Figure S4. Characterization of Ad-HEX-transduced hepatoblasts.
Figure S5. Progression of differentiation of the definitive endoderm to hepatoblasts.
Figure S6. X-gal staining of human iPSC (Tic)-derived definitive endoderms transduced with a conventional or a fiber-modified Ad vector containing the EF-1α promoter.
Figure S7. *HEX* promotes the differentiation into the hepatic lineage, not from undifferentiated iPSCs (Tic), but from iPSC (Tic)-derived definitive endoderm.
Table S1. List of Taqman gene expression assays and primers.
Table S2. List of antibodies used.
Materials and Methods.

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Chemical inhibition of sulfation accelerates neural differentiation of mouse embryonic stem cells and human induced pluripotent stem cells

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ABSTRACT

Pluripotency of embryonic stem cells (ESCs) is maintained by the balancing of several signaling pathways, such as Wnt, BMP, and FGF, and differentiation of ESCs into a specific lineage is induced by the disruption of this balance. Sulfated glycans are considered to play important roles in lineage choice of ESC differentiation by regulating several signalings. We examined whether reduction of sulfation by treatment with the chemical inhibitor chlorate can affect differentiation of ESCs. Chlorate treatment inhibited mesodermal differentiation of mouse ESCs, and then induced ectodermal differentiation and accelerated further neural differentiation. This could be explained by the finding that several signaling pathways involved in the induction of mesodermal differentiation (Wnt, BMP, and FGF) or inhibition of neural differentiation (Wnt and BMP) were inhibited in chlorate-treated embryoid bodies, presumably due to reduced sulfation on heparan sulfate and chondroitin sulfate. Furthermore, neural differentiation of human induced pluripotent stem cells (hiPSCs) was also accelerated by chlorate treatment. We propose that chlorate could be used to induce efficient neural differentiation of hiPSCs instead of specific signaling inhibitors, such as Noggin.

1. Introduction

Embryonic stem cells (ESCs) [1–3] are promising tools for biotechnology and possess key features that should allow their exploitation in the development of cell replacement therapies [4]. Extrinsic signaling pathways are key mechanisms for determining ESC cell fate, and sulfated glycans, such as heparan sulfate (HS), are well known regulators of signal transduction [5]. HS chains are present abundantly on the cell surface of undifferentiated mouse ESCs (mESCs) and functional roles of HS chains have been demonstrated [6,7]. Thus, the modification of sulfated glycans is an attractive approach for developing methods to regulate ESC differentiation.

Sulfated glycans are synthesized in the Golgi as follows. The high energy sulfate donor 3′-phosphoadenosine 5′-phosphosulfate (PAPS), which is required as a substrate for sulfation, is synthesized in the cytosol and nucleus by PAPS synthetase [8], and subsequently is translocated via the PAPS transporter (PAPST) into the Golgi [9–13], where it is used by sulfotransferases to sulfate glycans. Recently, we have demonstrated that the reduction of sulfation by knockdown of *PAPST* using vector-based RNA interference (RNAi) promotes neurogenesis of mESCs [13]. However, the rapid, simple, and safety method for modification of sulfated glycans instead of gene transfer is desired particularly for application of human ESCs (hESCs) to regenerative medicine.

Chlorate inhibits ATP sulfurylase activity of PAPS synthetases by competing with sulfate ions in binding to ATP-sulfurylase resulting in reduction of PAPS [14]. Thus, inhibition of PAPS synthesis by chlorate leads to reduction of sulfation on several sulfated proteins, glycoproteins, glycolipids, and proteoglycans [15,16].

Since induced pluripotent stem cells (iPSCs) were reported [17], the application of human iPSCs (hiPSCs) to regenerative medicine has been expected. In particular, neural induction of hiPSCs is an important research tool for several neural diseases and has been applied to cell replacement therapies. Therefore, methods of efficient and rapid neural induction are required [18]. In this study, we examined whether neural differentiation of hiPSCs in addition to mESCs was enhanced by the chemical down-regulation of sulfation with chlorate.

2. Materials and methods

2.1. Cell culture

R1 [19] and E14TG2a [20] mESC lines were maintained on mouse embryonic fibroblasts (MEFs) inactivated with 10 μg/ml

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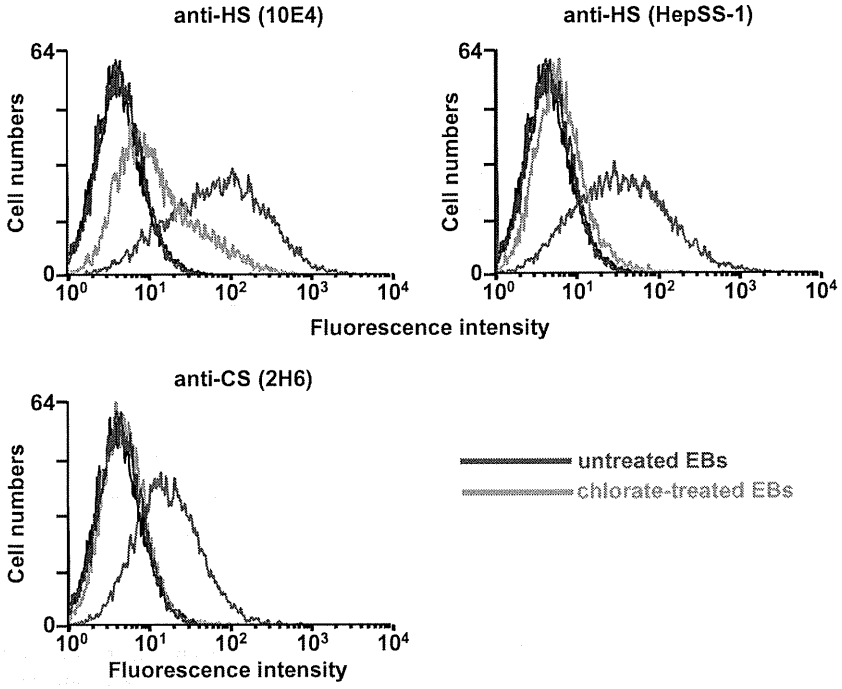

Fig. 1. Chlorate treatment reduces sulfation of HS and CS in EBs. FACS analysis of cells 3 days after EB formation using an anti-HS antibody (10E4 or HeppSS-1) or an anti-CS antibody (2H6) (black and blue lines represent the IgM isotype control for untreated and chlorate-treated EBs, respectively). Chlorate treatment was performed for 24 h from 2 days after EB formation. Three independent experiments were performed and representative results are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mitomycin C (Sigma) in ESC medium (DMEM supplemented with 15% FBS {Hyclone}, 1% penicillin/streptomycin {Gibco}, 0.1 mM 2-mercaptoethanol {Gibco}, and 0.1 mM non-essential amino acids {Gibco}) with 1000 U/ml LIF (Chemicon). hiPSC clones (MRC-hiPS_Fetch {NIHS0604} and MRC-hiPS_Tic {JCRB1331}) [21] were maintained on inactivated MEFs in iPSellon (Cell-Sight) with 10 ng/ml bFGF (Wako). All mESC experiments were performed using the R1 line and most results were confirmed using the E14TG2a line.

For embryoid body (EB) formation, mESCs or hiPSCs were transferred to low cell binding 60 mm dishes (Nunc) and cultured in ESC medium without LIF or iPSellon without bFGF, respectively. Before EB formation, hiPSCs were preplated on gelatin-coated dishes to remove feeder cells. For neuronal differentiation, 1 μM all-trans retinoic acid (RA) (Sigma) was added 4 days after EB formation. Then, 5 days after EB formation, EBs were plated onto PDL/laminin-coated 60 mm dishes (Becton Dickinson) in DMEM-F12 containing N2 supplement (Gibco).

To down-regulate sulfation, 50 mM chlorate (Sigma) was added from 2 days after EB formation throughout EB culture.

2.2. FACS analysis

FACS analysis was performed 3 days after EB formation. After EDTA treatment, the cell suspension was incubated with primary antibodies diluted in FACS buffer (0.5% bovine serum albumin and 0.1% sodium azide in PBS). After washing, the cell suspension was incubated with FITC-conjugated secondary antibody (Sigma) diluted in FACS buffer. Cell sorting and analysis were performed using a FACSAria Cell Sorter (Becton Dickinson). Primary antibodies were as follows: mouse IgM isotype control (Chemicon), the anti-HS antibody 10E4 (Seikagaku Corp.), the anti-HS antibody HepSS-1 (Seikagaku Corp.), the anti-chondroitin sulfate (CS) antibody 2H6 (Seikagaku Corp.).

2.3. Immunoblotting

Cells were lysed with lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM Na_3VO_4, 10 mM NaF, protease inhibitors). Isolation of nuclear extracts was performed as described previously [6]. Samples containing 5 μg of cell lysate or nuclear extract were separated by 10% SDS–PAGE and transferred onto PVDF membranes (Millipore). After blocking, the membranes were incubated with primary antibodies as follows: anti-ERK1/2 (Cell Signaling Technology), anti-phosphorylated ERK1/2 (Thr183/185; Cell Signaling Technology), anti-phosphorylated Smad1 (Ser463/465; Cell Signaling Technology), anti-β-actin (Sigma), anti-β-catenin (Cell Signaling Technology), anti-lamin B_1 (Zymed), and anti-βIII-tubulin (Chemicon). The membranes were then incubated with the appropriate peroxidase-conjugated secondary antibodies (Cell Signaling Technology), washed and developed with ECL Plus reagents (GE Healthcare).

2.4. Immunostaining

After neural differentiation on PLL/laminin-coated glass chamber slides (Iwaki), cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% saponin. After washing and subsequent blocking, cells were stained with an anti-βIII-Tubulin antibody. After washing, cells were stained with an FITC-conjugated secondary antibody and counterstained with propidium iodide (PI). Immunofluorescence images were obtained using an LSM5Pascal confocal laser scanning microscope (Carl Zeiss).

2.5. Real-time PCR

Real-time PCR was performed as described previously [6]. For hiPSCs, FastStart Universal SYBR Green Master (Roche) was used. Primer sets and probes are listed in Supplementary Table 1.

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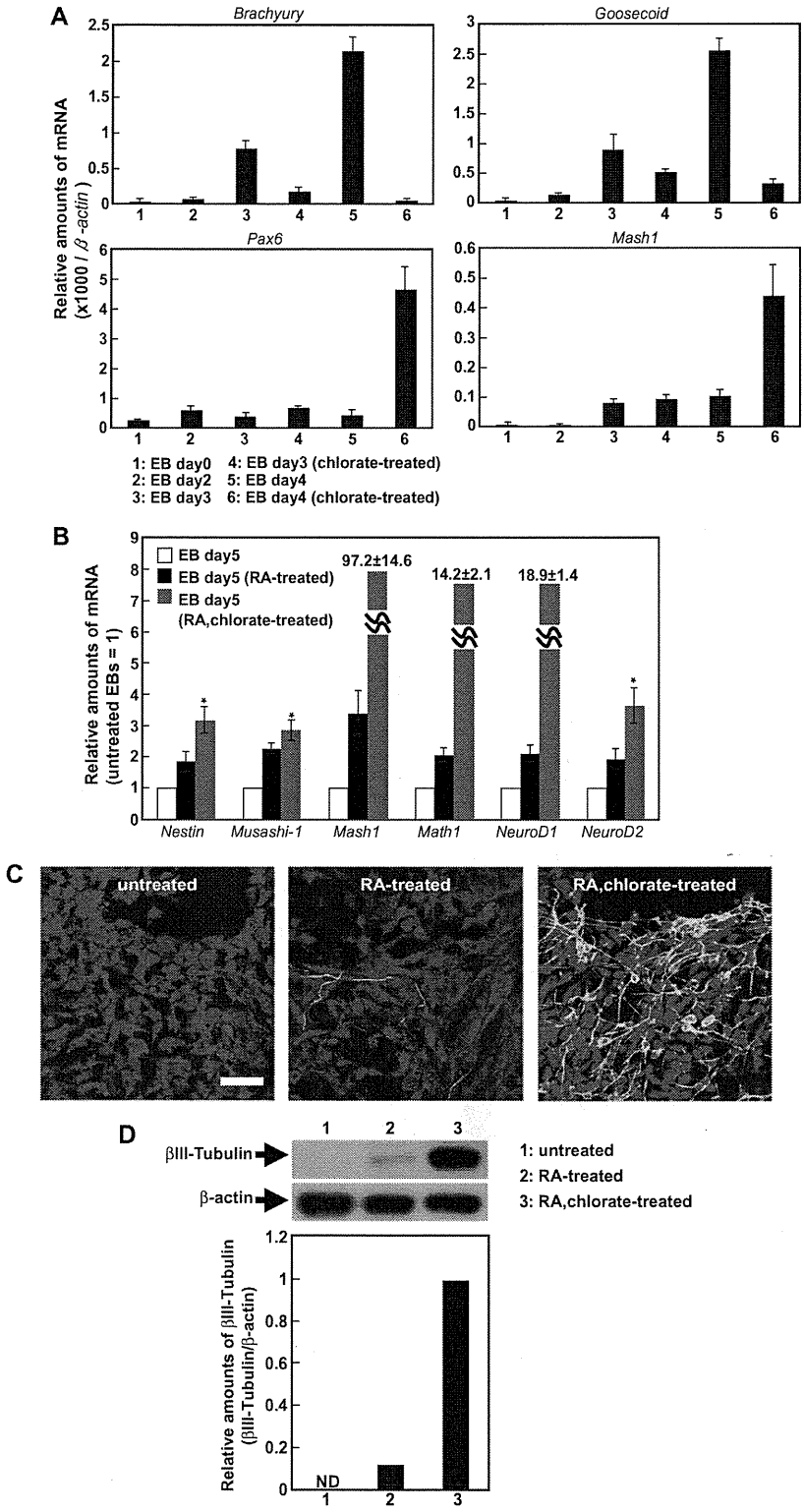

Fig. 2. Reduction of sulfation accelerates neural differentiation of mESCs. (A) Real-time PCR analysis of markers of mesoderm (*Brachyury*, *Goosecoid*) and ectoderm (*Mash1*, *Pax6*) in EBs harvested on each day shown. Relative amounts of each mRNA were calculated after normalization to *β-actin* mRNA. The values shown are the means ± SD of three independent experiments. (B) Real-time PCR analysis of neural markers, such as neural stem/progenitor cell markers (*Nestin*, *Musashi-1*) and proneural markers (*Mash1*, *Math1*, *NeuroD1* and *NeuroD2*), 5 days after EB formation. The results are shown after normalization against the values obtained with untreated EBs (value = 1). The values shown are the means ± SD of duplicate measurements from two independent experiments and significant values are indicated; $^{*}P < 0.05$, compared with the RA-treated EBs. (C) Immunocytochemical staining 2 days after replating of EBs. Representative confocal images from two independent experiments are shown (βIII-tubulin, green; PI, red). Scale bar, 20 μm. (D) Western blotting 2 days after replating of EBs. Representative immunoblots from two independent experiments are shown. The histogram shows mean densitometric readings of βIII-tubulin/loading controls. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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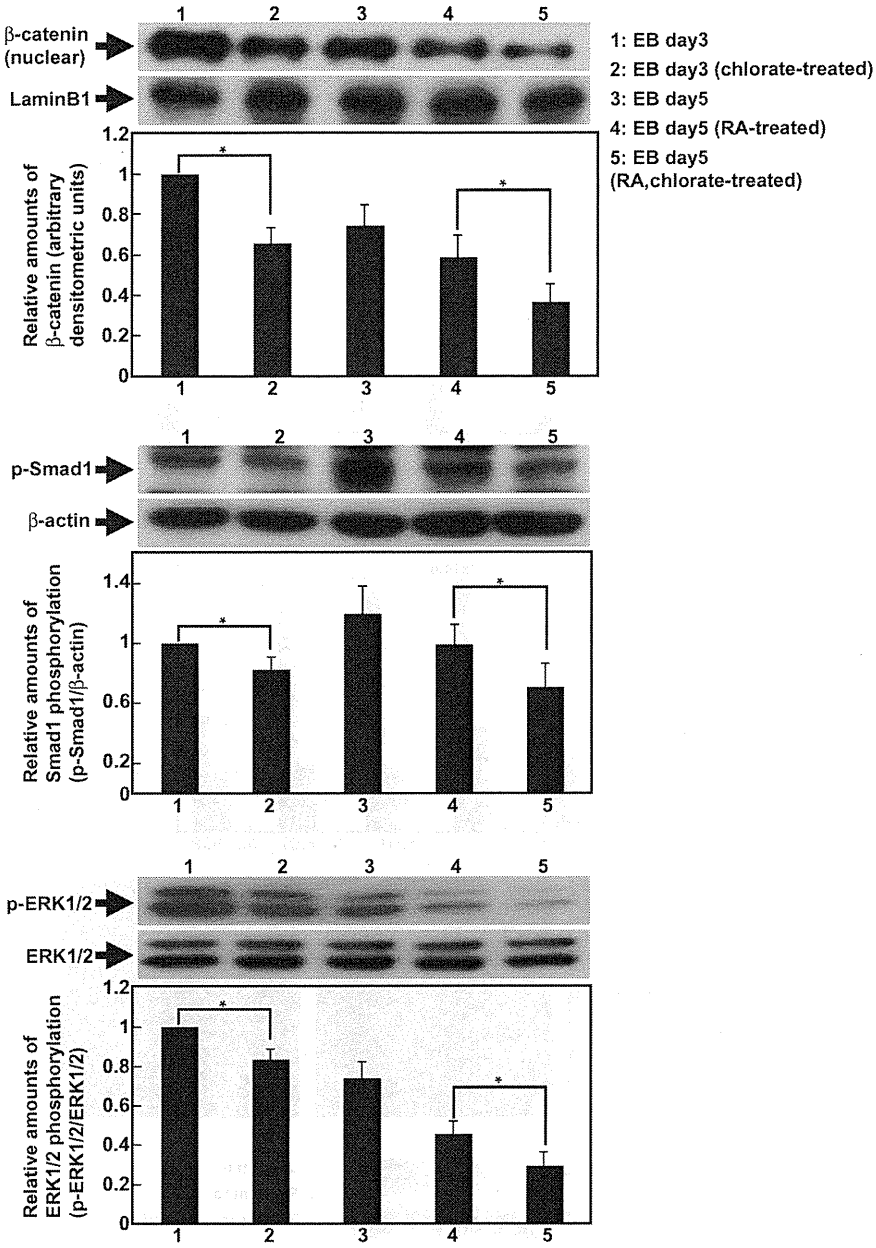

Fig. 3. Signaling via several pathways is decreased in chlorate-treated EBs. Western blotting of downstream signaling components 3 or 5 days after EB formation. Representative immunoblots from two independent experiments are shown. The histograms show mean densitometric readings ± SD of β-catenin or the phosphorylated proteins/loading controls after normalization against the values obtained with untreated EBs cultured for 3 days (value = 1). Values were obtained from duplicate measurements of two independent experiments and significant values are indicated; $^*P < 0.05$.

3. Results and discussion

Firstly, we examined whether sulfation of glycans in EBs was reduced by chlorate treatment. HS and CS are functionally important sulfated glycans that are expressed until at least 8 days after EB formation in mESCs [13]. Thus, we focused on HS and CS in this study. Three days after EB formation, FACS analysis of chlorate-treated EBs revealed poor staining by the anti-HS and the anti-CS antibodies, indicating that sulfation on HS and CS was reduced (Fig. 1).

Signaling by both Wnt and BMP is essential for the choice of lineage between mesoderm and ectoderm: the reduction of both Wnt and BMP signaling inhibits mesodermal differentiation and enhances ectodermal differentiation [22,23]. Thus, we assumed that a reduction in Wnt and BMP signaling at the point when mesoderm starts to differentiate (2–4 days after EB formation) would accelerate neural differentiation. Therefore, we examined EB differentiation in response to chlorate treatment 2 days after EB formation. Real-time PCR analysis at 0–4 days after EB formation showed that the expression of early mesoderm markers (*Brachyury*, *Goosecoid*) did not increase in a time-dependent manner in chlorate-treated EBs, although it did in untreated EBs (Fig. 2A). In contrast, expression of ectoderm markers (*Mash1*, *Pax6*) was higher in chlorate-treated EBs than in untreated EBs at 4 days after EB formation (Fig. 2A). These results indicate that chlorate treatment from 2 days after EB formation affected lineage choice between mesoderm and ectoderm: it inhibited mesodermal differentiation but induced ectodermal differentiation. As shown in

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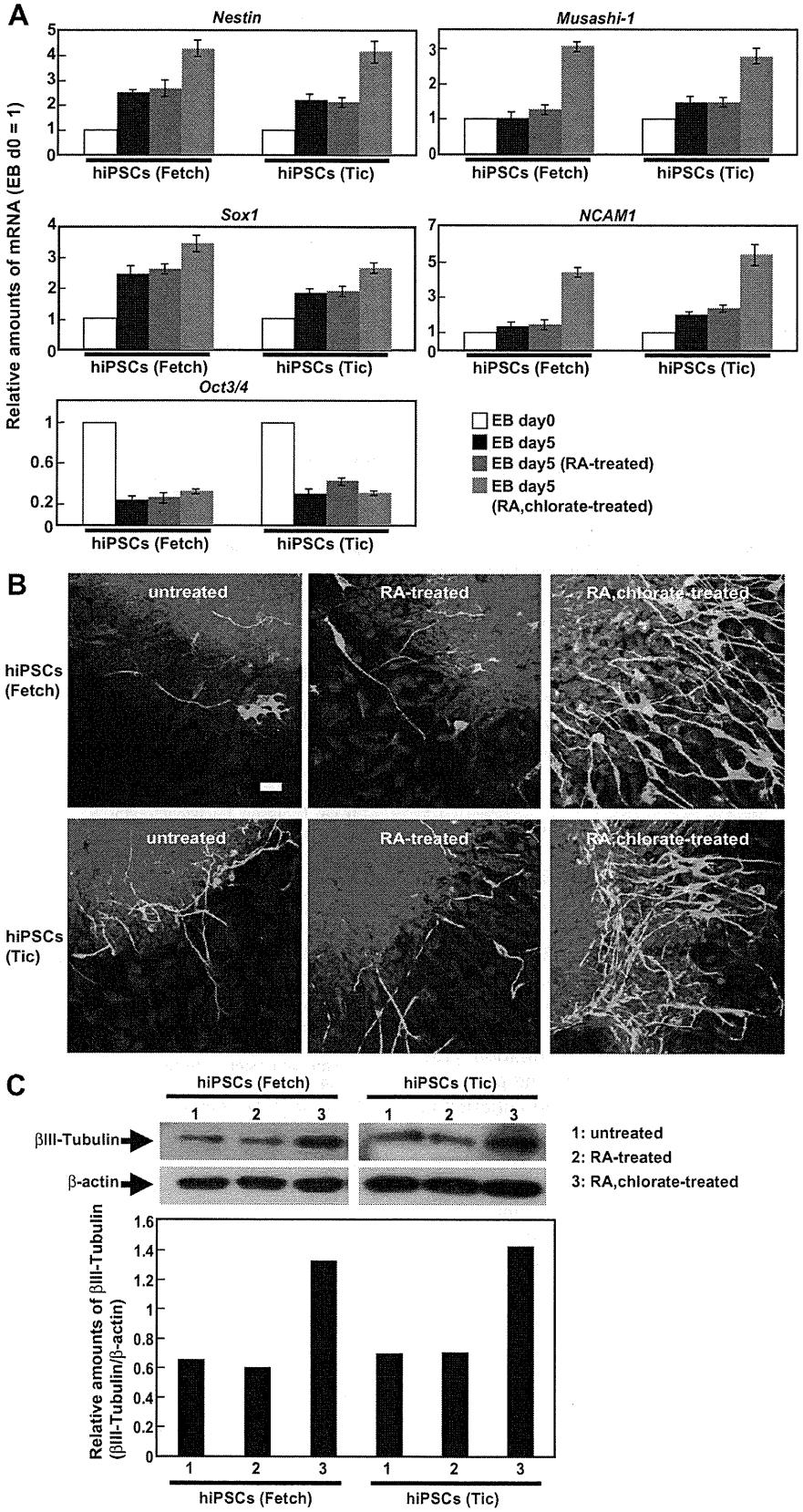

Fig. 4. Reduction of sulfation accelerates neural differentiation of hiPSCs. (A) Real-time PCR analysis of neural markers (*Nestin*, *Musashi-1*, *NCAM1* and *Sox1*) and an undifferentiated cell marker (*Oct3/4*) 5 days after EB formation. The relative amounts of each mRNA were normalized by *GAPDH* mRNA. The results are shown after normalization against the values obtained with undifferentiated hiPSCs (value = 1). The values shown are the means ± SD of duplicate measurements from representative experiments. Two independent experiments were performed. (B) Immunocytochemical staining 2 days after replating of EBs. Representative confocal images from two independent experiments are shown (βIII-tubulin, green; PI, red). Scale bar, 20 μm. (C) Western blotting 2 days after replating of EBs. Representative immunoblots from two independent experiments are shown. The histogram shows mean densitometric readings ± SD of βIII-tubulin/loading controls. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3, Wnt/β-catenin, BMP/Smad, and FGF/ERK signaling, which are involved in the mesodermal differentiation of mESCs [24], were reduced in chlorate-treated EBs. Therefore, the inhibition of these signaling pathways by chlorate treatment seemed to cause defects in mesodermal differentiation and induced ectodermal differentiation. Chlorate treatment from the start of EB formation inhibited initial differentiation, as reported previously (data not shown, [25]). Thus, chlorate treatment from the appropriate time induces differentiation into a specific lineage, in particular neural differentiation.

Next, we investigated further the neural differentiation of chlorate-treated EBs. Firstly, we examined the expression of neural differentiation markers (Fig. 2B). Even at 5 days after EB formation, the expression levels of these markers were significantly higher in chlorate-treated EBs than in EBs only treated with RA, which indicated that differentiation into neural stem/neural progenitor cells and neural precursor cells was accelerated by chlorate treatment. These results were thought to be due to the reduction of Wnt and BMP signaling as shown in Fig. 3. Because, signaling by BMP and Wnt inhibits the neurogenesis of mESCs via EB formation [26,27]. Secondly, we examined the ability of chlorate-treated EBs to differentiate into mature neurons. Immunocytochemical staining for the mature neuron marker βIII-tubulin showed that only 2 days after plating, chlorate-treated EBs generated extremely dense networks of neurite outgrowths, which were not seen with EBs only treated with RA (Fig. 2C). We confirmed by western blotting that the level of βIII-tubulin in chlorate-treated cells was significantly higher than that in cells only treated with RA (Fig. 2D). Taken together, these results demonstrated that the reduction of sulfation by chlorate treatment accelerated the neurogenesis of mESCs.

As described above, the reduction of sulfation on HS and CS by chlorate treatment (Fig. 1) caused defects in Wnt/β-catenin, BMP/Smad, and FGF/ERK signaling during EB formation (Fig. 3), which was followed by the acceleration of neural differentiation (Fig. 2). These results are consistent with previous report [13], in which reduction of PAPST-dependent sulfation promotes neurogenesis of mESCs due to the decreased sulfation on HS and CS.

To date, several signaling pathways have been shown to contribute to the neural differentiation of hESCs and hiPSCs [18,28]. The BMP inhibitor Noggin and the inhibitor of the Lefty/Activin/TGFβ pathways SB431542 enhance neural differentiation [18]. Thus, we examined whether the inhibition of signaling pathways by chlorate treatment also enhanced the neural differentiation of hiPSCs. Real-time PCR analysis 5 days after EB formation showed that the expression of several neural stem/progenitor cell markers was higher in chlorate-treated EBs than in EBs only treated with RA, whereas expression of the marker of undifferentiated cells, *Oct3/4*, was reduced equally in both types of EBs (Fig. 4A). These results indicate that chlorate treatment accelerated the neural differentiation of hiPSCs. Furthermore, as shown in Fig. 4B and C, differentiation into mature neurons was induced markedly in chlorate-treated EBs.

In conclusion, we have demonstrated that the down-regulation of sulfation by chlorate treatment could accelerate neural differentiation of hiPSCs as well as mESCs. This acceleration was induced by the addition of chlorate at the correct time, and involved the inhibition of signaling pathways involved in the induction of mesodermal differentiation (Wnt, BMP, and FGF) and inhibition of neural differentiation (Wnt and BMP). This study as well as our previous report [13] have demonstrated that reduction of sulfation is effective to induce neural differentiation compared with the standard method using RA. In our previous report [13], we used RNAi method using retrovirus vector for long-term neural differentiation. This method requires great care and may give damages to the transfected cells. Moreover, the reduction of sulfation is more drastic in chlorate-treated cells than in *PAPST*-knockdown cells. This reflects more rapid induction of neural differentiation in chlorate-treated cells as shown in this report than in *PAPST*-knockdown cells. Furthermore, in hiPSCs including hESCs, efficiency of gene transfer is very low and for clinical application gene transfer should be avoided. Therefore, chlorate is useful for rapid, simple, and safety reduction of sulfation for rapid neural differentiation of hiPSCs, possibly including hESCs. Taken together, we propose that chlorate, which is available at low cost, could be used to achieve efficient and rapid neural induction of hiPSCs as well as mESCs in place of expensive signaling inhibitors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.09.085.

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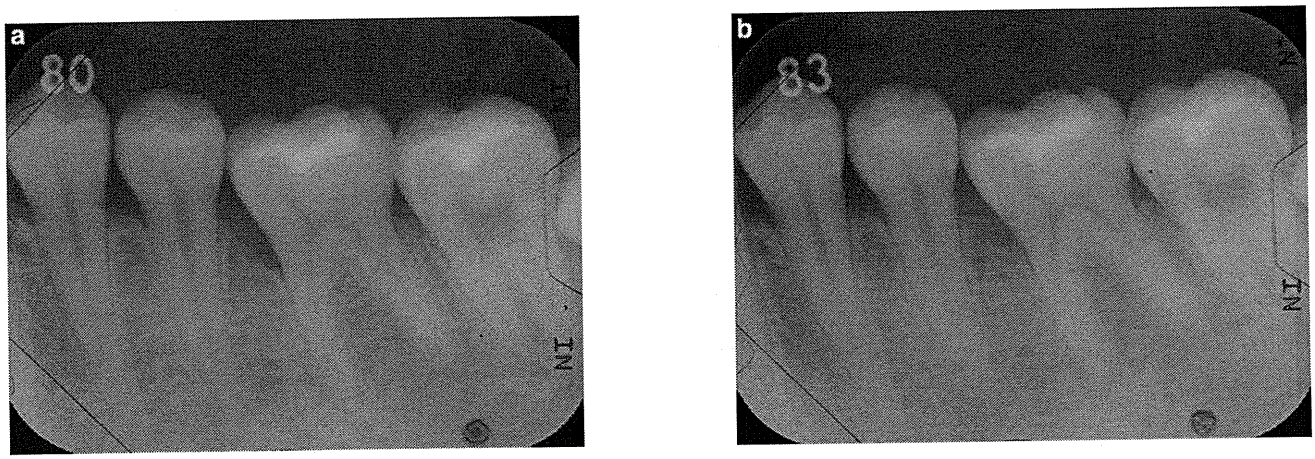
平成24(2012)年 5月

Fibroblast Growth Factor-2 Stimulates Periodontal Tissue Regeneration

Shinya Murakami,* Satoru Yamada,* Takenori Nozaki,* and Masahiro Kitamura*



Introduction: An ideal goal of periodontal therapy is to achieve the complete regeneration of periodontal tissue destroyed by the progression of periodontal diseases. The use of recombinant cytokines is one of the physiologically efficient procedures to stimulate periodontal tissue regeneration. Recently, the safety and efficacy of topical application of basic fibroblast growth factor (FGF-2) to regenerate 2- or 3-walled vertical bone defects was evaluated in Phase 2 multicenter clinical trials in Japan. The results revealed a significant difference in the percentage of increase in alveolar bone height at 36 weeks after administration between the placebo group and the 0.3% FGF-2 group.

Case Presentation: Two cases of intraosseous bone defects, which were treated with a topical application of FGF-2, are presented. To our knowledge, this is the first case report of FGF-2-induced periodontal tissue regeneration. The flap operation was conducted in accordance with the modified Widman procedure, and recombinant human FGF-2 with 3% hydroxypropylcellulose was used as a vehicle and topically applied to the vertical bone defects. A standardized dental radiograph revealed a considerable increase in radiopacity at the bone defects 36 weeks after administration; also, no serious adverse events attributable to the drug were identified in either case.

Conclusion: These results strongly suggest that topical application of 0.3% FGF-2 can be efficacious in an increase in the height of alveolar bone that has been destroyed by periodontitis. *Clin Adv Periodontics* 2011;1:95-99.

Key Words: Case report; fibroblast growth factor 2; periodontitis; regeneration.

Background

Recent progress in periodontal biology has demonstrated that undifferentiated somatic tissue stem cells and progenitor cells exist within the periodontal ligament.[1] Accordingly, improving or enhancing the biologic potential of these cells can facilitate more effective periodontal tissue regeneration. We have been working on establishing new treatments to accelerate the regeneration of periodontal tissue by topical application of the human recombinant fibroblast growth factor-2 (FGF-2).[2-5] Recently, randomized controlled Phase 2 multicenter clinical trials were conducted in Japan to evaluate the safety and efficacy of the FGF-2 drug for use in periodontal tissue regeneration.[6,7] To improve the operability of drug administration to the alveolar bone defect, we mixed freeze-dried recombinant human FGF-2 with 3% hydroxypropylcellulose (HPC), a colorless and viscid vehicle solution, and prepared the gel-like investigational drug. Periodontitis patients with 2- or 3-walled vertical bone defects ≥3 mm from the top of the remaining alveolar bone crest were registered. The primary outcome was the percentage of increase in alveolar bone height at the bone defect, to which the investigational drug was applied. The exploratory Phase 2A clinical trial, which involved 13 dental hospitals and 74 patients, revealed that 0.3% FGF-2

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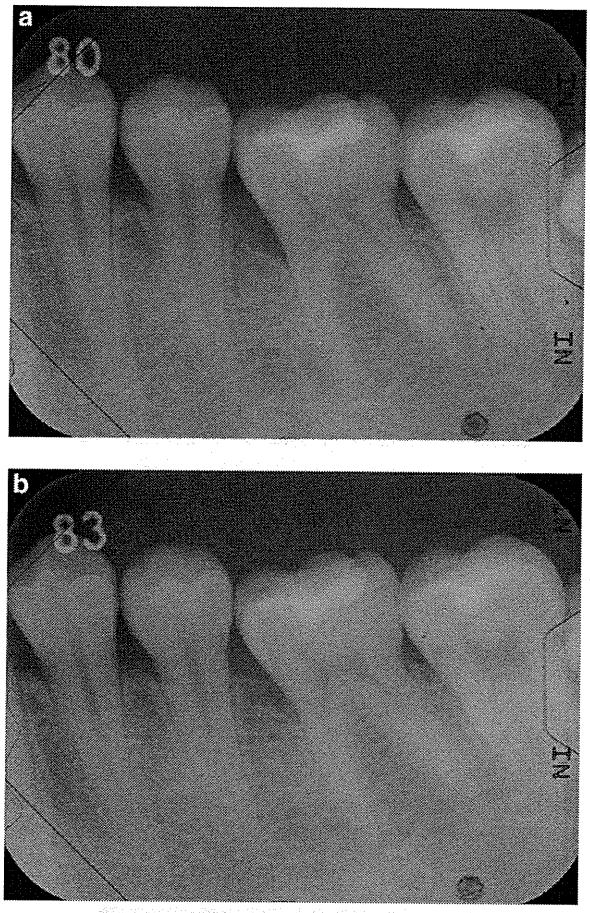

FIGURE 1a Case 1. Standardized dental radiograph before administration. **1b** 36 weeks after administration of 0.3% FGF-2.

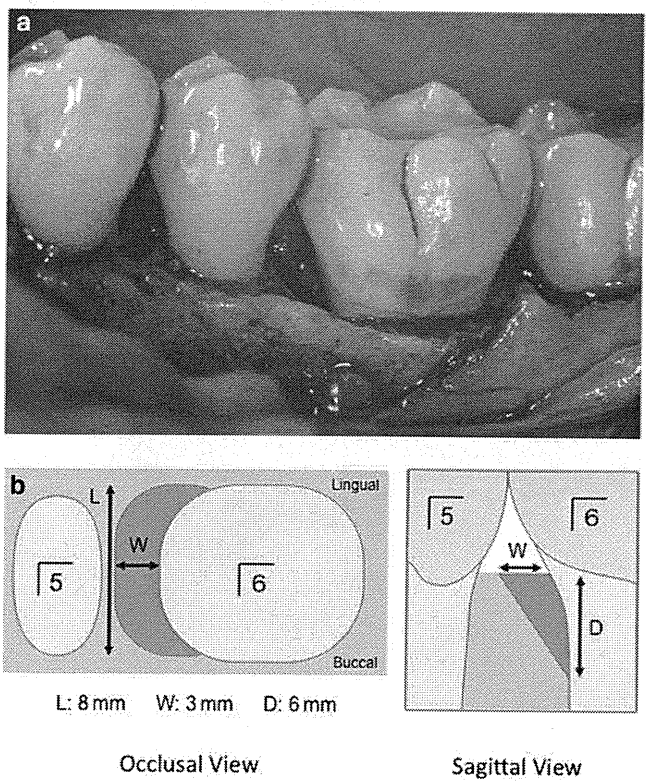

FIGURE 2a Case 1. Image during surgery. **2b** Illustration of the size and shape of the 3-walled bone defect.

significantly improved the percentage of increase in alveolar bone height compared to the vehicle alone, with an ≈2 mm clinical attachment level (CAL) regained.[6] Based on these results, a randomized, double-masked, placebo-controlled Phase 2B clinical trial involving 24 dental hospitals was designed and conducted on 253 periodontitis patients.[7] In this clinical trial, 200 μL of the investigational drug containing 0%, 0.2%, 0.3%, or 0.4% FGF-2 was administered to the bony defects. Each dose of FGF-2 showed a significant increase in the percentage of increase in alveolar bone height at 36 weeks after administration, and the percentage peaked (50.6%) in the 0.3% FGF-2 group. The CAL regained in each group was >2 mm, and no significant difference was observed between the groups. Furthermore, no serious adverse effects attributable to the FGF-2 drug were identified in either clinical trial.

In this case report, we present two cases in which 0.3% FGF-2 was administered to the vertical bone defects. The concentration of FGF-2 was revealed when the clinical trial was completed and the mask was removed.

Clinical Presentation, Management, and Outcomes

Case 1

The patient was a systemically healthy, 24-year-old male. After the initial preparation, a 7-mm periodontal pocket

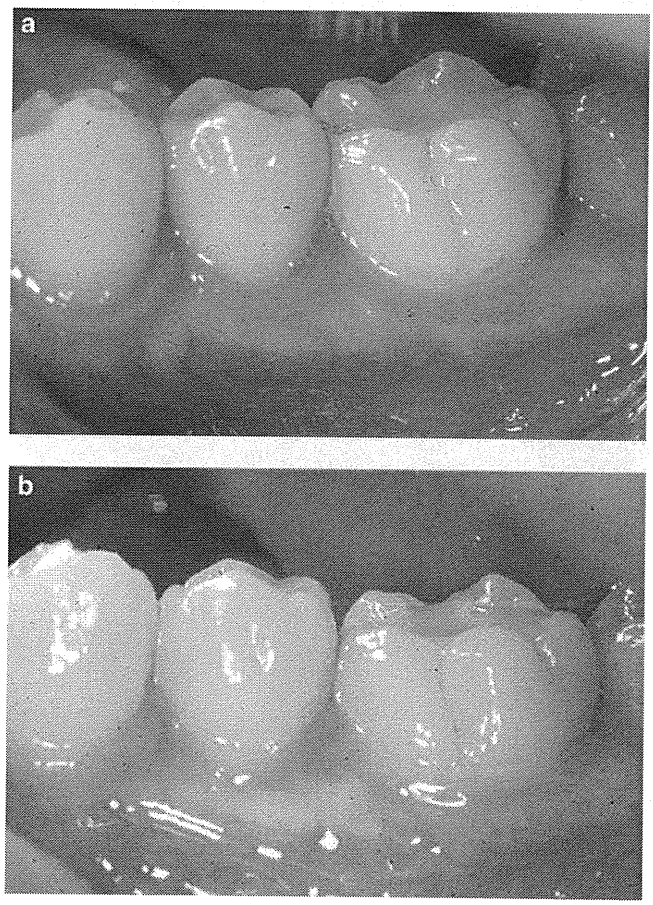

FIGURE 3a Case 1. Preoperative image of the site. **3b** Postoperative image of the FGF-2-treated site.

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remained at the mesio-buccal site of the lower left first molar. The tooth mobility was 0, and the width of the keratinized gingiva was 5 mm. The standardized dental radiograph taken before the surgery shows the vertical bony defect at the mesial site of the tooth (Fig. 1). Because this patient provided written informed consent for this clinical trial, we conducted a flap operation at this site in accordance with the modified Widman procedure.[8] All granulation tissues associated with the bone defect were removed, and soft and hard deposits on the root surface were also removed to ensure thorough degranulation and root planing. Before application of 200 μL of the investigational drug (0.3% FGF-2 plus 3% HPC), the sutures were prepared. They were then closed after the application of the drug to the bone defect. No specific root conditioning was performed. Figure 2 illustrates the size and shape of the 3-walled bone defect that was confirmed during the surgery. Figure 3 shows the images of the test site before administration and again 36 weeks after administration. At 36 weeks after administration, the probing depth of the test site was 3 mm, and the CAL regained was 4 mm. The geometric standardized radiography used photograph indicators. The tooth axis height at the baseline between the remaining alveolar bone crest and the bottom of

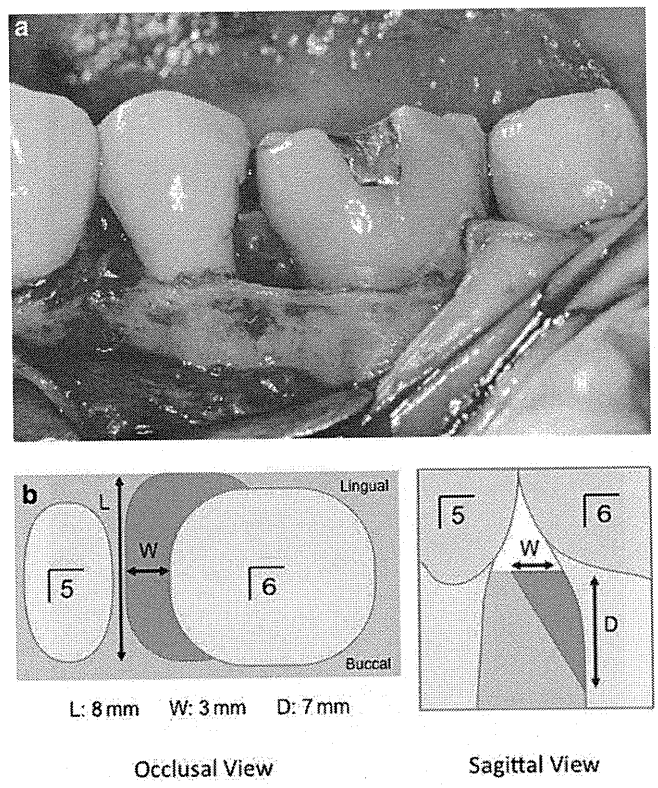

FIGURE 5a Case 2. Image during surgery. **5b** Illustration of the size and shape of the 2-walled bone defect.

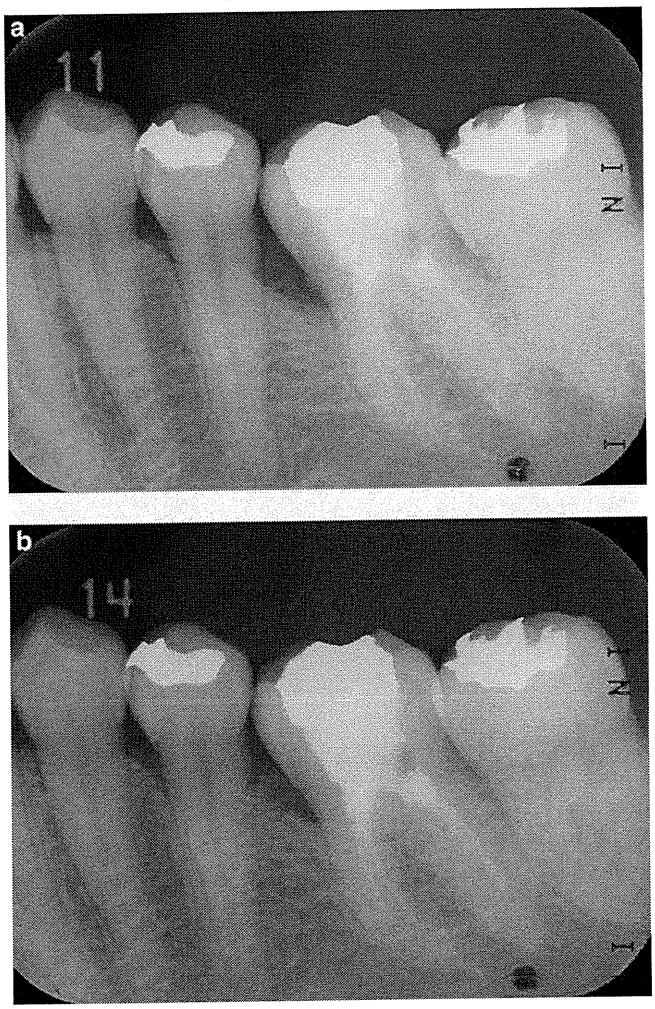

FIGURE 4a Case 2. Standardized dental radiograph before administration. **4b** 36 weeks after administration of 0.3% FGF-2.

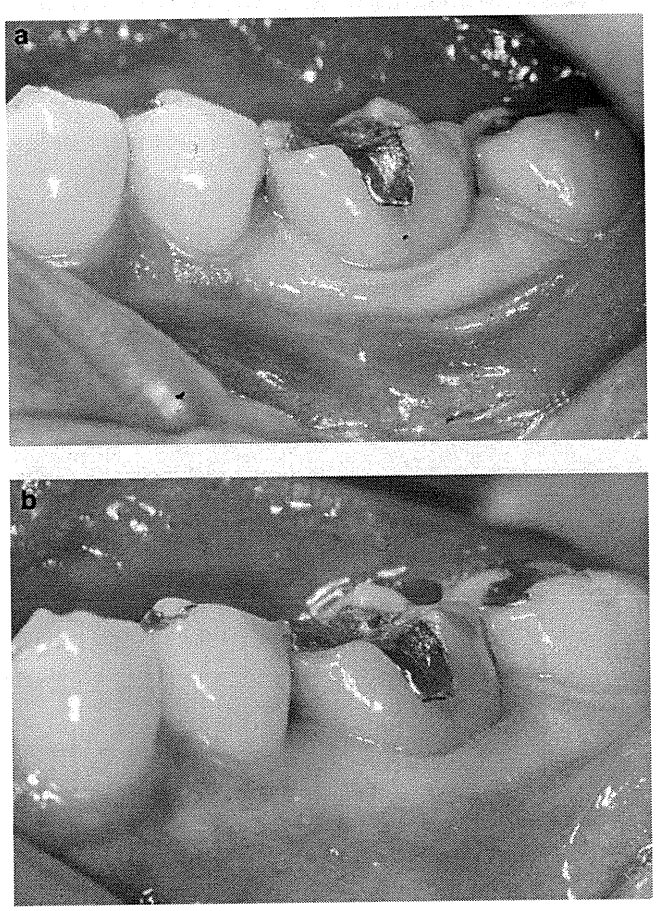

FIGURE 6a Case 2. Preoperative image of the site. **6b** Postoperative image of the FGF-2-treated site.

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the bone defect was regarded as 100%. Five doctors (Toshi Furuuchi, Dai Onodera, Naoyuki Kurihara, Yoichi Shimeno, and Ikuho Kojma, Tohoku University Dental Hospital, Sendai, Japan) specializing in dental radiology independently measured the percentage of increase in alveolar bone height using the methods described previously.[6,7] The median of the five measurements taken from the same image was then selected. The standardized dental radiograph shows the increase in bone mineral content at the test site (Fig. 1); the rate of increase in alveolar bone height was 59.7%.

Case 2

The patient was a systemically healthy, 54-year-old female. After the initial preparation, a 9-mm periodontal pocket remained at the mesio-lingual site of the lower left first molar. The tooth mobility was 0, and the width of the keratinized gingiva was 5 mm. The standardized dental radiograph taken before the surgery shows the vertical bone defect at the mesial site (Fig. 4). Because this patient also provided written informed consent for this clinical trial, we conducted the same surgical procedure at this site, and the investigational drug (0.3% FGF-2 plus 3% HPC) was administered to the bone defect. Figure 5 illustrates the size and shape of the 2-walled bone defect that was confirmed during the surgery. Figure 6 shows the images of the test site before administration and 36 weeks after administration. At 36 weeks after administration, the probing depth of the test site was 3 mm, and the CAL regained was 5 mm. The standardized dental radiograph shows the increase in bone mineral content at the test site (Fig. 4); the rate of increase in alveolar bone height was 101%.

Discussion

The results of these clinical trials strongly suggest that topical application of FGF-2 can be efficacious in alveolar bone height at 2- or 3-walled bone defects and moderate intraosseous bone defects. HPC is merely a vehicle and does not function as an osteoconductive material or space maker. Thus, it is essential in the future to introduce the concept of a "scaffold" into the carrier of the FGF-2 drug to treat severe bony defects or horizontal bone destruction with FGF-2. Interestingly, a recent study using a beagle dog 1-wall defect model indicated the efficacy of concomitant use of FGF-2 and β-tricalcium phosphate for periodontal regeneration after severe destruction.[9] Development of an FGF-2 drug carrier that could provide a formable and osteoconductive scaffold for undifferentiated progenitor cells in the near future would dramatically promote the application of the FGF-2 drug. ■

Summary

Why is this case new information?	To our knowledge, this is the first case report showing that topical application of 0.3% FGF-2 can be efficacious in the increase in alveolar bone height.
What are the keys to successful management of this case?	It is important to ensure thorough degranulation and root planing at the bony defect and to prepare the sutures before application of FGF-2.
What are the primary limitations to success in this case?	Topical application of 0.3% FGF-2 can be efficacious at moderate intraosseous bone defects. However, development of a new scaffold customized for the FGF-2 drug carrier is desirable to treat severe bony defects or horizontal bone destruction with FGF-2.

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○ indicates key references.

原　著

歯周炎罹患歯に対するFGF-2投与の長期的効果および安全性の検討

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Long-term Benefits of Regenerative Therapy Using FGF-2

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Abstract：Basic fibroblast growth factor (FGF-2) is one of the major candidates as a periodontal tissue regenerating agent. A series of animal studies and clinical trials have demonstrated its efficacy and safety. In the present study, we surveyed the eight-year periodontal treatment and symptom records of 79 patients who had been administered investigational drugs containing 0% (placebo; vehicle alone), 0.03%, 0.1% or 0.3% human recombinant FGF-2 (Code No. KCB-1D) in the exploratory phase II clinical trial, to evaluate the long-term benefits of regenerative therapy using FGF-2. The treatments and symptoms caused by progression of local periodontitis and those not related to periodontitis were categorized as "events" or "censored", respectively. The number of events was 14, and survival analysis (generalized Wilcoxon test) revealed that 0.3% FGF-2 significantly prolonged the time to "event" as compared with vehicle alone ($p=0.0345$). In this study, no safety problem was observed

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Key words：regenerative therapy, basic fibroblast growth factor, long-term follow up, retrospective study, survival analysis

要旨：塩基性線維芽細胞増殖因子（FGF-2：basic fibroblast growth factor）は，歯周組織再生誘導薬の有力な候補の一つと期待されており，動物実験および臨床治験によって，その有効性と安全性が明らかにされている。本研究では，KCB-1D（歯周病を対象とした遺伝子組み換えヒト型FGF-2の治験薬コード）を用いた探索的第II相臨床治験に参加した79名の被験者を対象として，フラップ手術時にプラセボ（0%）あるいは0.03%，0.1%，0.3%の何れか濃度のFGF-2を投与した被験歯の長期経過を調査した。すなわち，診療録などの診療情報から，臨床治験最終観察日から本研究調査実施日までの間（約8年間）に，各種濃度のFGF-2あるいはプラセボを投与された被験歯に対して行なわれた治療や，被験歯に出現した症状の内容と年月日を調査した。そして，これらのうち，治験薬投与部位における歯周炎の悪化に起因すると判定された治療や症状をイベント，それ以外を打ち切りとして，生存時間解析を行った。その結果，発生した全イベントは14例で，生存時間解析の結果，0.3% FGF-2投与群はフラップ手術を単独で施行したプラセボ群に比べてイベント発生までの期間の有意な延長が認められた（一般化Wilcoxon検定：$p=0.0345$）。また，本研究の観察期間を含めてFGF-2投与の安全性に関する問題は認めなかった。

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キーワード：歯周組織再生，塩基性線維芽細胞増殖因子，歯の長期的予後，後ろ向き観察研究，生存時間解析

緒　言

超高齢化社会を迎え，歯の喪失に伴う様々なQOLの低下が社会的問題となっている我国で，成人が歯を喪失する第一の原因は歯周炎である[1]。そのため，歯周炎によって失われた歯周組織を再生させることにより歯の喪失を防ぐことができれば，生涯を通じて自分の歯で咀嚼することが可能となり国民のQOLの向上，さらにはより良い全身状態の維持に寄与するものと考えられる。そこで現在，歯周炎による歯の喪失を減少させるため，重度歯周炎に対する標準的治療法となりうる歯周組織再生誘導薬の開発が強く求められている。

塩基性線維芽細胞増殖因子（FGF-2：basic fibroblast growth factor）は，歯周組織再生誘導薬の有力な候補の一つとして期待され，ビーグル犬[2,3]やカニクイザル[4]を用いた動物実験および3，2壁性歯槽骨欠損を有すると診断された歯周炎患者を対象とした第II相臨床治験[5,6]で，その歯周組織再生誘導能と安全性が明らかになってきている。これらの第II相臨床治験では，FGF-2投与36週後の時点で，臨床的な付着を獲得しつつ，通常のフラップ手術に比べて統計学的に有意な新生歯槽骨の増加がもたらされることが証明されている。しかしながら，歯周組織再生療法の真の目的が長期的な歯の保存であることを考慮すると，歯の予後を含めたFGF-2投与のさらに長期的な効果と安全性を検討することは大きな意義があると考えられる。そこで，本研究では，上記のFGF-2を用いた新規歯周組織再生療法の開発に係る第II相臨床治験のなかでも，早期に実施され長期の術後観察が可能である探索的試験[5]に参加した被験者を対象として，各種濃度のFGF-2あるいはプラセボを投与した歯の長期経過を調査し，歯周炎に対するFGF-2を用いた歯周

組織再生療法の長期的な効果と安全性を検討した。

材料および方法

1. 被験者

対象は，KCB-1D（歯周病を対象とした遺伝子組み換えヒトFGF-2の治験薬コード）探索的試験[5]で試験対象の3壁もしくは2壁性の骨欠損形態を有すると診断された部位（各被験者につき1部位）に治験薬が投与された被験者79名とした。同治験は二重盲検・多施設共同・無作為化・プラセボ対照のデザインで，医薬品の臨床試験の実施に関する基準（GCP：Good Clinical Practice）遵守下で，科研製薬株式会社の依頼に基づき2001年～2004年に実施された。被験歯には，ハイドロキシプロピルセルロース（HPC）を基材としたプラセボ（0%），0.03%，0.1%，0.3%の何れかの濃度のFGF-2を含有する治験薬がフラップ手術時に単回投与され，それぞれの歯周組織再生状態が比較・検討され，0.3% FGF-2投与群で統計学的に有意な歯槽骨の増加が認められている[5]。

2. 研究組織および研究デザイン

KCB-1D探索的試験[5]を実施した13施設の研究責任者が臨床研究施設の被験者の診療録などの診療情報から，臨床治験最終観察日から本研究調査実施日までの間に被験歯にみられた歯周病の再発や予後に関わる各種事象の発生日と発生状況を追跡可能な限り調査した。その後，研究代表者（村上伸也）が各施設の研究責任者から匿名化されたデータの提供を受け，各種濃度のFGF-2あるいはプラセボが投与された被験歯における各種事象の発生頻度や程度を比較・解析した。なお，本研究に必要なKCB-1D探索的試験[5]の成績は，科研製薬株式会社から研究代表者に提供された。

3. 調査実施手順

各臨床研究施設の研究責任者あるいは主治医等の研究分担者は，所属研究施設の診療録等の診療情報より，治験薬投与36週後のKCB-1D探索的試験[5]の最終観察日から本研究調査日までの期間に被験歯（治験薬を投与した歯）に対して行った治療もしくは被験歯に生じた事象（以下，治療等）のうち(1)～(6)に該当するものとその時期（年月日）を調査した。そして，さらに，(1)～(5)については，当該治療等の発生が治験薬投与部位の歯周炎の悪化に起因するか否かを各臨床研究施設の研究責任者あるいは研究分担者が判定した。調査内容は調査票に記載され，匿名化されて研究代表者へ提出された。なお，各施設の研究責任者あるいは研究分担者の判定の妥当性に関しては別途設置した委員会で評価・確認された。

(1) 抜歯（治験薬投与部位を有する歯根の抜去を含む）
(2) 歯周組織再生療法（エナメルマトリクスタンパク：EMDを用いた歯周組織再生療法，歯周組織再生誘導法：GTR法等）
(3) 歯周組織再生療法を除く歯周外科手術
(4) 積極的な介入をした非外科的歯周治療（歯肉縁下の処置を目的としたスケーリング・ルートプレーニング，局所抗菌薬投与等）
(5) その他，歯周炎の進行が原因となって生じた事象（逆行性歯髄炎等）
(6) 異常な歯周組織の治癒（歯肉増殖等）が疑われる所見

4. 統計解析

上記の(1)～(5)の治療等うち，治験薬投与部位の歯周炎の悪化に起因する治療等であると判定されたものをイベント，それ以外を打ち切りとして，生存時間解析を行った。なお，観察期間内に同一被験歯に治療等が複数存在する場合には，最初に発生した治療等をイベントもしくは打ち切りとして扱った。また，観察期間内に治療等が認められなかった被験者のうち，現在は来院していない被験者は診療情報が最後に得られた日を打ち切り日に，そして，現在も来院している被験者は調査実施日を打ち切り日とした。

5. 倫理的対応

本研究は，「臨床研究に関する倫理指針（平成20年7月31日厚生労働省告示第415号）」を遵守して実施され，実施前に各研究施設の倫理委員会もしくはそれに相当する組織の承認を得た。本研究は被験者に対する介入を伴わない既存の診療情報のみを用いた観察研究であるため，被験者に対するインフォームド・コンセントは各研究施設が必要と判断した場合にのみ取得した。そして，インフォームド・コンセントの取得が必要ではないと判断された場合においては，本研究の概要を各臨床研究施設のホームページ等で公開した。

結　果

1. 被験者の構成

被験者構成を図1に示す。治験薬投与が行われた被験者79名の中で，25名は転院等によって調査実施日までの診療情報の一部が得らなかったため治療等が発