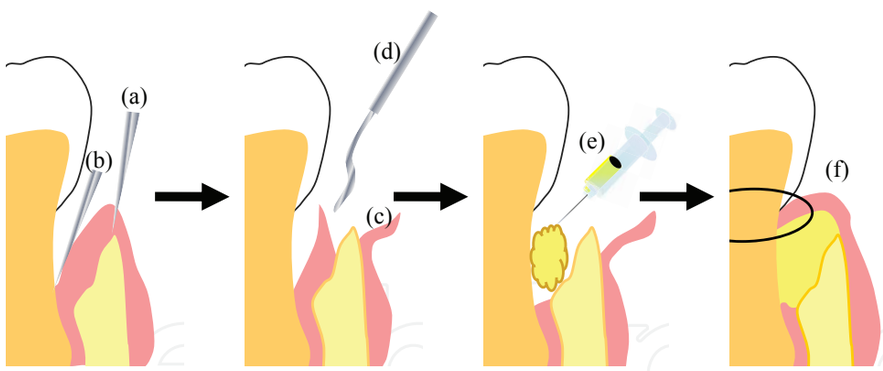

Fig. 19. Protocol of tissue engineered bone for periodontal treatment. (a) Internal bevel incision. (b) Intersulcular incision. (c) Flap elevation. (d) Scaling and root planning. (e) Injection with tissue engineered bone. (f) Suture (From Yamada et al. 2006).

Application to alveolar cleft defects

The reconstruction of alveolar cleft defects is well established, with the most widely accepted approach being secondary alveolar cleft osteoplasty in the mixed dentition phase with autologous bone grafting [62, 63]. The source material for most bone grafts has been particulate marrow harvested from the anterior iliac crest, and this represents the standard material with which other materials from rib, mandible, calvarium, and tibia are compared

[62-64]. Donor site morbidity is an important factor in deciding the site for harvesting cancellous bone. Allogenic or xenogenic materials can eliminate this concern but not the risk of disease transmission. As another solution, the use of injectable tissue-engineered bone in bone augmentation procedures as a replacement for autologous bone grafts, offers predictable results with minimal donor-site morbidity [23, 65]. We considered that tissue-engineered bone is beneficial material for alveolar cleft osteoplasty, and applied this treatment.

Surgical techniques

Following a 3-cm-long mucosal incision at the level of the labiogingival junction, dissections were made in the ingrown scar tissue to reach the bony surface of the cleft walls. The tissue was then elevated in the subperiosteal plane to the levels of the anterior nasal spine anteriorly, the lateral piriform rim superiorly and to the alveolar ridges inferiorly, while taking care not to damage the unerupted teeth and the content of the incisive canal. The flaps of the nasal floor and the oral mucosa formed the ceiling and the floor of the cleft cavity, respectively. The ceiling, floor and front walls of the defect were supported with a 0.1-mm-thick titanium-mesh plate. The thus-created pouch was filled with all the prepared TEOM through a syringe using a packer. Following release incisions in the periosteum and the scar tissue of the flaps and to allow them to cover the grafted area, the wound was closed without tension.

Postoperative course

Before this treatment, a 3-month-old female patient born with a congenital left unilateral cleft lip and alveolus underwent a cheiloplasty that resulted in no remaining oronasal fistula. At 9 years of age, computed tomograms (CTs) revealed that the left maxillary canine, lateral, and supernumerary incisors had formed half of their roots, and had closely surrounded the alveolar cleft bony defect which was 10 mm wide and 13 mm deep anteroposteriorly. The left central incisor was orthodontically overcorrected due to previous severe rotation and distal location (Fig. 20).

The patient exhibited an uneventful postoperative course. The radiopacity of serial CTs slicing the middle level of the alveolar cleft in the grafted region increased gradually over time. Dome-shaped radiopaque images with 233 Hounsfield units (HU) faced one another, extended from the cleft bony walls inside the cavity after 3 months, and were fused together into an image with 324 HU after 6 months. The image increased in radiopacity to 447 HU in 9 months, and at the bony bridge the lateral and supernumerary incisors horizontally migrated from their original positions in the respective major and minor segments. The incisive canal was reconstructed just medial to the bridge. The erupting canine and lateral incisor pushed the mesh plate vertically, and the mucosa covering the cleft consequently swelled and thinned. A mucosal cut was made in the crest of the alveolar ridge over these teeth, and the part with the plate overlying the teeth was removed under local anesthesia. The canine and the lateral incisor then erupted approximately at the same time (Fig. 21).

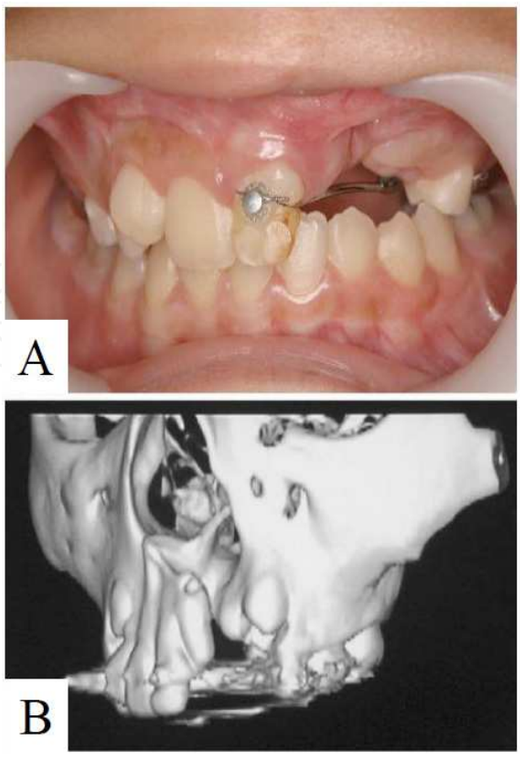

Fig. 20. The left unilateral cleft of the alveolus of our 9-year-old female patient. A: Intraoral view. B: Three-dimensional computed tomogram demonstrating the left maxillary canine and the alveolar bony defect (From Hibi et al. 2006. Reprinted with permission).

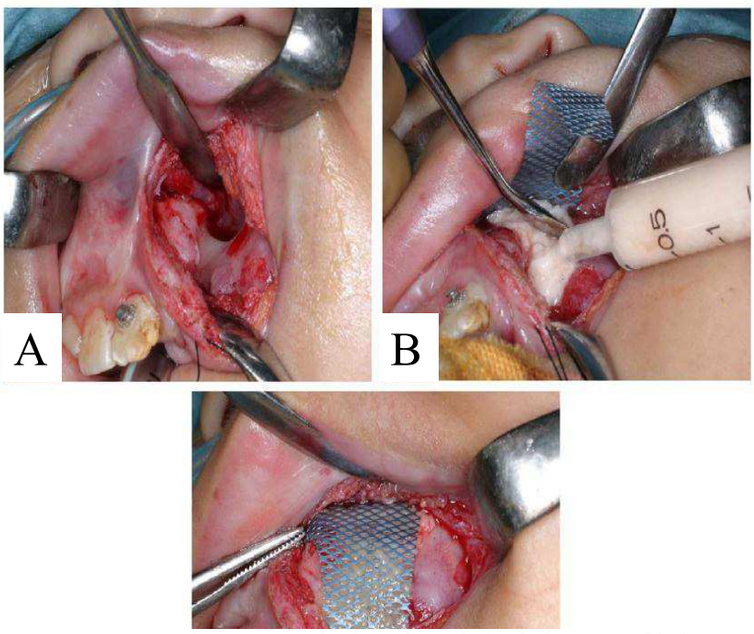

Fig. 21. Intraoperative views: A: Exposed alveolar cleft defect. B: Cleft cavity grafted with the tissue-engineered osteogenic material. C: Graft covered with the titanium mesh plate (From Hibi et al. 2006. Reprinted with permission).

This material regenerated the bone in the alveolar cleft defect without donor-site morbidity resulting from the autologous bone graft. Grafted bone remodels new bone due to apposition following resorption, and Van der Meij et al. reported that 1-year postoperative volumetric rates were approximately 70% for secondary bone grafts before canine eruption [66]. Using their measuring method [67] at 9 months postoperatively the present case showed 79.1% regenerated bone. The same authors indicated that the eruption of the canine generally occurred 2 years after bone graft if the patient was 9 years old. High resorbability of the bone in the grafted region may result in the early eruption of canine (Fig. 22).

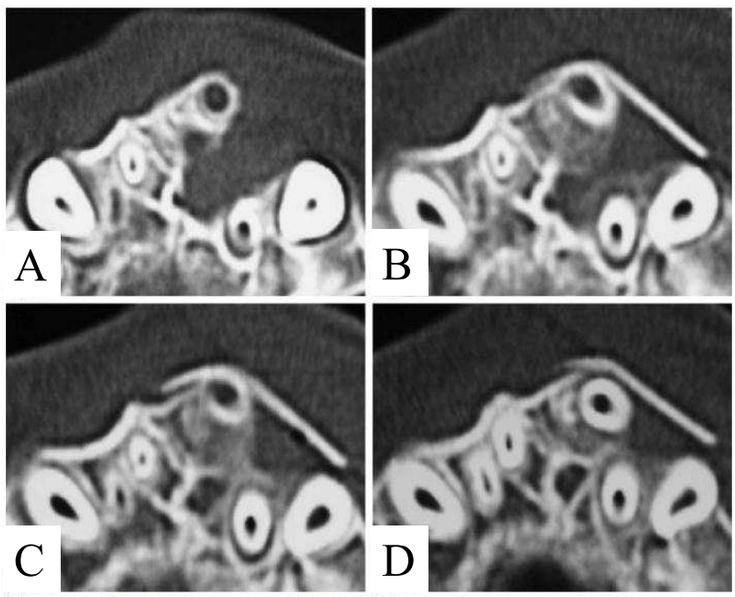

Fig. 22. Serial computed tomograms slicing the middle level of the alveolar cleft. A: Preoperation. B: Three months postoperation. Dome-shaped radiopaque images facing together and extending from the cleft bony walls inside the cavity. C: Six months postoperation. Fused image in the cleft cavity. D: Nine months postoperation. The lateral and supernumerary incisors are approximated in the bony bridge lateral to the reconstructed incisive canal (From Hibi et al. 2006. Reprinted with permission).

In the present case the canine coronally forced the mesh plate at 9 months postoperatively, which was earlier than expected. As the bone regenerated in the cleft defect, the ingrowing bone seemed to accompany the roots of not only the canine but also the lateral and supernumerary incisors, which consequently approximated and erupted. Bone regeneration with the injectable tissue-engineered may therefore have helped to induce teeth to reposition properly in the horizontal and vertical planes. Distraction of the transport bony segment has been attempted for closing alveolar defects. The defects are actually only reduced and not eliminated, and the teeth in the transport segment also were moved unintentionally according to the distraction. Some alteration in teeth positions may be beneficial, but others compromise crown morphology or require its recontouring. The bone transport in repair of the alveolar cleft therefore remains controversial (Fig. 23). Injectable tissue-engineered bone thus has a promising future. Its repeatability will also facilitate the sequential treatments of cleft palate patients.

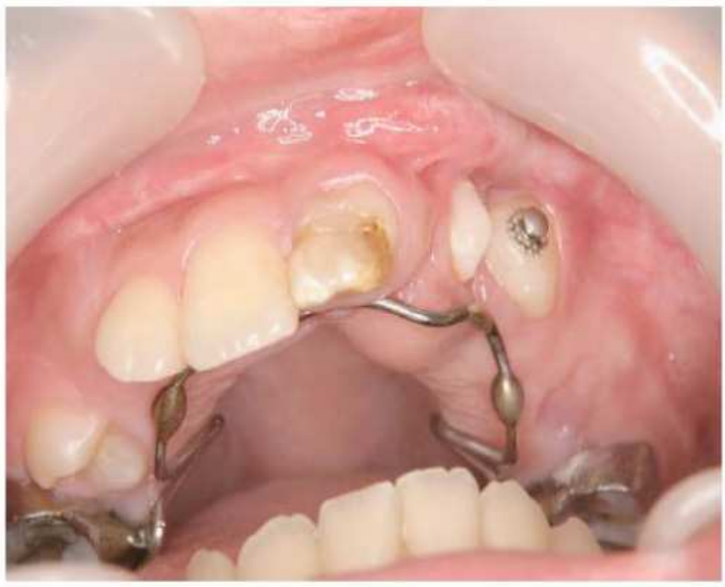

Fig. 23. The canine and the lateral incisor erupting in the reconstructed alveolar ridge (From Hibi et al. 2006. Reprinted with permission).

Taken together injectable tissue-engineered bone would provide a further option as a graft material for maxillary sinus floor augmentation, periodontal treatment, and distraction. The use of injectable tissue-engineered bone may well decrease healing time in days to come. Further, a tissue-engineered bone-induced increase in bone mass would potentially provide a great benefit to patients in cranio-maxillofacial and plastic surgery and to the bone reconstruction of other parts. Future research must address the long-term success rates of implants, the stability of tissue-engineered bone, and the application of the therapy to a less vascularized environment. Based on the present findings, future clinical trials are warranted.

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(Ito K, Yamada Y, Naiki T, Usami K, Mizuno H, Okada K, Narita Y, Aoki M, Kondo T, Mizuno D, Mase J, Nishiguchi H, Kagami H, Kinoshita K, Hibi H, Nagasaka T, Ueda M)

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Applied Tissue Engineering

Edited by

ISBN 978-953-307-689-8
Hard cover, 76 pages
Publisher InTech
Published online 08, June, 2011
Published in print edition June, 2011

Tissue engineering, which aims at regenerating new tissues, as well as substituting lost organs by making use of autogenic or allogenic cells in combination with biomaterials, is an emerging biomedical engineering field. There are several driving forces that presently make tissue engineering very challenging and important: 1) the limitations in biological functions of current artificial tissues and organs made from man-made materials alone, 2) the shortage of donor tissue and organs for organs transplantation, 3) recent remarkable advances in regeneration mechanisms made by molecular biologists, as well as 4) achievements in modern biotechnology for large-scale tissue culture and growth factor production.

This book was edited by collecting all the achievement performed in the laboratory of oral and maxillofacial surgery and it brings together the specific experiences of the scientific community in these experiences of our scientific community in this field as well as the clinical experiences of the most renowned experts in the fields from all over Nagoya University. The editors are especially proud of bringing together the leading biologists and material scientists together with dentist, plastic surgeons, cardiovascular surgery and doctors of all specialties from all department of the medical school of Nagoya University. Taken together, this unique collection of world-wide expert achievement and experiences represents the current spectrum of possibilities in tissue engineered substitution.

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