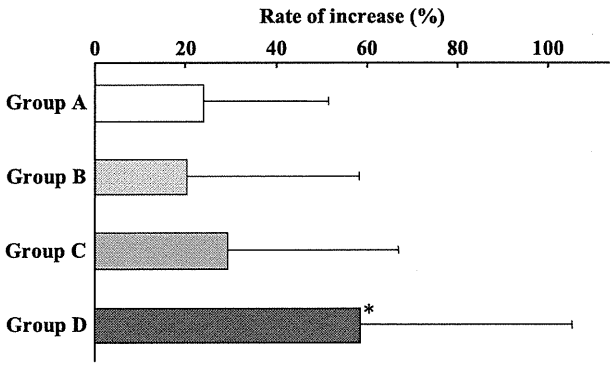

Fig. 4. Rates of increased alveolar bone heights. Placebo (group A) and recombinant human basic fibroblast growth factor at 0.03% (group B), 0.1% (group C) and 0.3% (group D) were applied to root surfaces associated with infrabony defects. Standardized radiographs were compared pre- and post-treatment (9 months) and the increased bone heights were assessed by five individual oral radiologists for increased alveolar bone heights. No statistical differences were noted with the exception of group D (*P = 0.02).

Application of tissue engineering principles: implant site preparation

A second area of focus is applying tissue engineering principles for implant site preparation. Whereas the challenge for periodontal regeneration is the simultaneous regeneration of the three tissues to reconstruct the periodontal apparatus, the challenge for implant site preparation is to regenerate adequate volume of hard and possibly soft tissue. Though much of the focus in this field has centered on the use of recombinant human bone morphogenetic protein, some preliminary data are available which suggest that recombinant human platelet-derived growth factor-BB may be used for this purpose.

The use of recombinant human platelet-derived growth factor for implant site preparation

Preliminary data are now available which suggest that recombinant human platelet-derived growth factor-BB may be used for implant site preparation. In a standardized dog model, recombinant human platelet-derived growth factor was used in conjunction with an anorganic bone block for vertical ridge augmentation (149). Surgically created defects were grafted with block grafts infused with recombinant human platelet-derived growth factor with and without collagen membrane. Better healing and an increased amount of regenerated bone were observed in sites grafted with recombinant human platelet-derived growth factor-infused block in the absence of a barrier membrane. When these blocks were analyzed utilizing backscattered electron microscopy, the percentage of weight and volume calcium:phosphorus ratios of the regenerated and native bone were found to be similar (125). This suggests that the regenerated bone would have a similar bone-implant interface compared with native bone.

Recently, recombinant human platelet-derived growth factor-BB [from a GEM-21® kit (Osteohealth, Shirley, NY)] was used in conjunction with freeze-dried bone allograft and a barrier membrane to augment both hard and soft tissues simultaneously in preparation for implant placement (29). Following

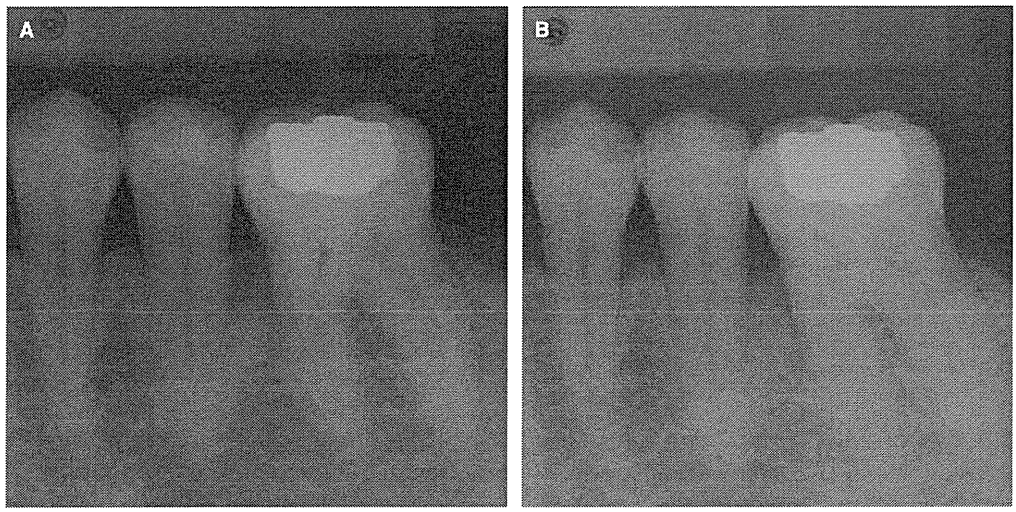

Fig. 5. (A) Tooth #20 presented with a clinical attachment loss of 10 mm and a pocket depth of 5 mm. The defect was treated with 0.3% recombinant human fibroblast growth factor. (B) After 9 months post-surgically, the clinical attachment loss was 7 mm and pocket depth was 2 mm (photographs courtesy of Dr Matsuyama, Kagoshima University, Kagoshima, Japan).

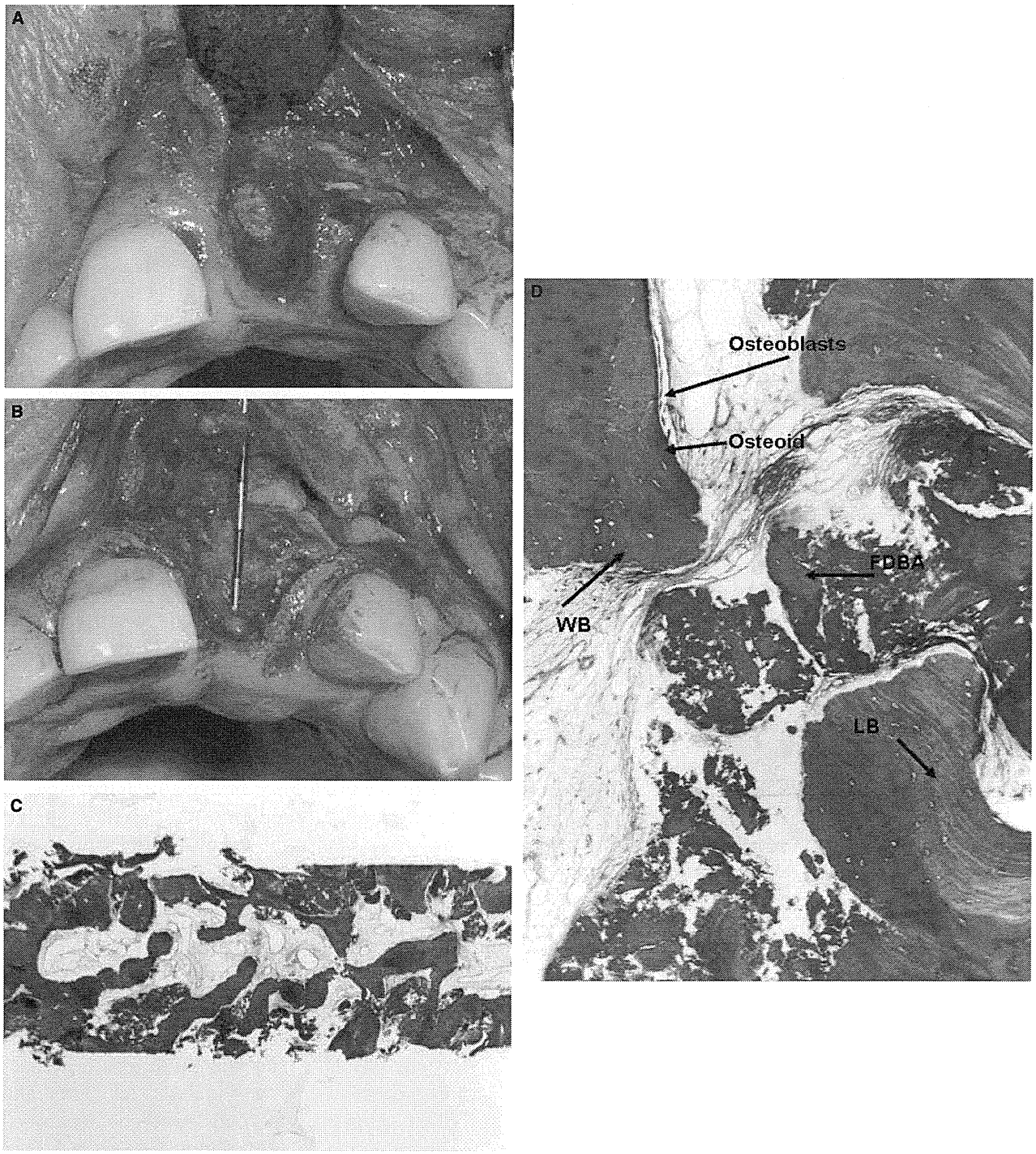

Fig. 6. The extraction defect for tooth #9 was treated with recombinant human platelet-derived growth factor-freeze-dried bone allograft and a titanium-reinforced membrane (A). On re-entry, the defect was filled with bone (B). Histologic analysis (C, D) showed the new bone formation. FDBA, freeze-dried bone allograft; LB, linear bone formation; WB, woven bone.

extraction, the bony defect was filled with recombinant human platelet-derived growth factor-freeze-dried bone allograft and covered with expanded polytetrafluoroethylene membrane (Fig. 6A,B). The soft-tissue deficiency was grafted with a pediculated graft from the palate and the soft tissue site was irrigated with recombinant human platelet-derived growth factor prior to closure. After healing, the amount of both bone and soft tissue volume increased. At the time of implant placement, the site was trephined for histological analysis, which revealed the presence of bone regeneration. The

microscopic field contained approximately 48% woven bone and 19% mineralizing osteoid (Fig. 6C,D). This report emphasizes the potential of the use of recombinant human platelet-derived growth factor for simultaneous soft and hard tissue implant site preparation.

The use of bone morphogenetic protein for implant site preparation

Recent attention has focused on recombinant human bone morphogenetic protein-2 as a replacement for autogenous bone grafts because it reliably induces bone formation and large quantities can be produced using recombinant DNA technology (167). Using various animal species, including nonhuman primates, recombinant human bone morphogenetic protein-2 absorbed into bovine type-1 collagen sponges consistently induced bone at graft sites (16, 38, 107, 160, 171). The recombinant human bone morphogenetic protein-2-soaked collagen sponges reliably induced bone formation in critical sized defects, whereas the defects repaired with sponges without recombinant human bone morphogenetic protein-2 did not fill with bone. Continuity defects and deficient alveolar ridges and maxillary sinuses were successfully reconstructed with recombinant human bone morphogenetic protein-2 (16, 107). Much higher concentrations of recombinant human bone morphogenetic protein-2 were needed to induce bone formation in nonhuman primates than in rodents and rabbits.

Human clinical trials were initiated following the promising results in animals. A feasibility study using an open-label clinical trial demonstrated that recombinant human bone morphogenetic protein-2 soaked into a collagen sponge and placed on the maxillary sinus floor stimulated bone formation (18). A randomized prospective muticenter clinical trial (17) was initiated following the open-label study. Implant survival in the maxillary sinuses augmented with recombinant human bone morphogenetic protein-2 absorbed into collagen sponges was similar to survival of implants placed in sinuses augmented with autografts. A dose–response performed as part of the study showed that the greatest bone induction occurred when 1.5 mg/ml of recombinant human bone morphogenetic protein-2 was used.

Biopsies of the bone induced by the recombinant human bone morphogenetic protein-2 were taken after approximately 7 months of healing. Histological examination revealed that the recombinant human bone morphogenetic protein-2 induced new bone formation. The collagen sponges were no longer present and woven, and lamellar bone filled the grafted sinus floors. The study demonstrated that recombinant human bone morphogenetic protein-2 could induce adequate bone for the placement and functional loading of endosseous implants.

The efficacy of bone morphogenetic protein-2 to augment deficient alveolar ridges has also been evaluated. A preliminary feasibility and safety study demonstrated that recombinant human bone morphogenetic protein-2 on a collagen sponge was safe and might be useful for alveolar ridge augmentation and preservation of bone following tooth extraction (68). The preliminary study was followed by a randomized prospective clinical trial that recruited patients requiring alveolar ridge augmentation following tooth extraction (31). The recombinant human bone morphogenetic protein-2 on the collagen sponge induced more alveolar bone than the collagen sponge alone.

Based on the animal studies and the human clinical trials, the Food and Drug Administration approved recombinant human bone morphogenetic protein-2 (INFUSE®) for use 'as an alternative to autogenous bone graft for sinus augmentations, and for localized alveolar ridge defects associated with extraction sockets' in March 2007 (1). However, the cost of treatment with recombinant human bone morphogenetic protein-2 is high, and less-expensive augmentation materials may be equally as effective as recombinant human bone morphogenetic protein-2 for the augmentation of the maxillary sinuses and ridge preservation following the extraction of teeth (2).

A systematic review of the evidence established predictable augmentation, by alloplasts, allografts, combinations of allografts and alloplasts, and barrier membranes, of maxillary sinus and alveolar ridges for implants (2). Similarly to the biopsies of the sites augmented with recombinant human bone morphogenetic protein-2, biopsies taken from maxillary sinuses augmented with alloplasts/allografts and combinations of materials consistently demonstrated bone induction (132, 136). Figure 7 shows a biopsy taken 6 months after augmentation of a maxillary sinus with freeze-dried cancellous bone allograft (Northwest Tissue Bank, Seattle, WA) combined with hydroxyapatite (Interpore®; Interpore Cross International, Irvine, CA); new bone formation adjacent to the hydroxyapatite and cancellous particles is evident.

Further studies comparing recombinant human bone morphogenetic protein-2 with alloplasts and allographs and combinations of materials are needed to determine if less-expensive materials are equally

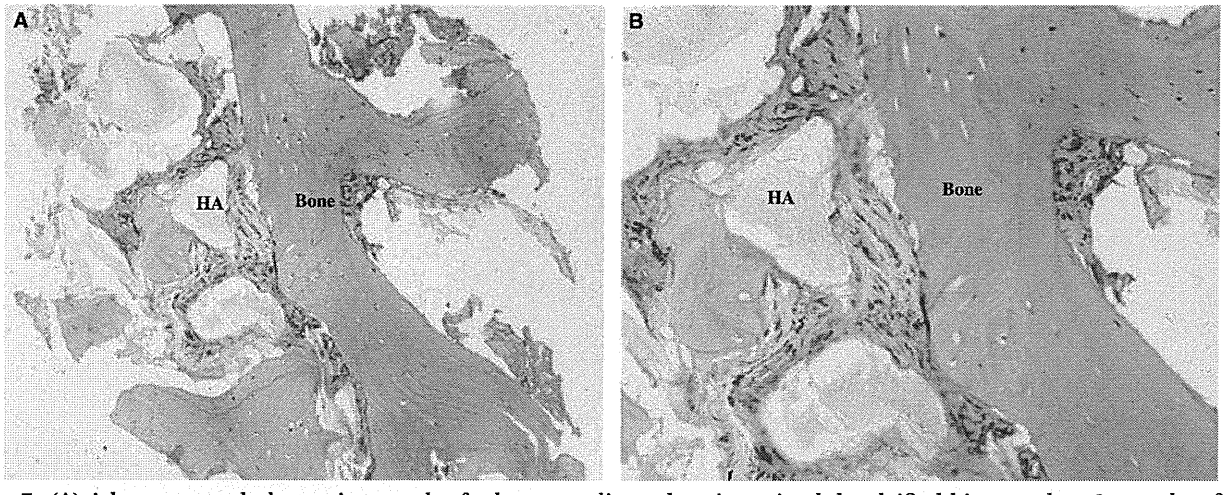

Fig. 7. (A) A low-powered photomicrograph of a hematoxylin and eosin-stained decalcified biopsy taken 6 months after grafting the sinus floor with hydroxyapatite combined with freeze-dried cancellous bone chips. (B) A higher magnification of the same biopsy. New bone (Bone) can be seen adjacent to the hydroxyapatite / cancellous chip. HA, hydroxyapatite.

as effective as recombinant human bone morphogenetic protein-2 before routinely using recombinant human bone morphogenetic protein-2 to augment sites with inadequate bone for the placement of endosseous implants.

Application of tissue-engineering principles: maxillofacial surgical procedures

Tissue regeneration for maxillofacial congenital and acquired defects involves several elements. Cells, scaffolds and growth factors are needed to regenerate a functional replacement for the missing tissue (20, 80). The ideal combination of elements is the current focus of research related to maxillofacial tissue engineering. This discussion will focus on reconstruction of bone defects.

Continuity defects of the mandible frequently develop following trauma or removal of tumors. Large defects are usually repaired using autogenous bone harvested from the iliac crest (80). The autogenous graft is considered the 'gold standard' because it contains three essential elements that are needed to regenerate bone: osteoblasts and osteoprogenitor cells; osteoinductive proteins; and a scaffold of organic and inorganic extracelluar matrix. Tissue-engineered replacements ideally should contain the three elements present in the autogenous bone graft (20, 78). Synthetic scaffolds that replace the structural integrity of the missing bone can be fabricated from several different materials. However, the scaffold must be resorbed and replaced with normal bone because the synthetic scaffold will eventually fatigue and fracture from long-term loading. The scaffolds should be porous to allow adherence of cells and osteogenic proteins as well as vascular invasion into the matrix. Allografts have been used as scaffolds; however, they are not ideal because they have the potential to be immunogenic and to transfer infection (20, 78). The ideal synthetic scaffold should not be immunogenic or have the potential to transfer / support infection. Hollister et al. (64) described an approach to engineer a craniofacial scaffold that used computational design, scaffold fabrication, scaffold structural and mechanical evaluation, and *in vivo* tissue-regeneration tests to develop scaffolds that meet anatomical, load-bearing and tissue-regeneration requirements.

Cells capable of replacing missing tissue are an essential component of engineered tissue replacements. Osteoblasts and mesenchymal stem cells that can differentiate into bone-forming cells are found in bone, bone marrow and periosteum (33, 76, 175). Unlike osteoclasts, which are derived from circulating cells, the osteoprogenitor cells must be present at the graft site or be part of the tissue-engineered graft. Bone marrow aspirate contains adult mesenchymal stem cells that can be induced to transform into osteoblasts and to replace bone (20, 85). Mesenchymal stem cells can be harvested from bone marrow or periosteium and expanded in culture using specific growth media. Stem cells have also been isolated and cultured from dental pulp (85). Large bone defects that no longer have their associated periosteum have few cells capable of transforming into osteoblasts and frequently cannot be repaired without including osteogenic cells in the graft.

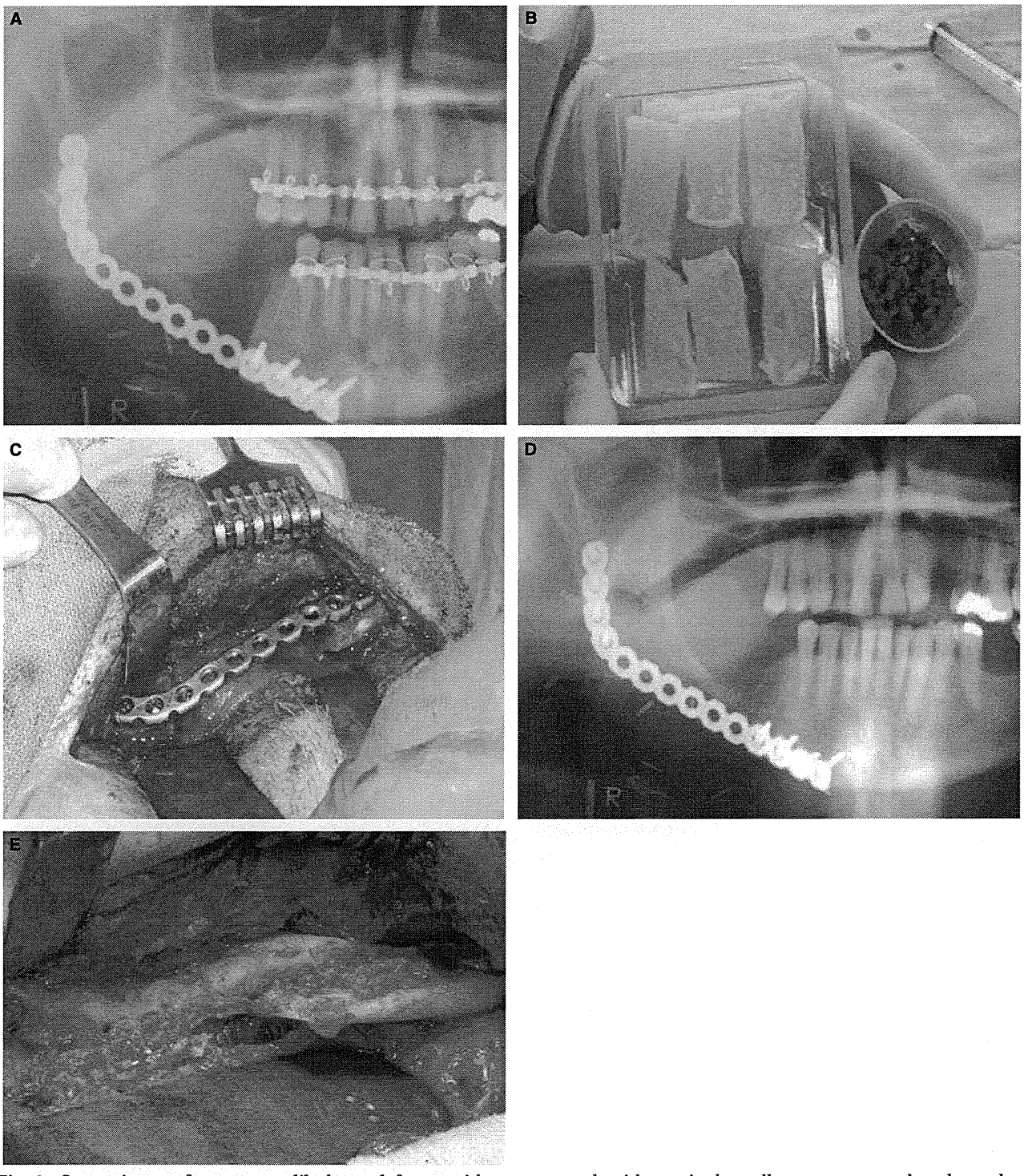

Fig. 8. Correction of a mandibular defect with recombinant human bone morphogenetic protein. (A) Panoramic radiograph of the continuity defect of the right mandible following resection of the patient's infected bone. (B) The six sponges contain a total of 12 mg of recombinant human bone morphogenetic protein. The cup contains cortical cancellous bone chips mixed with bone marrow aspirate from the iliac crest. (C) The defect is exposed with a single collagen sponge placed at the medial aspect of the mandible. The wrapped bone chip / cell mix sponges were placed between the plate and the collagen sponge. (D) Panoramic radiograph of the repair after 8 months. The radiograph is suggestive of bone bridging the defect. (E) Re-entry for the removal of the reconstruction plate indicates bone regeneration in the defect.

Growth factors and morphogens are needed to stimulate cell differentiation and regeneration of tissue (20, 78). Many signaling factors contribute to tissue regeneration. Further studies are needed to determine which factors are essential to regenerate a defect rapidly. These factors do not appear at the same time during healing and it may be necessary to deliver different signaling proteins at particular points during the healing process to regenerate the missing tissue reliably.

Currently, some reports of off-label use of osteogenic proteins, such as recombinant human bone morphogenetic protein-2, show promising results (21, 62, 85). Fig. 8A shows a bone defect resulting from an infection in a fractured mandible. The patient lost teeth, and ultimately part of his right mandible was resected to resolve his infection. An autogenous graft from the iliac crest was recommended for repair of the defect. Because the patient was concerned about the recovery from harvesting bone from the iliac crest, he was informed that recombinant human bone morphogenetic protein-2, which was not approved by the Food and Drug Administration for the reconstruction of mandibular continuity defects, might stimulate bone healing in the defect. Because of a previous failure using recombinant human bone morphogenetic protein-2 alone, a combination graft was used. Bone marrow cells were aspirated from the patient's hip (autogenous mesenchymal stem cells) and mixed with freeze-dried cancellous bone chips (allograft scaffold). The collagen sponges impregnated with 1.5 mg/ml of recombinant human bone morphogenetic protein-2 were wrapped around the bone chip/cell mix. Fig. 8B shows the impregnated sponges and the bone/chip/cell mix, and Fig. 8C shows the repair of the defect. Several months after reconstruction, mandibular continuity was restored (Fig. 8D). The patient developed pain at the reconstructed site and the plate was removed 10 months after surgery using recombinant human bone morphogenetic protein-2. Fig. 8E shows the bone that bridged the defect. There was inadequate bone height for implant reconstruction and additional grafting will be required if the patient is interested in implant placement.

Reconstruction of continuity defects in the mandible with recombinant human bone morphogenetic protein-2 is not predictable (21). Herford et al. have reported several cases of successful reconstruction of cleft palates, mandibular continuity defects and atrophic alveolar ridge defects using recombinant human bone morphogenetic protein-2 alone (62, 63). A combination of osteoinductive proteins, mesenchymal stem cells and synthetic scaffolds must be developed before reconstruction of bone defects using tissue-engineering methods will provide predictable results. Preliminary case reports of off-label use of the recombinant human bone morphogenetic protein-2 demonstrate that it is possible to reconstruct bone defects with an osteoinductive protein produced using recombinant DNA technology (21, 62, 63). However, more studies are needed to design the ideal combination of factors, cells and scaffolds to reconstruct bony defects using tissue engineering in a reliable manner.

Are we there yet?

As reports of tissue-engineering successes become more prevalent, clinicians increasingly demand predictable and faster treatment modalities. But, have we reached that point? In this article, some of the concerns regarding tissue engineering are discussed. Clinicians should be aware of these to understand in more detail the results they will obtain in practice.

This review should help practitioners understand that there are many variables of tissue engineering that need further investigation. Throughout the investigations conducted to identify the ideal concentration of recombinant human platelet-derived growth factor for periodontal regeneration and recombinant human bone morphogenetic protein for implant site preparation kits, several concentrations were used (Table 3). In the case of recombinant human platelet-derived growth factor, doses that are too high or too low resulted in little or no significant amount of periodontal regeneration. A similar pattern was observed with recombinant human bone morphogenetic protein studies. This raises concerns of whether each different type of surgical procedure will require a different concentration for an optimal response.

Another issue is what are the ideal properties for the scaffold? Although most clinicians have focused on the signaling molecules or biologic mediators, the scaffold may be just as critical in determining the volume and shape of the regenerated tissue. This is of paramount importance for implant site preparation because the desired volume and vertical proportions are a key to success.

Does the regenerated tissue behave and act like the original tissue? In a vertical bone-grafting study utilizing recombinant human platelet-derived growth factor-BB, the investigators analyzed the chemical

Table 3. In search of the optimal dosage for therapeutic use of signaling molecules

Signaling molecule	Dose	Regenerative response	Reference
• rhPDGF-BB	0.05 mg/ml	0	Howell et al. (67)
	0.15 mg/ml	+	Howell et al. (67)
	0.30 mg/ml	+++	Nevins et al. (108)
	1.00 mg/ml	++	Nevins et al. (108)
• rhBMP	0.43 mg/ml	+	Cochran et al. (23)
	0.75 mg/ml	+	Fiorellini et al. (31)
	1.50 mg/ml	+++	Fiorellini et al. (31)

rhBMP, recombinant human bone morphogenetic protein; rhPDGF-BB, recombinant human plate-derived growth factor-BB.

qualities of the regenerated bone and compared them with native bone to ensure they were similar to the implant–bone interface (125). Similarly, clinical regeneration induced by enamel matrix derivative, recombinant human platelet-derived growth factor and recombinant human basic fibroblast growth factor was confirmed histologically.

Are the regenerated tissues sustainable? For regenerated periodontium, the confirmatory evidence would be long-term studies of the treated defects. At this point, 5-year data are available for enamel matrix derivative and 2-year data are available for recombinant human platelet-derived growth factor. For bone regeneration for implant placement, the concerns would be twofold. First, is the quality of bone that forms similar to that of native bone? This would be important to achieve a similar bone–implant interface with regenerated bone compared with native bone. The ultimate test would be whether the survival rates of implants placed in tissue-engineered bone are the same as those for implants placed in native bone. The second issue is whether tissue-engineering applications are critical for wound healing. Given the added expense, will the application of tissue engineering speed the healing process and ensure final healing results to the point where the added expense is justified? By contrast, the regeneration of infrabony defects is a competition between cells that will result in healing vs. regeneration, and the cells involved in the healing and the healing results of an extraction socket or a sinus will normally result in bone formation. Will the addition of a biologic mediator result in a higher quality of bone, increase the rate of bone formation, or ensure regeneration that otherwise would not have occurred? The challenge will be to have each claim of superior results justified by a comparative study where tissue engineering was not applied.

Can the level of response for tissue engineering be increased by adding multiple signaling molecules (as suggested by the enamel matrix derivative phenomenon), improving the scaffold, or with cell therapy? As we add each of these variables, the number of studies increases. The potential projects for researchers are almost limitless. Clinicians can help direct these investigations by being good observers of both positive and negative therapeutic responses. These observations can help to determine the design of randomized clinical trials that can improve our current therapeutic approach.

All of the aforementioned issues require significant funding for investigation. Although the National Institutes of Health have funded early investigational efforts, there is currently minimal funding in this area. As a result, much of the research is currently being underwritten by the company developing the product. This ultimately raises the cost of these materials and limits these investigations, which may lead to improved application protocols. The concern is that when the cost is high and the protocol is not perfect, there is a likelihood that several therapeutic approaches may not be accepted by the profession. This, in turn, could lead to an abandonment of tissue-engineering approaches. Of all the surgical fields where tissue engineering can be applied, the oral environment is probably the most challenging and yet a developmental area where limited success, or even failure, is not life-altering or life-threatening. If tissue-engineering approaches are to be refined, the oral environment is the perfect model for this development.

Summary

Over the past three decades, the dental literature has been filled with reports related to the regeneration of

periodontal tissues. This therapeutic goal, although ideal, has been difficult to achieve. A variety of new regenerative strategies utilizing tissue-engineering principles are now available. Despite certain limitations, our ability to provide regenerative therapeutics continues to evolve. As we do so, we continue to improve our understanding of the physical and biologic requirements necessary for specific tissue regeneration. This understanding will help us to improve our manipulation of the various elements of tissue engineering (signaling molecules, scaffold and cells) to generate specific regenerative responses. This knowledge will help us develop better therapeutic approaches so tissues will regenerate faster and provide our patients with more predictable outcomes.

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Nicotine can skew the characterization of the macrophage type-1 (MΦ1) phenotype differentiated with granulocyte-macrophage colony-stimulating factor to the MΦ2 phenotype

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ABSTRACT

Macrophages (MΦs) exhibit functional heterogeneity and plasticity in the local microenvironment. Recently, it was reported that MΦs can be divided into proinflammatory MΦs (MΦ1) and anti-inflammatory MΦs (MΦ2) based on their polarized functional properties. Here, we report that nicotine, the major ingredient of cigarette smoke, can modulate the characteristics of MΦ1. Granulocyte-macrophage colony-stimulating factor-driven MΦ1 with nicotine (Ni-MΦ1) showed the phenotypic characteristics of MΦ2. Like MΦ2, Ni-MΦ1 exhibited antigen-uptake activities. Ni-MΦ1 suppressed IL-12, but maintained IL-10 and produced high amounts of MCP-1 upon lipopolysaccharide stimulation compared with MΦ1. Moreover, we observed strong proliferative responses of T cells to lipopolysaccharide-stimulated MΦ1, whereas Ni-MΦ1 reduced T cell proliferation and inhibited IFN-γ production by T cells. These results suggest that nicotine can change the functional characteristics of MΦ and skew the MΦ1 phenotype to MΦ2. We propose that nicotine is a potent regulator that modulates immune responses in microenvironments.

Introduction

Macrophages (MΦs) exhibit many biological functions. The functional heterogeneity and plasticity of MΦs depend on the local microenvironment [1,2]. MΦs have polarized functional properties and can be classified into at least two types, namely classical MΦ (MΦ1) and nonclassical MΦ (MΦ2) [3,4]. MΦ1 are differentiated by granulocyte-macrophage colony-stimulating factor (GM-CSF), have IL-12^{high}IL-10^{low} phenotype, participate in resistance against microorganisms and tumors, and are involved in Th1 immune responses. In contrast, MΦ2 are induced by macrophage colony-forming factor (M-CSF), produce IL-10 but not IL-12, and promote anti-inflammatory responses, tissue remodeling and angiogenesis.

Tobacco smoking is associated with increased incidences of numerous diseases such as cancers, vascular diseases, chronic obstructive pulmonary diseases and periodontal diseases [5–7]. For example, smoking-induced immunosuppression, reduction of natural killer cell cytotoxicity, and inhibition of proinflammatory cytokine production and the microbicidal activity of alveolar macrophages [8,9], have been implicated in the immunopathogenesis of these diseases, although tobacco smoke may also ameliorate inflammation [10–12]. Nicotine is one of the main components of tobacco smoke and a selective agonist of nicotinic acetylcholine receptors (nAChRs). Although the expression of nAChRs was first discovered in the central nervous system, nAChRs are also present in non-neuronal cells. A recent study suggested that acetylcholine produced after vagus nerve stimulation inhibits the release of proinflammatory cytokines from MΦs, and that nAChRα7 is essential for the attenuation of proinflammatory cytokine production [13].

In this study, we hypothesized that nicotine exposure can modulate the differentiation of MΦs. We demonstrate that nicotine promotes monocyte differentiation into IL-12^{low} MΦ1 (Ni-MΦ1) with MΦ2 features. Ni-MΦ1 are associated with reduced allogenic T cell stimulatory capacity and Th1 responses, but generate IL-10-producing T cells. Our findings suggest the possibility that nicotine exposure is involved in the heterogeneity and plasticity of the monocyte-macrophage lineage.

Materials and methods

Isolation of monocytes, and generation of MΦ1 and MΦ2. The protocol for this study was reviewed and approved by the Institutional Review Board of the Osaka University Graduate School of Dentistry. All the subjects participated in the study after providing informed consent. Human monocytes were purified from peripheral blood mononuclear cells (PBMCs) isolated from healthy volunteers by

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standard density gradient centrifugation using Histo-Paque 1077 (Sigma–Aldrich, St. Louis, MO). The cells were further purified by magnetic cell sorting with anti-CD14 microbeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. The purity of the $CD14^+$ monocytes was more than 98%. MΦ1 and MΦ2 were generated in complete RPMI-10 (RPMI-1640 containing 10% heat-inactivated fetal calf serum, 20 mM Hepes, 50 μg/ml gentamicin, 100 U/ml penicillin and 100 μg/ml streptomycin) supplemented with GM-CSF (5 ng/ml) or M-CSF (25 ng/ml) for 5 or 6 days, respectively. In some experiments, MΦ1 and MΦ2 were generated in the presence of nicotine (10^{-3} M). Nicotine was prepared in PBS and neutralized to pH 7.2. $CD4^+$ naive T cells were obtained from PBMC-isolated CD4 T cells using a Human $CD4^+$ T cell isolation kit (Miltenyi Biotec). $CD45RA^+$ cells were isolated from $CD4^+$ T cells using CD45RO Microbeads (Miltenyi Biotec).

Analysis of MΦ1 and MΦ2 surface molecules by flow cytometry. The expressions of surface molecules were evaluated by flow cytometry. Briefly, the cells were incubated with fluorescently labeled monoclonal antibodies at 10 μg/ml or isotype-matched control antibodies for 30 min at 4 °C in the dark. The FITC-conjugated antibodies (anti-CD14 and anti-CD163) and PE-conjugated antibodies (anti-CD1a, anti-CD11b, anti-CD16, and anti-CD206) used were obtained from BD Biosciences (San Jose, CA). The cells were washed twice and data were acquired using a FACSCalibur (BD Biosciences). Analyses of viable cells were performed using the CELLQuest™ software (BD Biosciences).

Assays for antigen-uptake activity. To examine the endocytic activity of MΦ1 and MΦ2, the cells were incubated with 0.1 mg/ml FITC-dextran (Sigma–Aldrich) or Lucifer yellow (Sigma-Aldrich) for 1 h at 4 °C or 37 °C. Cells were washed with PBS and analyzed by flow cytometry.

T cell proliferation assay. MΦs were cultured under the above-described conditions for 24 h, treated with mitomycin (50 μg/ml) for 1 h and cocultured with 1×10^5 allogeneic naive $CD4\ T^+$ cells for 6 days. The cells were pulsed with 0.5 μCi/well of ^{3}H-labeled thymidine (Amersham Pharmacia, Buckinghamshire, UK) for the last 8 h of the 6-day culture period, followed by scintillation counting. The results were calculated as the mean cpm values ± SD obtained from triplicate cultures.

Assays for cytokine and chemokine production. To measure cytokine secretion, monocytes, MΦ1 and MΦ2 were stimulated with 10 ng/ml lipopolysaccharide (LPS; *Salmonella minnesota*; List Biological Laboratories Inc., Campbell, CA) for 24 h, and the supernatants were harvested. The cytokine levels in the supernatants were measured using IL-8, IL-10, IL-12 and MCP-1 ELISA kits (Pierce Endogen, Rockford, IL). Each sample was assayed in triplicate. The supernatants harvested from the above-described allogeneic T cell proliferation assays were measured for their IFN-γ and IL-10 levels. In some experiments, T cells were restimulated with a plate-bound anti-human CD3 antibody (2 μg/ml; BD Biosciences) for 24 h.

Results and discussion

Characteristics of surface markers of MΦ1 and MΦ2 in the presence or absence of nicotine

Monocytes were differentiated into MΦs in the presence of GM-CSF or M-CSF for MΦ1 or MΦ2, and GM-CSF or M-CSF plus nicotine

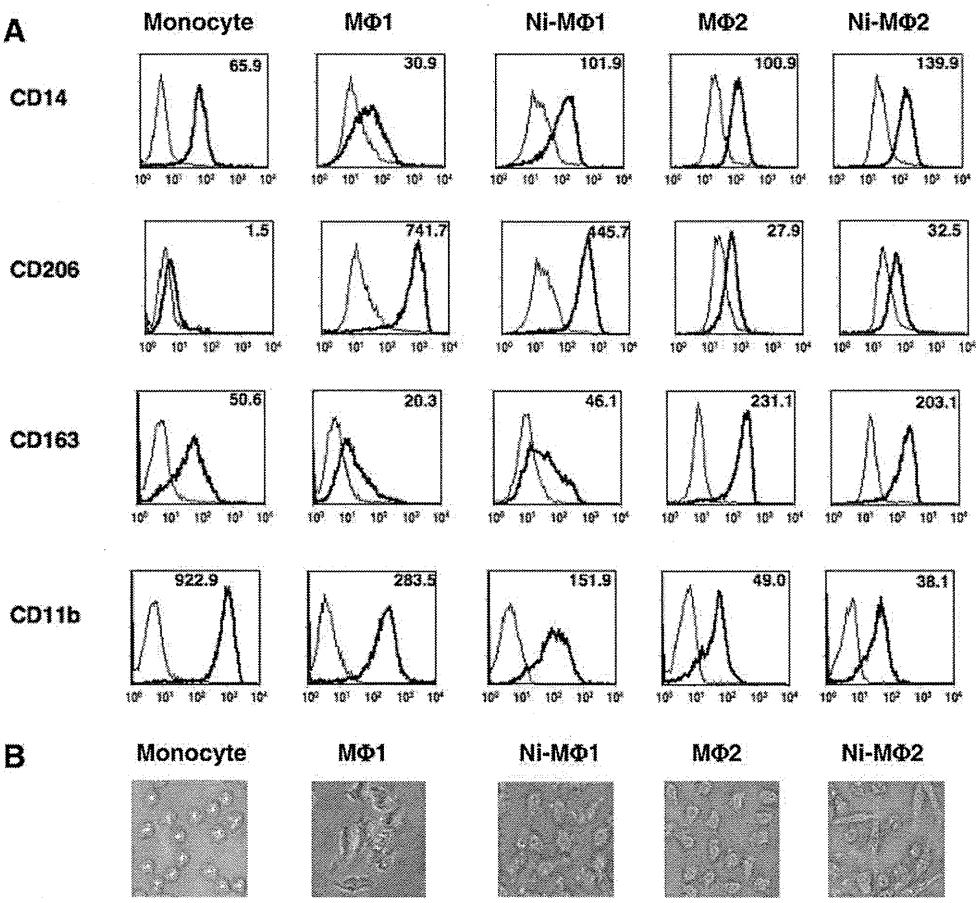

Fig. 1. Characterization of monocytes, MΦ1, Ni-MΦ1, MΦ2, and Ni-MΦ2. Monocytes were isolated from PBMCs, and MΦ1, Ni-MΦ1, MΦ2, and Ni-MΦ2 were generated in parallel from the same donor by culture for 6 days. (A) The surface molecule expressions of CD14, CD206, CD163, and CD11b on the cells were determined by flow cytometry (thick lines). The thin lines represent the isotype-matched control antibodies. Data are representative of 3–6 independent experiments. Monocytes were analyzed at day 0. (B) Morphologies of monocytes, MΦ1, Ni-MΦ1, MΦ2, and Ni-MΦ2. Data are representative of three independent experiments.

for Ni-MΦ1 or Ni-MΦ2, respectively. Following a previously described method for differentiation [14], we reproduced similar characteristics of MΦ1 and MΦ2. MΦ1 and MΦ2 shared the typical macrophage phenotype of $CD1a^-$ (data not shown), $CD11b^+$ and $CD14^+$. MΦ1 expressed consistently lower levels of CD14 and CD163 than MΦ2, but higher levels of CD206 and CD11b (Fig. 1A). Although some review papers have documented that the expression level of CD206 is higher in MΦ2 than in MΦ1 [1,2], it seems to depend on the environmental conditions for differentiation. After GM-CSF-mediated differentiation, MΦ1 express high levels of CD206 [14,15]. In contrast, M-CSF-derived MΦ2 express very low levels of CD206 [14]. CD163, a hemoglobin scavenger receptor, is associated with the nonclassical MΦ2 phenotype [16]. In the presence of nicotine, the phenotype of the surface molecules of MΦ1 (Ni-MΦ1) was somewhat similar to that of MΦ2. Ni-MΦ1 expressed higher levels of CD14 and CD163 than MΦ1, but expressed lower levels of CD206 and CD11b (Fig. 1A). The morphological findings for monocytes, MΦ1, Ni-MΦ1, MΦ2 and Ni-MΦ2 are shown in Fig. 1B. MΦ2 were less adherent, while Ni-MΦ2 exhibited a stretched spindle-like morphology. On the contrary, Ni-MΦ1 were adherent, but had rounder and more irregular shapes than MΦ1.

The findings shown in Fig. 1 suggested that Ni-MΦ1 retained the MΦ1 phenotype but were partially skewed to obtain the characteristics of the MΦ2 phenotype. Therefore, we speculated that nicotine could promote MΦs to obtain MΦ2 properties.

Effects of nicotine on antigen-uptake by Ni-MΦ1

To evaluate the antigen-uptake ability of MΦs, lectin-mediated endocytosis and macropinocytosis were examined using FITC-dextran and Lucifer yellow, respectively. Although Ni-MΦ1 expressed a lower level of CD206 than MΦ1, their uptake of FITC-dextran was more efficient than that of MΦ1 (Fig. 2A). A recent study showed that MΦ1 expressed a higher level of CD206 than MΦ2, but their uptake of FITC-dextran was comparable to that of MΦ2 owing to the involvement of lectin-independent mechanisms such as macropinocytosis [14]. The uptake of Lucifer yellow by Ni-MΦ1 was also more efficient than that of MΦ1 (Fig. 2A). Although the reason why Ni-MΦ1 were able to uptake FITC-dextran is unclear, nicotine may induce presently unidentified molecules involved in receptor-mediated endocytosis. MΦ2 have a higher capacity for Lucifer yellow uptake than MΦ1 [14]. Our data confirm that MΦ2 are active in macropinocytosis-mediated uptake of Lucifer yellow, and that Ni-MΦ1 share a strong phagocytic function with MΦ2. Unlike MΦ1, which have a low capacity for antigen-uptake, Ni-MΦ1 not only had a similar capacity to MΦ2 for macropinocytosis but also had strong characteristics for endocytosis.

The cytokine profile of Ni-MΦ1 differs from that of MΦ1

To investigate the effects of nicotine on cytokine production by MΦs, the cytokine production capacities of LPS-stimulated MΦs were examined. MΦ1 have been reported to produce large amounts of IL-12, whereas IL-10 and MCP-1 are hardly produced [2,3]. Ni-MΦ1 produced significantly lower amounts of IL-12 than MΦ1, but maintained the production of comparable levels of IL-10. Furthermore, Ni-MΦ1 showed a high MCP-1-producing capacity, which is typical of MΦ2. IL-8 was produced at constitutively high levels by all cell types. These data suggest that Ni-MΦ1 may not be as completely polarized as MΦ2, since Ni-MΦ1 were unable to produce IL-10 to the same extent as MΦ2. However, Ni-MΦ1 pro-

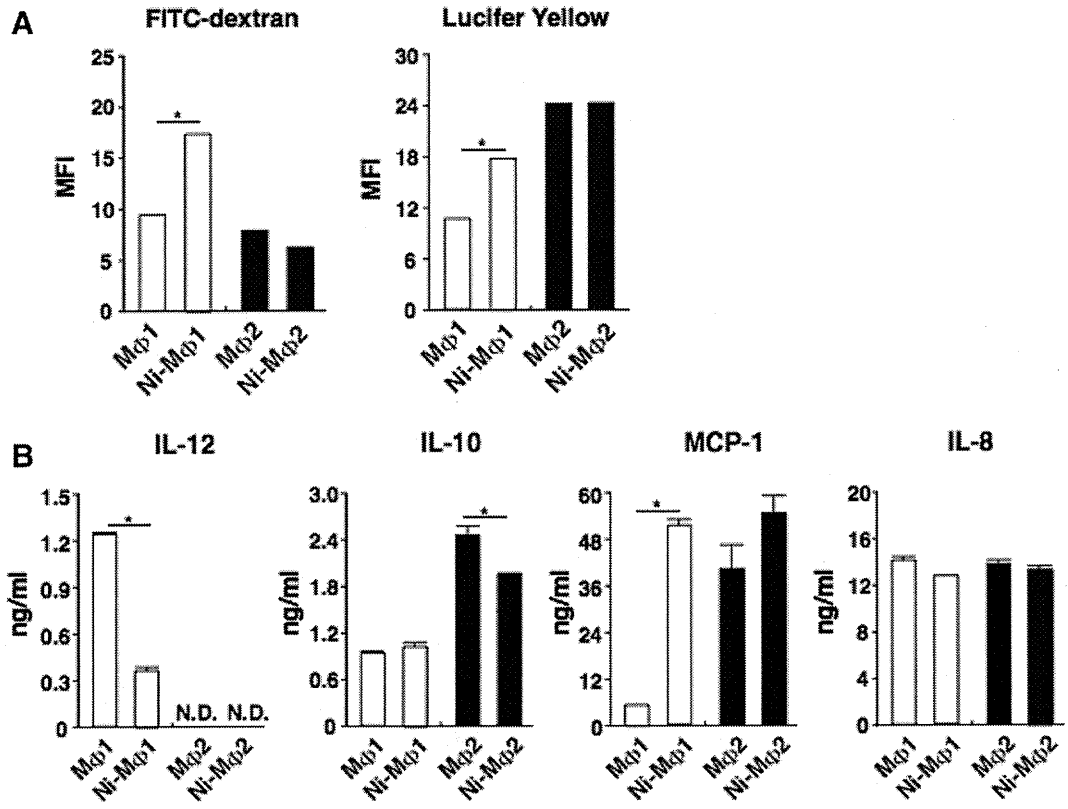

Fig. 2. Effects of nicotine on antigen-uptake and cytokine production by MΦ1, Ni-MΦ1, MΦ2, and Ni-MΦ2. (A) Uptakes of FITC-dextran (0.1 mg/ml) and Lucifer yellow (0.1 mg/ml) by MΦ1, Ni-MΦ1, MΦ2, and Ni-MΦ2 after 1 h. The results are shown as the mean fluorescence intensity (MFI) values ± SD obtained from three independent experiments. The MFI values were calculated as the MFI value at 37 °C minus the MFI value at 4 °C. *$P < 0.05$ compared with MΦs without nicotine. (B) Cytokine productions by MΦ1, Ni-MΦ1, MΦ2, and Ni-MΦ2 in the presence or absence of LPS. The data are represent the means ± SD from triplicate cultures. The data shown were obtained in one of three or four independent experiments. *$P < 0.05$ compared with MΦs without nicotine.

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duced low amounts of IL-12 and large amounts of MCP-1, suggesting that Ni-MΦ1 share anti-inflammatory properties with MΦ2.

Ni-MΦ1 show hampered T cell stimulatory activities

Next, we examined the induction of T cell proliferation by MΦs. LPS-stimulated MΦ1 induced strong allogeneic T cell proliferation, compared with MΦ2. However, LPS-stimulated Ni-MΦ1 resulted in significantly reduced T cell proliferation (Fig. 3A).

IFN-γ production by activated T cells cocultured with LPS-stimulated Ni-MΦ1 was reduced to almost half the level produced by MΦ1 (Fig. 3B). In contrast, Ni-MΦ1 exhibited reduced IL-10 production in the presence or absence of LPS stimulation, while MΦ1 failed to produce IL-10 (Fig. 3B). MΦ2 and Ni-MΦ2 were unable to induce IFN-γ production, but produced the same levels of IL-10 after LPS stimulation. Taken together, Ni-MΦ1 and MΦ2 had similar characteristics with respect to the reduction of T cell proliferation and induction of IL-10 production.

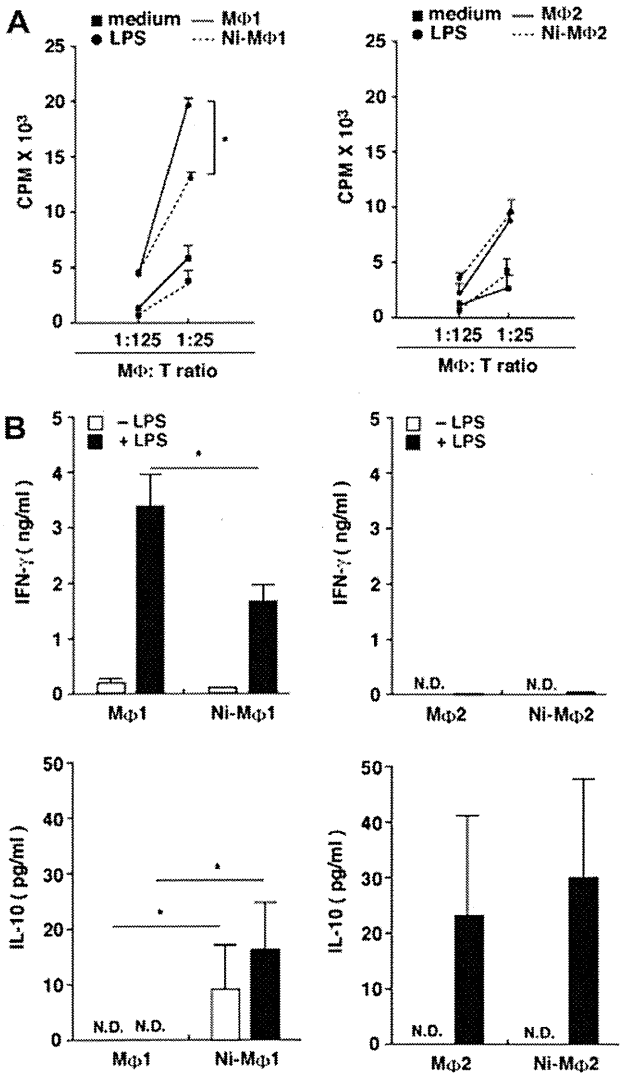

Fig. 3. T cell stimulation capacities of MΦ1, Ni-MΦ1, MΦ2, and Ni-MΦ2. (A) Comparisons of the effects of MΦ1, Ni-MΦ1, MΦ2, and Ni-MΦ2 on T cell proliferation. The results represent the mean cpm values ± SD obtained from triplicate cultures. The data shown were obtained in one of three independent experiments. *$P < 0.05$ compared with MΦ1 without nicotine. (B) Supernatants obtained from T cell proliferation assays were measured for their IFN-γ and IL-10 levels by ELISA. The results represent the mean values ± SD obtained from triplicate cultures. The data shown were obtained in one of three independent experiments *$P < 0.05$ compared with MΦ1 without nicotine.

M-CSF-derived MΦs have been reported to induce poor T cell proliferation and T cell anergy [17,18], suggesting that MΦ2 have anti-inflammatory effects and function in the maintenance of peripheral tolerance. We confirmed that Ni-MΦ1 suppressed T cell proliferation, similar to the case for MΦ2, and induced IL-10 production. In the steady-state condition in peripheral blood, GM-CSF, a proinflammatory cytokine, is hardly detected [19] whereas M-CSF is detectable [20]. As an inflammatory condition, ulcerative colitis (UC) is characterized by epithelial barrier disruption and abnormal immune responses, which induce the formation of ulcer-like lesions [21]. In UC patients, nicotine in cigarette smoke may be involved in ameliorating the disease severity, although the mechanisms remain unclear [10]. In the case of periodontal diseases, smokers tend to demonstrate reduced clinical inflammatory signs (bleeding on probing, tissue redness and edema) [11,12]. These findings for both UC and periodontal diseases suggest that cigarette smoke including nicotine can conceal the actual signs of disease severity, although nicotine may also contribute to UC remission. Our present data suggest that nicotine induces MΦs possessing anti-inflammatory and immunosuppressive properties in GM-CSF-dominant inflammatory regions. In this study, we have shown that nicotine modulates MΦ functions. However, the findings do not completely explain the effects of nicotine on human health because several kinds of cells express nAChRs and can respond to nicotine. Further studies are necessary to clarify the effects of nicotine.

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Fibroblast Growth Factor–2 Regulates the Cell Function of Human Dental Pulp Cells

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Abstract

Introduction: Homeostasis and tissue repair of dentin-pulp complex are attributed to dental pulp tissue and several growth factors. Dental pulp cells play a pivotal role in homeostasis of dentin-pulp complex and tissue responses after tooth injury. Among these cytokines, fibroblast growth factor (FGF)–2 has multifunctional biologic activity and is known as a signaling molecule that induces tissue regeneration. In this study, we examined the effects of FGF-2 on growth, migration, and differentiation of human dental pulp cells (HDPC). **Methods:** HDPC were isolated from healthy dental pulp. Cellular response was investigated by [^{3}H]-thymidine incorporation into DNA. Cytodifferentiation was examined by alkaline phosphatase (ALPase) assay and cytochemical staining of calcium by using alizarin red. Migratory activity was determined by counting the cells migrating into cleared area that had introduced with silicon block. **Results:** FGF-2 activated HDPC growth and migration but suppressed ALPase activity and calcified nodule formation. Interestingly, HDPC, which had been pretreated with FGF-2, showed increased ALPase activity and calcified nodule formation when subsequently cultured without FGF-2. These results suggest that FGF-2 potentiates cell growth and accumulation of HDPC that notably did not disturb cytodifferentiation of the cells later. Thus, FGF-2 is a favorable candidate for pulp capping agent. **Conclusions:** These results provide new evidence for the possible involvement of FGF-2 not only in homeostasis but also in regeneration of dentin-pulp complex. *(J Endod 2009;35:1529–1535)*

Key Words

Cell migration, cytodifferentiation, FGF-2, pulp cell

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Dental pulp tissue is a loose connective tissue comprising fibroblasts, blood vessels, nerves, odontoblasts, and extracellular matrix. The tissue has been recently demonstrated to include a population of putative postnatal stem cells (1–4). In fact, cultured dental pulp cells have an ability to form calcified tissue that is regulated by a complex sequence of cytokines *in vitro* (5–10). This suggests that cytokines induce regeneration of the injured dentin-pulp complex.

During development of a tooth, a plethora of cytokines contribute to the growth and differentiation of cells related to hard and soft tissue formation. Likewise, various kinds of cytokines and extracellular matrices participate during the tissue repair process after damage or injury caused by mechanical and chemical stimuli or disease processes. Among the cytokines, fibroblast growth factor (FGF)–2 is known to play an important role in the early phase of wound repair by influencing proliferation and migration and production of the extracellular matrix (11, 12).

Recently, FGF-2 localization in dentin was observed (13), suggesting that FGF-2 derived from injured dentin by bacterial, chemical, and mechanical stimuli (14) is released and might play a pivotal role in wound healing and dentin-pulp complex regeneration (15). After injury dental pulp fibroblasts release angiogenic growth factors including FGF-2 (16). FGF-2, which is embedded in the heparan sulfate matrix, is also released in dental pulp tissue during the wound healing process (17, 18). After injury of dental pulp tissue, inflammatory cell accumulation occurs, followed by migration of cells into the wound area that are responsible for tissue regeneration through interaction with a chemotactic factor and the extracellular matrix. Therefore, when dentin-pulp complex is injured and subsequent tissue repair events occur, human dental pulp cells (HDPC) can be exposed to FGF-2 and undergo activation by this cytokine. Recently, topical application of FGF-2 into experimental 3-wall bone defects or furcation defects was demonstrated to induce prominent regeneration (19–21), suggesting that FGF-2 potentiates cell activity of periodontal ligament and alveolar bone through their migration, proliferation, and cytodifferentiation. However, the effects of FGF-2 on the biologic functions in HDPC still remain to be clarified. On the basis of these findings, we hypothesized that FGF-2 plays a significant role in HDPC proliferation, migration, and mineralization. In this study, we investigated the influence of FGF-2 on cell proliferation, migration, and cytodifferentiation of HDPC.

Methods

Human Dental Pulp Cells

HDPC were isolated from healthy dental pulp of first premolar teeth of individuals undergoing tooth extraction for orthodontic treatment. All patients gave informed consent before providing samples. Healthy dental pulp tissue was removed after resection of the tooth and the center of the pulp tissue with a surgical scalpel. The tissue was minced and then transferred to plastic Leighton tubes (Costar, Cambridge, MA) with 2.5 μg/mL amphotericin B (22). The explants were cultured in α-MEM (ICN Biomedicals Inc, Costa Mesa, CA) supplemented with 10% fetal calf serum (FCS) (JRH Biosciences, Lenexa, KS), 60 μg/mL kanamycin (henceforth denoted standard medium), and medium was changed every 2 or 3 days. Cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. When cells growing from the explants had reached confluence, they were separated by treatment with trypsin