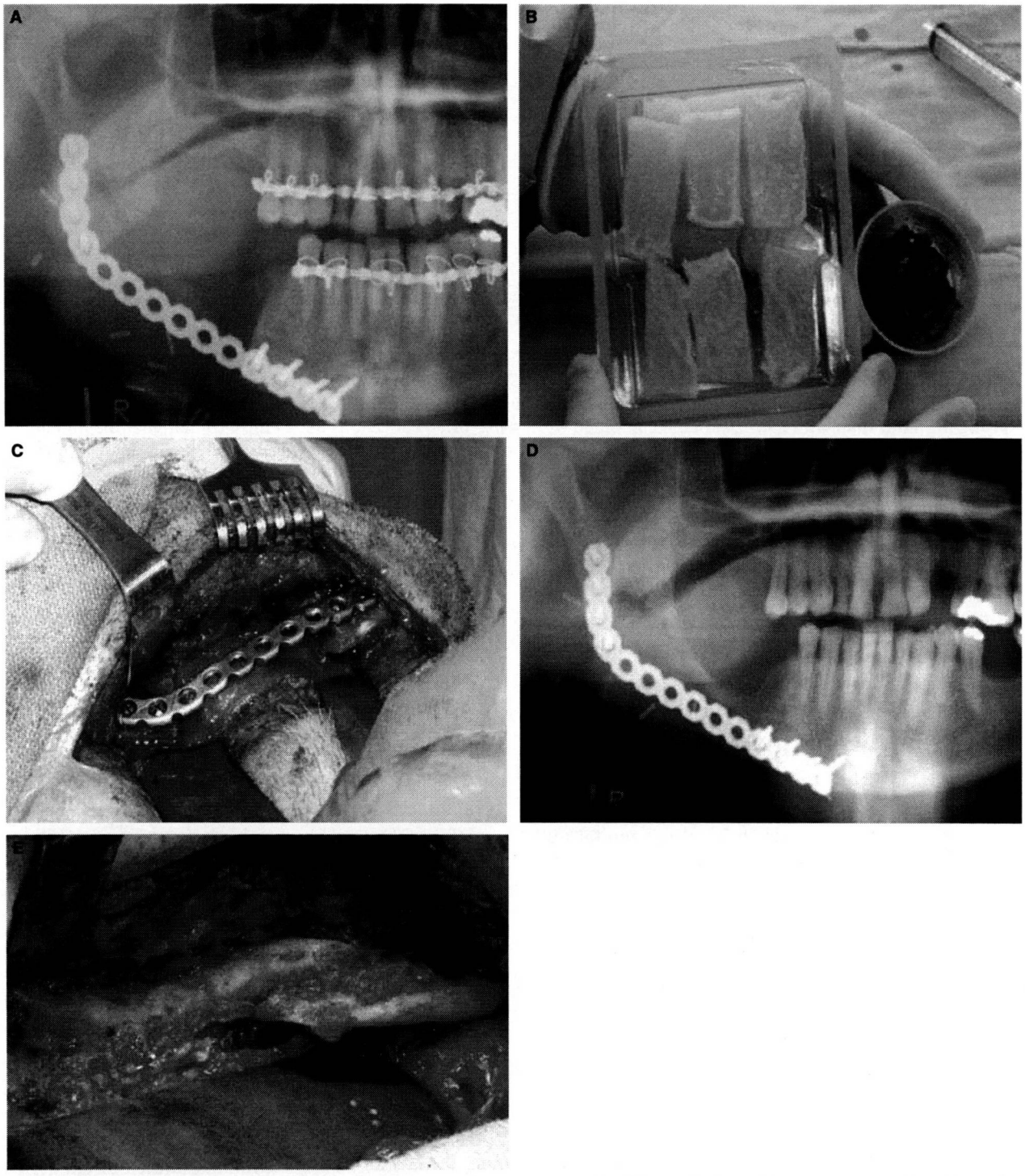

Fig. 8. Correction of a mandibular defect with recombinant human bone morphogenetic protein. (A) Panoramic radiograph of the continuity defect of the right mandible following resection of the patient's infected bone. (B) The six sponges contain a total of 12 mg of recombinant human bone morphogenetic 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

Manabu Yanagita, Ryohei Kobayashi, Shinya Murakami *

Department of Periodontology, Division of Oral Biology and Disease Control, Osaka University Graduate School of Dentistry, Osaka 565-0871, Japan

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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

* Corresponding author. Address: Department of Periodontology, Division of Oral Biology and Disease Control, Osaka University Graduate School of Dentistry, 1-8 Yamadaoka, Suita, Osaka, 565-0871, Japan. Fax: +81 6 6879 2934.
E-mail address: ipshinya@dent.osaka-u.ac.jp (S. Murakami).

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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 ^3H-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

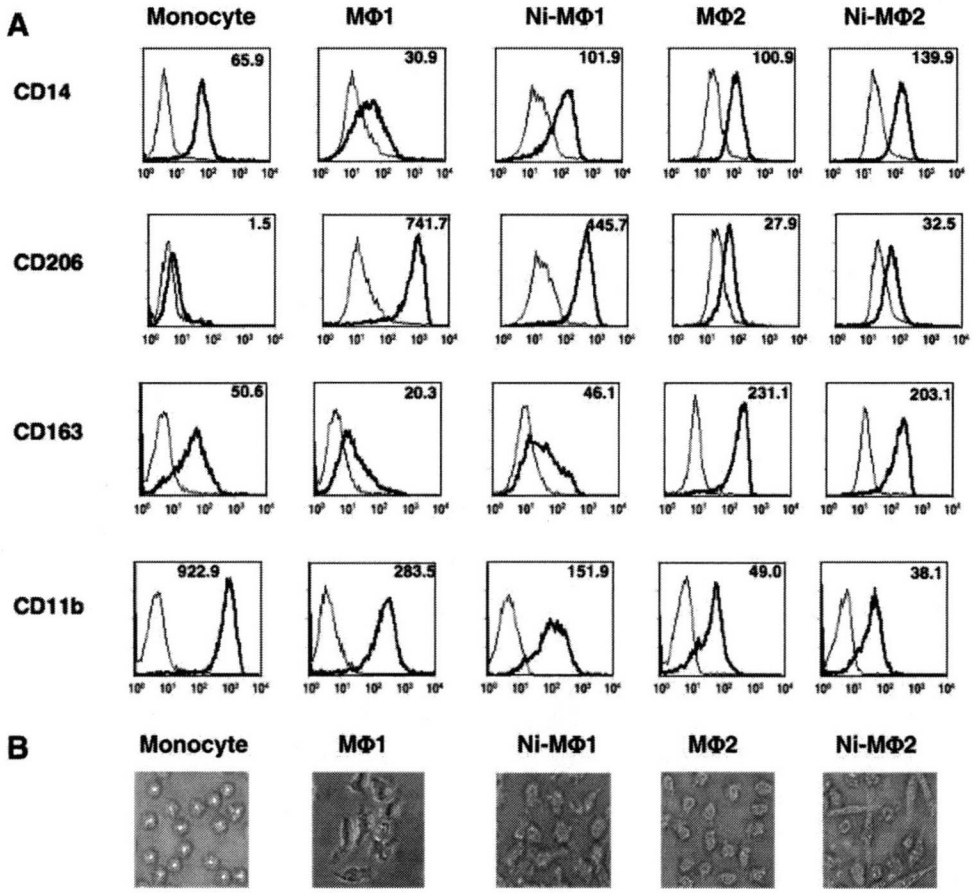

Fig. 1. Characterization of monocytes, MΦ1, Ni-MΦ1, MΦ2, and Ni-MΦ2. Monocytes were isolated from PBMCs, and MΦ1, Ni-MΦ1, MΦ2, and Ni-MΦ2 were generated in parallel from the same donor by culture for 6 days. (A) The surface molecule expressions of CD14, CD206, CD163, and CD11b on the cells were determined 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-

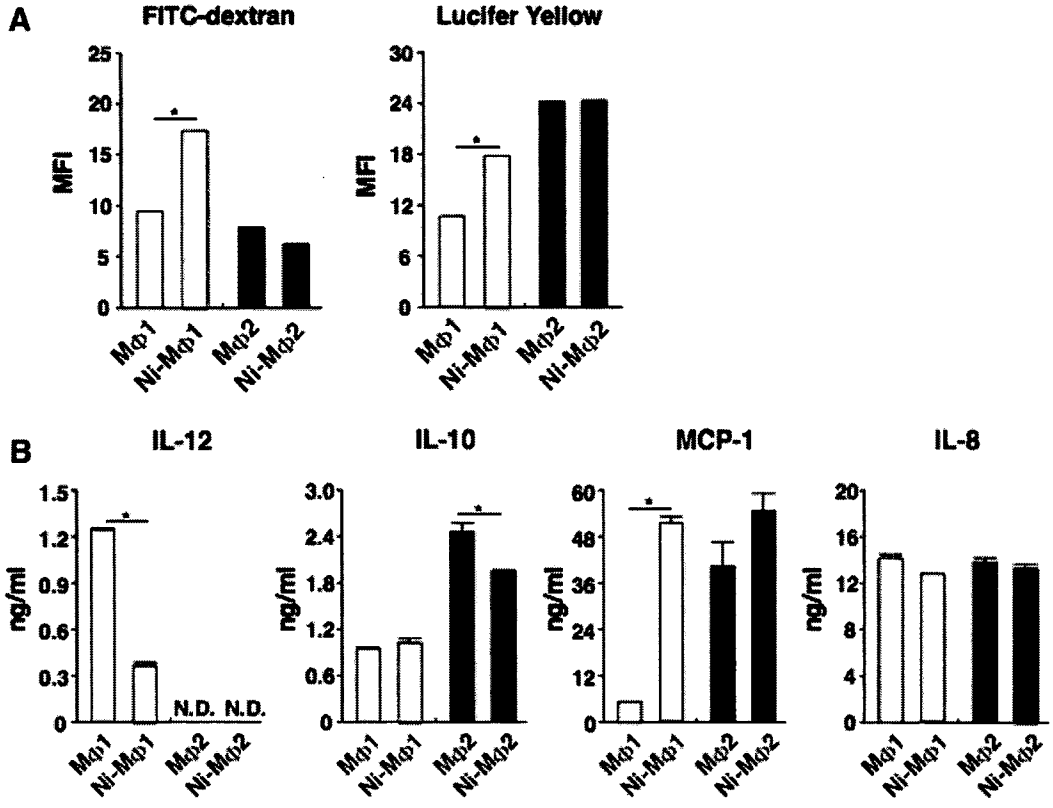

Fig. 2. Effects of nicotine on antigen-uptake and cytokine production by MΦ1, Ni-MΦ1, MΦ2, and Ni-MΦ2. (A) Uptakes of FITC-dextran (0.1 mg/ml) and Lucifer yellow (0.1 mg/ml) by MΦ1, Ni-MΦ1, MΦ2, and Ni-MΦ2 after 1 h. The results are shown as the mean fluorescence intensity (MFI) values ± SD obtained 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.

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.

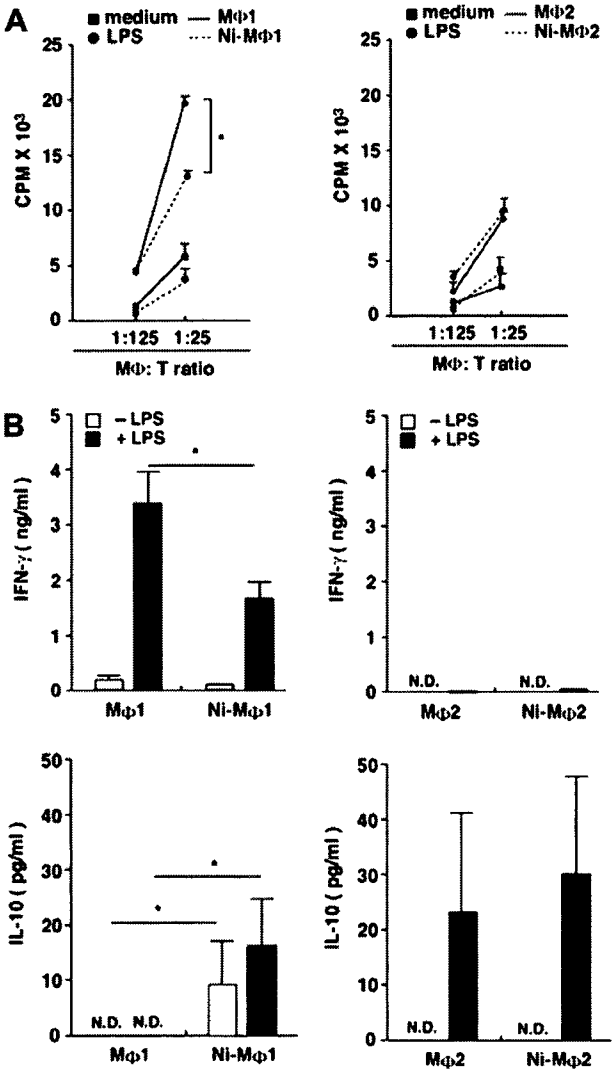

Fig. 3. T cell stimulation capacities of MΦ1, Ni-MΦ1, MΦ2, and Ni-MΦ2. (A) Comparisons of the effects of MΦ1, Ni-MΦ1, MΦ2, and Ni-MΦ2 on T cell proliferation. The results represent the mean cpm values ± SD obtained from triplicate cultures. The data shown were obtained in one of three independent experiments. *$P < 0.05$ 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

Yoshio Shimabukuro, PhD, DDS, Maki Ueda, PhD, DDS, Masao Ozasa, DDS, Jun Anzai, MPharm, Masahide Takedachi, PhD, DDS, Manabu Yanagita, PhD, DDS, Masako Ito, DDS, Tomoko Hashikawa, PhD, DDS, Satoru Yamada, PhD, DDS, and Shinya Murakami, PhD, DDS

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

From the Division of Oral Biology and Disease Control, Department of Periodontology, Osaka University Graduate School of Dentistry, Osaka, Japan.

Address requests for reprints to Dr Shinya Murakami, Department of Periodontology, Division of Oral Biology and Disease Control, Osaka University Graduate School of Dentistry, 1-8 Yamadaoka, Suita, Osaka 565-0871, Japan. E-mail address: ipshinya@dent.osaka-u.ac.jp.

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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

(0.05%)–ethylenediaminetetraacetic acid (0.53 mmol/L) solution, collected by centrifugation, and cultured on plastic culture dishes containing standard medium until confluent. The cells were then trypsinized at 1:3 split ratio. Experiments were carried out with cells from the fourth to fifth passages. In this study, we established 3 primary cell isolations from different volunteers, and all cell isolations were used in each experiment. All 3 primary cell isolations were examined for the several extracellular matrix proteins by using reverse transcription–polymerase chain reaction. We confirmed that the cells expressed mRNA of type I collagen, dentin matrix protein-1 (DMP-1), and dentin sialoprotein; the latter 2 proteins are known to be specific to dentin (23). All primary cell isolations provided similar results in each experiment of this study.

Proliferation Assay

HDPC (6×10^4 cells/well) were plated onto 24-well plates until confluence. The cells were made quiescent by replacing the medium with serum-free α-MEM for 24 hours, and the medium was replaced with or without 1% FCS α-modified MEM in the presence or absence of FGF-2 (10 ng/mL). In another experiment mitomycin C (MMC) (Kyowa Hakko Kogyo Co, Tokyo, Japan) treatment was carried out before FGF-2 stimulation to suppress cell proliferation. Proliferation was assayed by pulsing wells with 185 kBq/w of [^{3}H]-thymidine (ICN Radiochemicals; Irvine, CA) during the last 4 hours of the culture. At the end of the incubation, cultures were harvested by using a semiautomatic cell harvester (Labomash; LM101 Laboscience, Tokyo, Japan), and [^{3}H]-thymidine incorporation was measured in a liquid scintillation counter (Aloka Co Ltd, Tokyo, Japan).

Alkaline Phosphatase Activity Assay

Alkaline phosphatase (ALPase) activity was investigated according to the procedure of Bessay et al (24). The HDPC were seeded in 24-well culture dishes (6×10^4 cells/well) with standard medium containing 50 μg/mL ascorbic acid and 10 mmol/L beta-glycerophosphate (BGP) (Sigma, St Louis, MO). Cells were cultured for the number of indicated days in this medium, and medium was changed every 3 days. We investigated ALPase activity when the HDPC were incubated with 10 ng/mL FGF-2 for 8, 14, 20, or 26 days. To determine the reversibility of the FGF-2 effect on ALPase activity, the HDPC were cultured for 15 days in the presence of FGF-2. Cells were then subsequently cultured in the absence of FGF-2 in standard medium containing 50 μg/mL ascorbic acid and 10 mmol/L BGP. After washing twice with saline, the cells were homogenized in a glass homogenizer in 1 mL of 0.9% NaCl, 0.2% Triton X-100 at 0°C–4°C, and centrifuged for 15 minutes at 12,000*g*. ALPase activity in the supernatant was measured by using *p*-nitrophenyl phosphate (*p*NP) as substrate. The supernatant was assayed in a 0.5 mol/L Tris/HCl buffer (pH 9.0) containing 0.5 mmol/L *p*NP and 0.5 mmol/L $MgCl_2$. The reaction mixture was incubated at 37°C for 30 minutes and was stopped by addition of 0.25 volume of 1 N NaOH. Hydrolysis of *p*NP was monitored as change in A410 with a spectrometer (Hitachi, Tokyo, Japan). *p*-Nitrophenol was used as a standard. One unit of activity was defined as the amount hydrolyzing 1 nmol of *p*-NP in 30 minutes.

Cellular DNA Content

DNA content was measured by a modification of the method of Labarca and Paigen (25). The HDPC were seeded in 24-well culture dishes (6×10^4 cells/well) with standard medium containing 50 μg/mL ascorbic acid and 10 mmol/L BGP. They were cultured for the indicated days with this medium, which was changed every 3 days. Alteration of DNA content of the cells was determined as follows. The HDPC were washed with phosphate-buffered saline and then homogenized at 0°C–4°C in 1 mL of 2 mol/L NaCl/25 mmol/L Tris-HCl (pH 7.4). After centrifugation at 12,000*g* for 10 minutes, 25 μL of 5 μg/mL bisbenzimidazole (Sigma) was added to 100 μL of the supernatant. The fluorescent spectra at emission (458 nm) after excitation at 356 nm were monitored by a spectrophotometer. The concentration of DNA in the samples was determined by a standard curve made at various concentrations of calf thymus DNA.

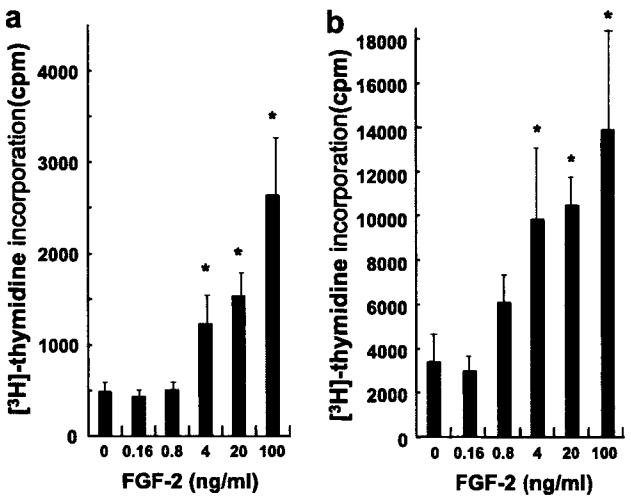

Figure 1. FGF-2 activates cell proliferation by HDPC. HDPC were incubated in 24-well plates until confluence. HDPC were made quiescent by replacing standard medium with serum-free alpha-MEM for 24 hours and cultured in the presence or absence of FGF-2 (0.16, 0.84, 4, 20, 100 ng/mL) without (*a*) or with (*b*) 1% FCS. Cells were pulsed with 185 kBq/w of [^{3}H]-thymidine during the last 4 hours of culture. At the end of the incubation, cultures were harvested, and the radioactivity was measured. Results of mean and standard error of the mean (SEM) of 3 identical experiments are shown. *$P < .05$.

Alizarin Red Staining

Cytochemical staining of calcium was performed by modification of the alizarin red staining method (26). HDPC were cultured on 6-well culture plates (4×10^5 cells/well). At the end of incubation with standard medium containing 50 μg/mL ascorbic acid and 10 mmol/L BGP for indicated time, cell layers were washed twice with saline and then fixed with dehydrated ethanol. After fixation, the cell layers were stained with 1% alizarin red S in 0.1% NH_4OH (pH 6.5) for 5 minutes and then washed with H_2O. Cells were observed under a microscope, and images were taken with a Nikon Coolpix (Nikon, Tokyo, Japan). Quantitative evaluation was determined by using image analysis with the WinRoof software program (Mitani Corporation, Fukui, Japan).

Migration Assay

Migration assays were performed after modifying the Teflon restraint migration assay (27). HDPC were seeded at 6×10^4 cells/glass bottom dish (Matsunami, Osaka, Japan), on which was placed a silicon block (30 mm × 70 mm; Togawa Rubber Co Ltd, Osaka, Japan) to introduce a cell-free area. Cells were grown until confluence, after which the silicon block was removed and the medium was replaced with 1% FCS α-MEM in the presence or absence of FGF-2 (10 ng/mL). The migration of HDPC to the cleared area was photographed immediately after removing the silicon block and 24 hours later by using a Nikon Eclipse TS100 inverted phase contrast microscope and Coolpix 990 (Nikon Corp). In each dish, 4 randomly chosen fields 1 mm in length were analyzed, and the number of cells that had translocated

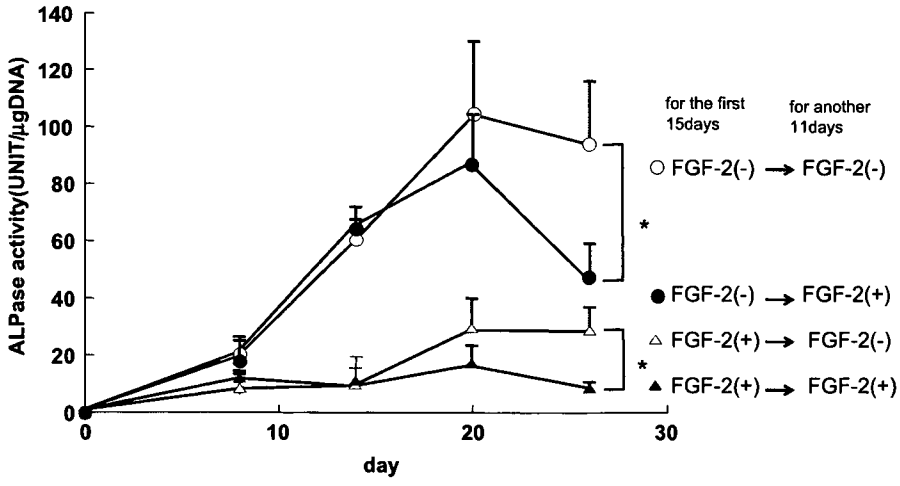

Figure 2. FGF-2 reversibly decreased ALPase activity by HDPC. HDPC were cultured with standard medium containing 50 μg/mL ascorbic acid and 10 mmol/L BGP in the presence or absence of FGF-2 (10 ng/mL). The 2 groups were then further subdivided, with the former cultures maintained in the continuous presence of FGF-2 (closed triangle) or FGF-2 was removed after 15 days, after which the culture continued to be incubated in the absence of FGF-2 (open triangle). The latter cultures were maintained in the absence of FGF-2 (open circle) or cultured with FGF-2 (closed circle). At the end of incubation, an ALPase assay was performed. Results of mean and SEM of 3 identical experiments are shown. *$P < .05$.

into the cleared area was determined. In some experiments HDPC were treated with 16 μg/mL of MMC for 2 hours at 37°C.

Statistical Analyses

All experiments were performed in triplicate, and results are expressed as mean ± standard deviation. Data were statistically analyzed between FGF-2–stimulated and unstimulated groups by using the Mann-Whitney rank sum test or Bonferroni ($P < .05$ was considered statistically significant).

Results

FGF-2 Increased HDPC Proliferation

Previous studies have revealed that FGF-2 has potential ability to promote cell proliferation in a variety of cell types (28). Here we examined the effects of FGF-2 on the proliferative responses of HDPC. Fig. 1 shows that FGF-2 increased the proliferative response of HDPC in a dose-dependent manner when the cells were cultured with or without 1% FCS. FGF-2, however, has little effect on the proliferative response of HDPC that were treated with α-MEM containing 10% FCS (data not shown). FCS is essential to maintain *in vitro* cell culture especially for a long term. However, addition of FCS affects the various cell responses because FCS contains a variety of growth factors. Therefore, to minimize the effect we used 1% FCS in the proliferation and migration assays. On the other hand, 10% FCS had to be added in ALPase and alizarin staining assays to maintain the long-term culture of HDPC.

FGF-2 Suppressed Cytodifferentiation of HDPC

ALPase assay and alizarin red staining were performed to investigate the influence of FGF-2 on cytodifferentiation of HDPC. Consistent with previous findings (6, 29, 30), HDPC have potential to produce a calcified nodule with elevated levels of ALPase activity when cultured with BGP and ascorbic acid. As shown in Figs. 2, 3, and 4, FGF-2 suppressed increased levels of ALPase activity and formation of calcified nodules. Also, FGF-2 decreased ALPase activity and formation of a calcified nodule by HDPC that had been activated with ascorbic acid and BGP for 15 days. Notably, the suppressive effects of FGF-2 on both ALPase activity and calcified nodule formation were abolished when FGF-2–treated HDPC were subsequently cultured in the absence of FGF-2. This suggests that FGF-2 reversibly inhibits the differentiation of HDPC.

FGF-2 Enhanced Cell Migration

Next, we carried out the migration assay to investigate the effect of FGF-2 on cell migration of HDPC. As shown in Fig. 5, FGF-2 treatment of HDPC activated cell migration. However, it is possible that the enhanced cell migration might be caused solely by FGF-2–dependent proliferation. To exclude this possibility, HDPC were treated with MMC (the replication inhibitor), and the migration assay was then performed. First, we confirmed the inhibitory potential of MMC. Fig. 6*a* and 6*b* showed that treatment of FGF-2– or unstimulated HDPC with 16 μg/mL of MMC resulted in little proliferative response (611 ± 251 or 437 ± 109 cpm, respectively), compared with that of the cells (1727 ± 423 cpm) cultured in the absence of FCS and FGF-2. Interestingly, FGF-2 increased cell migration of HDPC that had been treated with 16 μg/mL MMC (Fig. 6*c*).

Discussion

In human dental pulp, undifferentiated mesenchymal cells are observed, as are other tissues (1, 2, 31). The stem cells can form dentin-pulp complex *in vivo* when the tissue is pathophysiologically stimulated or damaged (1). Because the proportion of undifferentiated mesenchymal cells in dental pulp is higher than that in bone marrow cells (1), it is speculated that pulp tissues possess relatively high potential to regenerate themselves. In addition, the dental pulp cells exhibit various biologic functions in response to many stimuli to maintain the homeostasis of the dentin-pulp complex (32, 33).

During tooth development, FGF-2 is present on basement membrane between oral epithelium and mesenchymal tissue (34). FGF-2 regulates differentiation of odontoblasts and ameloblasts (35) and has been reported to induce the change from preodontoblast to odontoblast, in combination with transforming growth factor–β1 or insulin-like growth factor–1 (36). These observations suggest that FGF-2 plays important roles in cytodifferentiation of odontoblasts and in dentin formation.

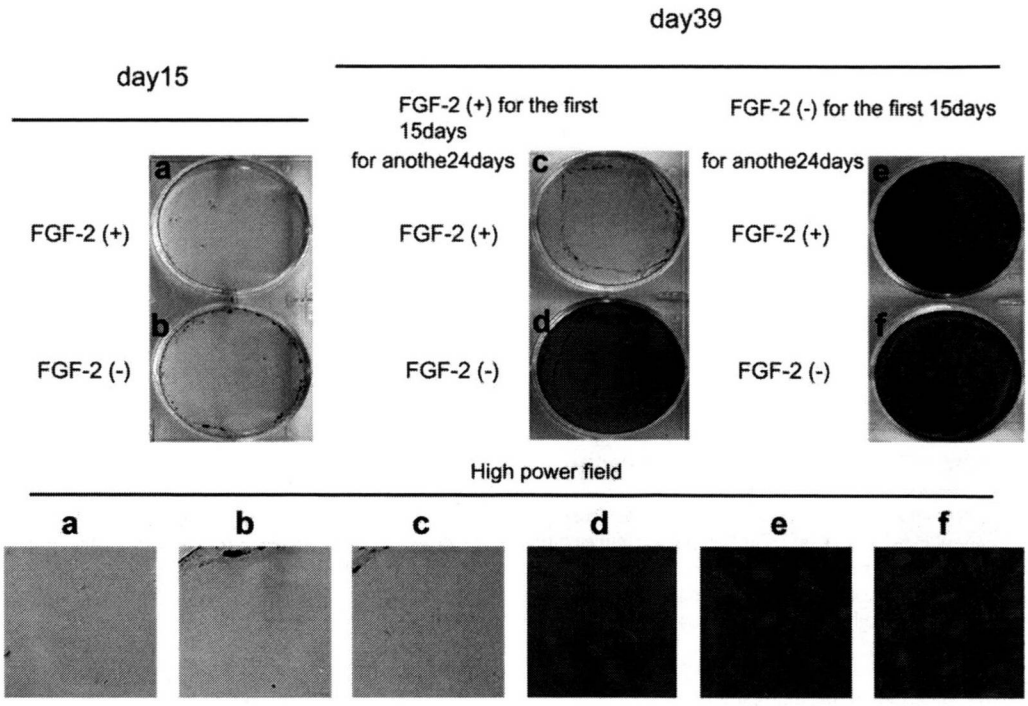

Figure 3. FGF-2 reversibly suppressed calcified nodule formation by HDPC. HDPC were cultured with standard medium containing 50 μg/mL ascorbic acid and 10 mmol/L BGP in the presence (*a*) or absence (*b*) of FGF-2 (10 ng/mL) for 15 days. The former cultures were kept in the continuous presence of FGF-2 for additional 24 days (*c*) or removed FGF-2 at the initial 15 days, after which the culture was maintained in the absence of FGF-2 (*d*). The latter culture was cultured for an additional 24 days in the absence of FGF-2 (*e*) or cultured with FGF-2 (*f*). At the end of incubation, alizarin red staining was performed. Results of 1 representative experiment among 3 identical experiments are shown.

Consistent with previous studies (37, 38), this study revealed that FGF-2 increased proliferation of HDPC (Fig. 1). Furthermore, FGF-2 activated cell migration and reversely suppressed ALPase activity and mineralization by HDPC (Figs. 2–4). The results presented in this study clearly support the hypothesis that FGF-2 modulates cell proliferation, migration, and mineralization of HDPC. FGF-2–stimulated migratory action observed in the migration assay (Fig. 5) is likely to be dependent on proliferative responses as FGF-2 has potential augmentation in DNA synthesis of HDPC (Fig. 1). Interestingly, FGF-2 stimulated migratory activity was increased even when the cell proliferation was inhibited by MMC (Fig. 6). Therefore, FGF-2 up-regulates cell migration of HDPC in addition to cell proliferative responses. Because cell proliferation and migration are critical events of wound healing and regeneration processes, cellular FGF-2–induced proliferation and migration contribute to favorable tissue repair of the dentin-pulp complex.

FGF-2 is localized in dentin and released after injury. Also, fibroblasts and endothelial cells in dental pulp produce FGF-2. Thus, HDPC

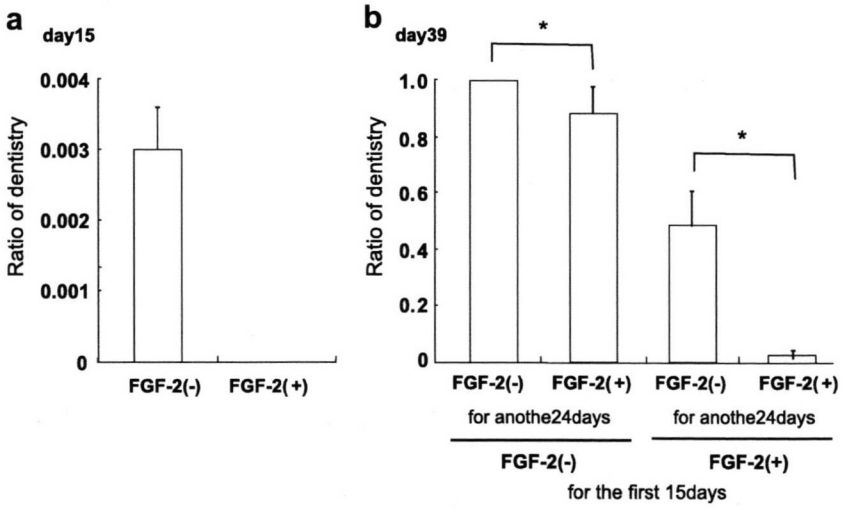

Figure 4. Quantitative evaluation on the effect of FGF-2 on calcified nodule formation by HDPC. Quantitative evaluation was determined by using image analysis with the WinRoof software program on alizarin red staining. (*a*) Results of mean and (*b*) SEM of 3 identical experiments are shown. *$P < .05$.

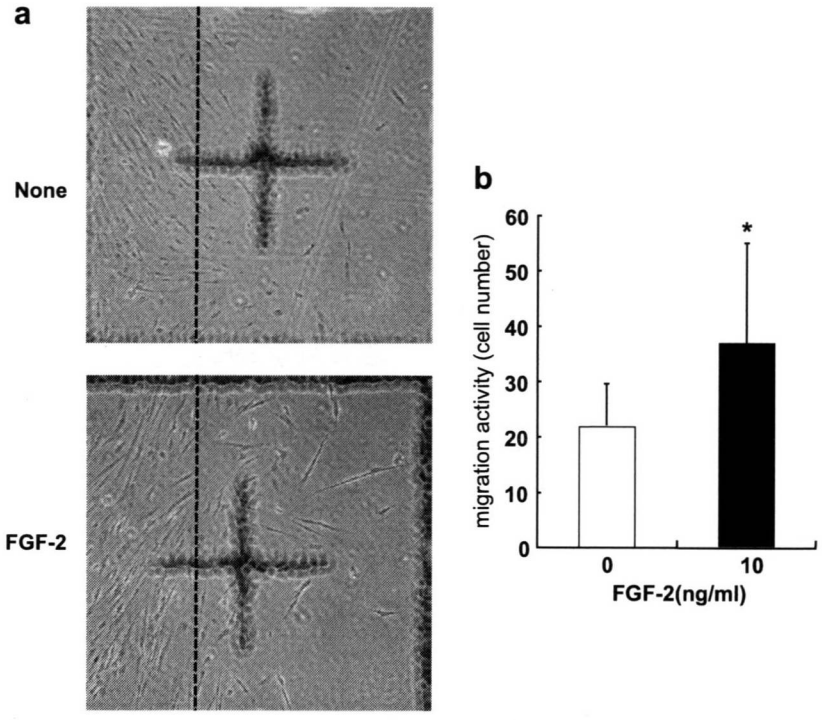

Figure 5. FGF-2 increased cell migration by HDPC. HDPC were seeded on a glass bottom dish, on which was placed a silicon block to introduce a cell-free area, and grown until confluence. The silicon block was then removed, and the medium was replaced with 1% FCS α-MEM in the presence or absence of FGF-2 (10 ng/mL). Migration of HDPC to the cleared area was photographed immediately after removing the silicon block and 24 hours later by using phase contrast microscopy. The dotted line is an edge of the cell layer just after removing silicon rubber. (*a*) Migratory activity was estimated by the assay described in Materials and Methods. (*b*) Results of mean and SEM of 3 identical experiments are shown. $*P < .05$.

are exposed to FGF-2 in physiologic and pathophysiologic conditions. HDPC expressed fibroblast growth factor receptor (FGFR) mRNA (data not shown). FGFRs are activated by its ligands such as FGF-2 and mediate cellular functions of the cells via various signaling pathways (Fig. 7).

The tissue repair process includes a sequence of functionally coordinated events involving cell proliferation and migration, release of extracellular matrix, and cytodifferentiation accompanying cell-signaling molecule interactions. Although suppression of FGF-2 for cell differentiation of HDPC was observed, HDPC, which had been pre-exposed to FGF-2 and then cultured without FGF-2, increased the ALPase activity and calcified nodule formation (Figs. 2, 3 and 4). The results emphasize the temporary suppression of differentiation by FGF-2 seen in the expanded dental pulp cells. Furthermore, HDPC are capable of cytodifferentiation at subsequent stages when other molecules distinct from FGF-2 play crucial roles. These results also 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.

FGF-2 enhanced pulp microvessel density (39). Revascularization is essential to wound repair. We have previously reported that FGF-2 increased hyaluronan expression by HDPC (40). Hyaluronan is capable of activating cell migration and, in turn, leads to accelerated tissue repair (41–47). Kikuchi et al (48) have demonstrated that application of FGF-2 to exposed pulp tissue leads to induced calcified particles and dentin bridge. Thus, the *in vivo* effects of FGF-2 appear to be due to multipotential actions of FGF-2 including cell proliferation, migration, and reversible suppression of cytodifferentiation observed in this study in addition to angiogenic activity and regulation of hyaluronan expression.

FGF-2 stimulates the proliferative responses but decreases the ALPase activity and mineralization of HDPC (Figs. 2, 3, and 4). At this growth stage, FGF-2 simultaneously down-regulates the mRNA expression of collagen type I, osteonectin, bone sialoprotein, DMP-1, and dentin sialophosphoprotein (data not shown), which are closely associated with the mineralization. Furthermore, the expression of decorin and biglycan is also decreased by FGF-2 (data not shown), both of which are main components of small leucin rich proteoglycan in dentin and play a critical role in mineralization of dentin and enamel (49). Thus, FGF-2–dependent modulation of extracellular matrix production can partly account for FGF-2–induced suppression of mineralization of HDPC.

Noteworthy is the fact that FGF-2–dependent inhibition of ALPase activity and calcified nodule formation was recovered when the pretreated HDPC were recultured in the absence of FGF-2 (Fig. 2△ vs ▲; Fig. 3 (*c*) vs (*d*)). The similar phenomenon was observed when periodontal ligament cells were stimulated with FGF-2. Importantly, it has also been revealed that topical application of FGF-2 induces significant periodontal regeneration (19–21). Thus, we deduce that topically applied FGF-2 during the early stages of wound healing increases the number of progenitor cells while suppressing differentiation into hard tissue–forming cells such as osteoblasts, cementoblasts, and probably odontoblasts. During the subsequent healing processes when FGF-2 activity disappears at the applied site, those progenitor cells start to differentiate, enhancing tissue regeneration. To evaluate the efficacy of FGF-2 for regeneration of dentin-pulp complex, *in vivo* experiments with beagles are in progress.