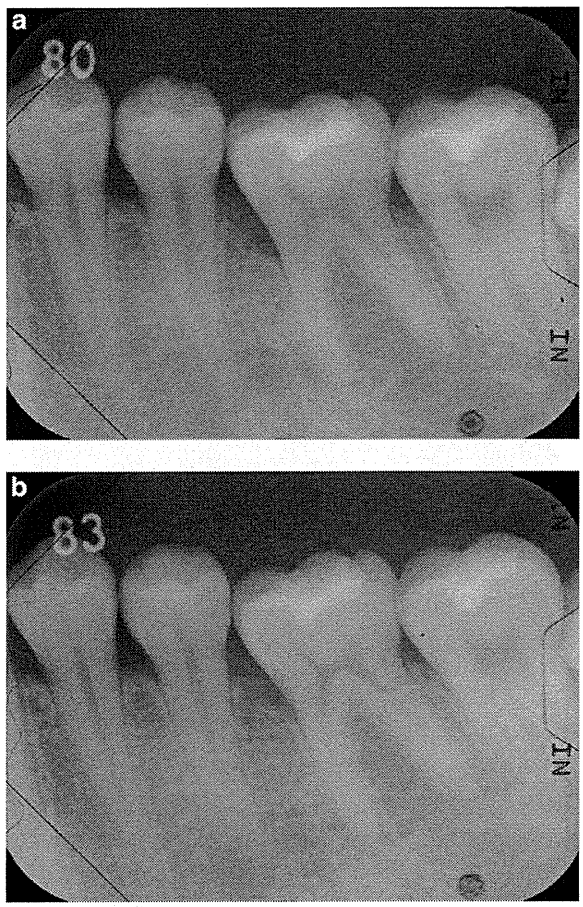

FIGURE 1a Case 1. Standardized dental radiograph before administration. **1b** 36 weeks after administration of 0.3% FGF-2.

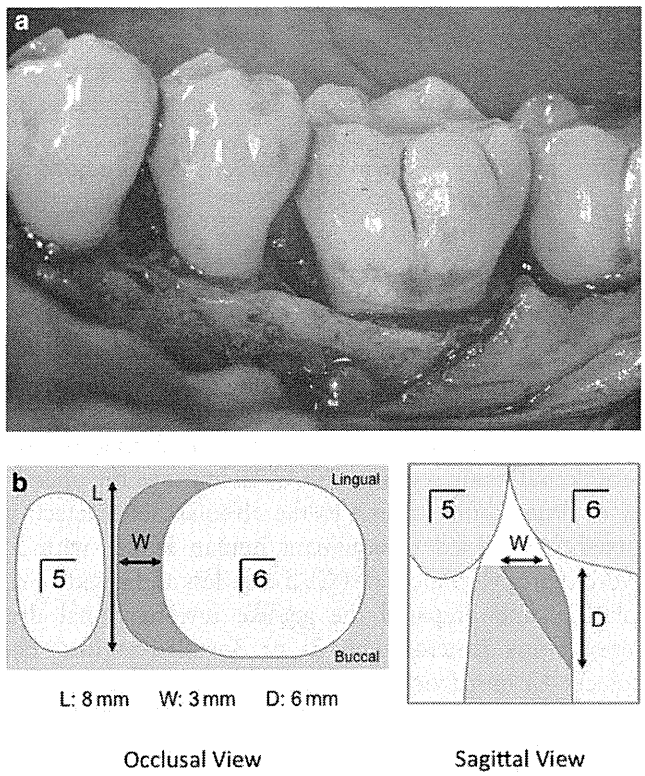

FIGURE 2a Case 1. Image during surgery. **2b** Illustration of the size and shape of the 3-walled bone defect.

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

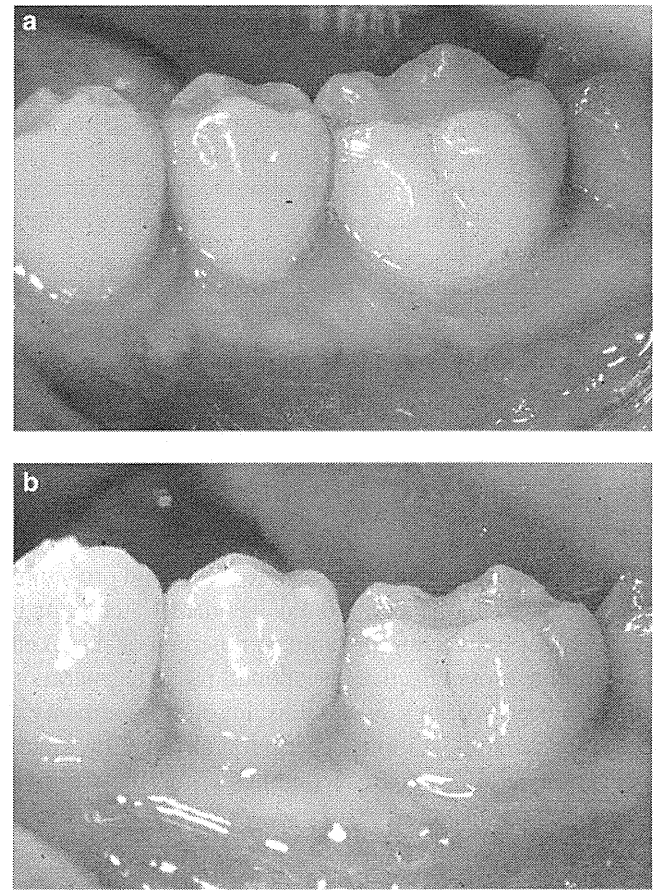

FIGURE 3a Case 1. Preoperative image of the site. **3b** Postoperative image of the FGF-2-treated site.

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

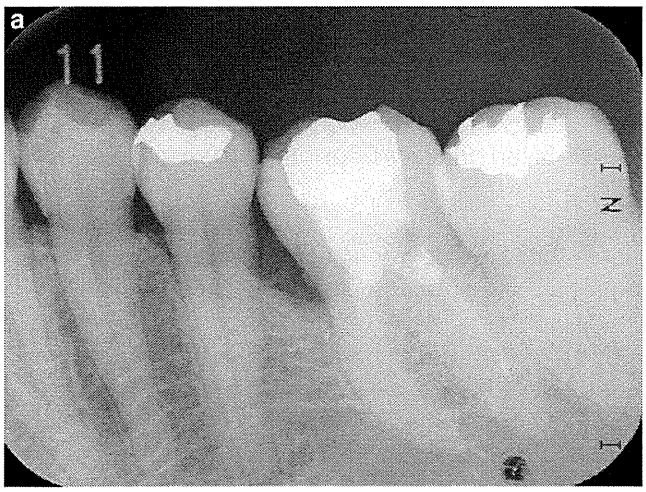

FIGURE 4a Case 2. Standardized dental radiograph before administration. **4b** 36 weeks after administration of 0.3% FGF-2.

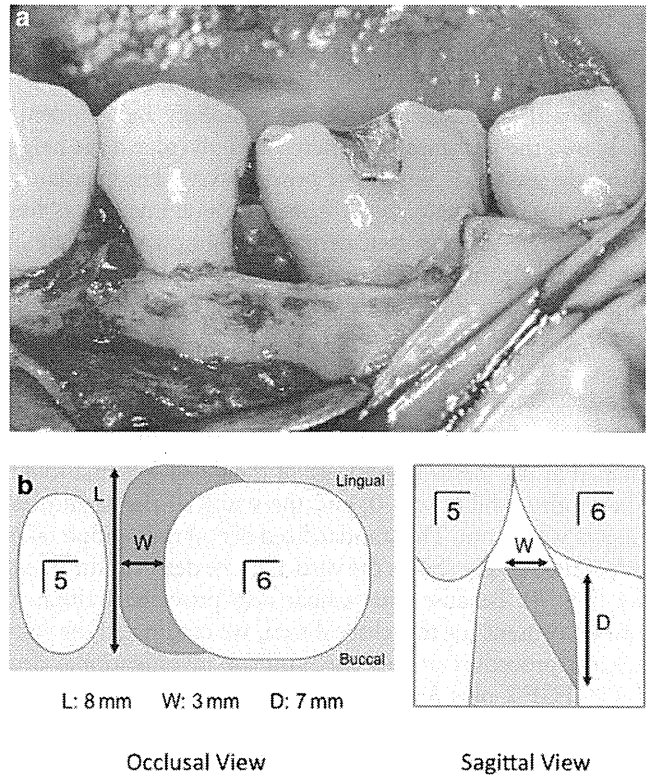

FIGURE 5a Case 2. Image during surgery. **5b** Illustration of the size and shape of the 2-walled bone defect.

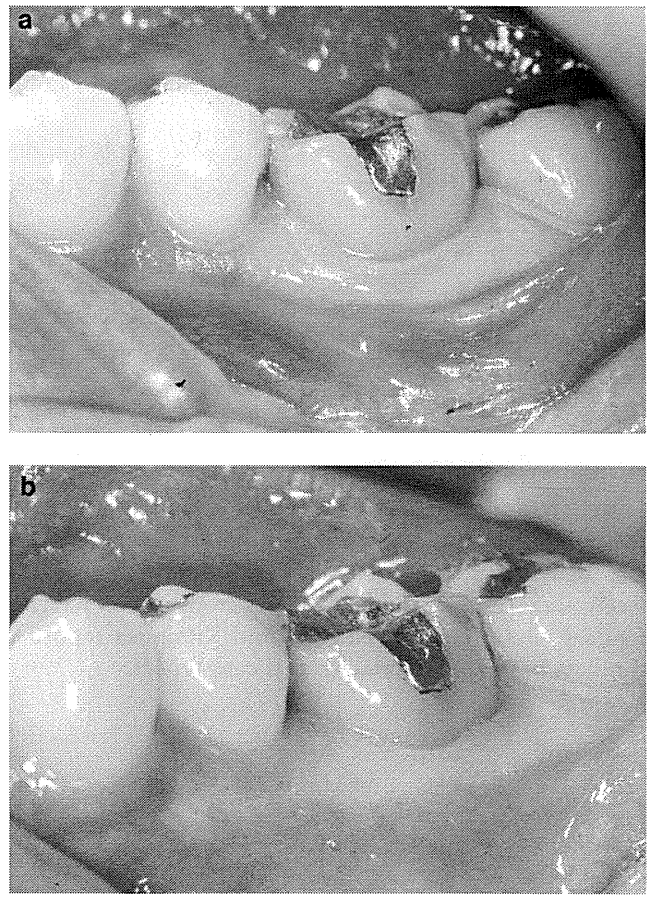

FIGURE 6a Case 2. Preoperative image of the site. **6b** Postoperative image of the FGF-2-treated site.

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.

Acknowledgments

The clinical trials were supported by Kaken Pharmaceutical (Tokyo, Japan), which has provided research grants (Dr. Murakami, J100801004). Dr. Murakami has also received consulting and lecture fees from Kaken Pharmaceutical. Drs. Yamada, Nozaki, and Kitamura report no conflicts of interest related to this case report.

CORRESPONDENCE:
Dr. Shinya Murakami, Department of Periodontology, Graduate School of Dentistry, Osaka University, 1-8 Yamadaoka, Suita, Osaka, 565-0871, Japan. E-mail: ipshinya@dent.osaka-u.ac.jp.

References

1. Seo BM, Miura M, Gronthos S, et al. Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet* 2004;364:149-155.
2. Kao RT, Murakami S, Beirne OR. The use of biologic mediators and tissue engineering in dentistry. *Periodontol 2000* 2009;50:127-153.
3. Murakami S. Periodontal tissue regeneration by signalling molecule(s): What role does fibroblast growth factor (FGF-2) have in periodontal therapy? *Periodontol 2000* 2011;56:188-208.
4. Takayama S, Murakami S, Shimabukuro Y, Kitamura M, Okada H. Periodontal regeneration by FGF-2 (bFGF) in primate models. *J Dent Res* 2001;80:2075-2079.
5. Murakami S, Takayama S, Kitamura M, et al. Recombinant human basic fibroblast growth factor (bFGF) stimulates periodontal regeneration in class II furcation defects created in beagle dogs. *J Periodontal Res* 2003; 38:97-103.
6. Kitamura M, Nakashima K, Kowashi Y, et al. Periodontal tissue regeneration using fibroblast growth factor-2: Randomized controlled phase II clinical trial. *PLoS One* 2008;3:e2611.
7. Kitamura M, Akamatsu M, Machigashira M, et al. FGF-2 stimulates periodontal regeneration: Results of a multi-center randomized clinical trial. *J Dent Res* 2011;90:35-40.
8. Ramfjord SP, Nissle RR. The modified Widman flap. *J Periodontol* 1974;45:601-607.
9. Anzai J, Kitamura M, Nozaki T, et al. Effects of concomitant use of fibroblast growth factor (FGF)-2 with beta-tricalcium phosphate (β-TCP) on the beagle dog 1-wall periodontal defect model. *Biochem Biophys Res Commun* 2010;403:345-350.

(1) 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

北村正博[*1]，古市保志[*2]，藤井健男[*3]，川浪雅光[*4]，國松和司[*5]，
島内英俊[*6]，山田　了[*7]，小方頼昌[*8]，和泉雄一[*9]，伊藤公一[*10]，
中川種昭[*11]，新井　高[*12]，山崎和久[*13]，吉江弘正[*14]，野口俊英[*15]，
渋谷俊昭[*16]，高柴正悟[*17]，栗原英見[*18]，永田俊彦[*19]，横田　誠[*20]，
前田勝正[*21]，廣藤卓雄[*22]，坂上竜資[*23]，原　宜興[*24]，野口和行[*25]，
小笠原健文[*26]，村上伸也[*1]

[*1]大阪大学大学院歯学研究科口腔分子免疫制御学講座歯周病分子病態学
[*2]北海道医療大学歯学部口腔機能修復・再建学系 歯周歯内治療学分野
[*3]北海道医療大学歯学部口腔機能修復・再建学系高度先進保存学分野
[*4]北海道大学大学院歯学研究科口腔健康科学講座歯周・歯内療法学教室
[*5]医療法人社団 顕正会歯科臨床研修センター
[*6]東北大学大学院歯学研究科口腔生物学講座歯内歯周治療学分野
[*7]東京歯科大学
[*8]日本大学松戸歯学部歯周治療学講座
[*9]東京医科歯科大学大学院医歯学総合研究科歯周病学分野
[*10]日本大学歯学部保存学教室歯周病学講座
[*11]慶應義塾大学病院歯科・口腔外科教室
[*12]鶴見大学歯学部第二歯科保存学教室
[*13]新潟大学大学院医歯学総合研究科口腔生命福祉学専攻口腔生命福祉学科口腔保健学講座
[*14]新潟大学大学院医歯学総合研究科摂食環境制御学講座歯周診断・再建学分野
[*15]愛知学院大学歯学部歯周病学講座
[*16]朝日大学歯学部口腔感染医療学講座歯周病学分野
[*17]岡山大学大学院医歯薬学総合研究科病態制御科学専攻病態機構学講座歯周病態学分野
[*18]広島大学大学院医歯薬学総合研究科先進医療開発科学講座歯周病態学分野
[*19]徳島大学大学院ヘルスバイオサイエンス研究部歯周歯内治療学分野
[*20]九州歯科大学
[*21]九州大学大学院歯学研究院口腔機能修復学講座歯周病学分野
[*22]福岡歯科大学総合歯科学講座総合歯科学分野
[*23]福岡歯科大学口腔治療学講座歯周病学分野
[*24]長崎大学大学院医歯薬学総合研究科医療科学専攻展開医療科学講座歯周病学分野
[*25]鹿児島大学大学院医歯学総合研究科先進治療科学専攻顎顔面機能再建学講座歯周病学分野
[*26]町田市民病院　歯科・歯科口腔外科

（受付日：2011年11月14日　受理日：2012年1月28日）

連絡先：村上伸也
〒565-0871 大阪府吹田市山田丘1-8
大阪大学大学院歯学研究科口腔分子免疫制御学講座歯周病分子病態学
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：ipshinya @dent.osaka-u.ac.jp

Long-term Benefits of Regenerative Therapy Using FGF-2

Masahiro Kitamura[*1], Yasushi Furuichi[*2], Takeo Fujii[*3], Masamitsu Kawanami[*4], Kazushi Kunimatsu[*5], Hidetoshi Shimauchi[*6], Satoru Yamada[*7], Yorimasa Ogata[*8], Yuichi Izumi[*9], Koichi Ito[*10], Taneaki Nakagawa[*11], Takashi Arai[*12], Kazuhisa Yamazaki[*13], Hiromasa Yoshie[*14], Toshihide Noguchi[*15], Toshiaki Sibutani[*16], Syogo Takashiba[*17], Hidemi Kurihara[*18], Toshihiko Nagata[*19], Makoto Yokota[*20], Katsumasa Maeda[*21], Takao Hirofuji[*22], Ryuji Sakagami[*23], Yoshitaka Hara[*24], Kazuyuki Noguchi[*25], Takefumi Ogasawara[*26] and Shinya Murakami[*1]

[*1]Department of Periodontology, Division of Oral Biology and Disease Control, Osaka University Graduate School of Dentistry, [*2]Department of Oral Rehabilitation, Division of Periodontology and Endodontology, School of Dentistry, Health Sciences University of Hokkaido, [*3]Department of Oral Rehabilitation, Division of Highly-advanced Conservative Dentistry, School of Dentistry, Health Sciences University of Hokkaido, [*4]Department of Periodontology and Endodontology, Division of Oral Health Science, Hokkaido University Graduate School of Dental Medicine, [*5] Dental Training Center, Kensei-kai Medical Incorporated Association, [*6] Division of Periodontology and Endodontology, Department of Oral Biology, Tohoku University Graduate School of Dentistry, [*7]Tokyo Dental College, [*8]Department of Periodontology, Nihon University School of Dentistry at Matsudo, [*9]Department Periodontology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, [*10]Department of Periodontology, Nihon University School of Dentistry, [*11]Department of Dentistry and Oral Surgery, School of Medicine, Keio University, [*12]Department of Periodontics and Endodontics, Tsurumi University of Dental Medicine, [*13]Laboratory of Periodontology and Immunology, Division of Oral Science for Health Promotion, Niigata University Graduate School of Medical and Dental Sciences, [*14]Department of Oral Biological Science, Division of Periodontology, Niigata University Graduate School of Medical and Dental Sciences, [*15]Department of Periodontology, School of Dentistry, Aichi-Gakuin University, [*16]Department of Periodontology, Division of Oral Infections and Health Science, School of Dentistry, Asahi University, [*17] Department of Pathophysiology-Periodontal Science, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, [*18]Department of Periodontal Medicine, Division of Frontier Medical Science, Hiroshima University Graduate School of Biomedical Sciences, [*19]Department of Periodontology and Endodontology, Institute of Health Biosciences, The University of Tokushima Graduate School, [*20]Kyushu Dental College, [*21] Section of Periodontology, Division of Oral Rehabilitation, Faculty of Dental Science, Kyushu University, [*22]Section of General Dentistry, Department of General Dentistry, Fukuoka Dental College, [*23] Section of Periodontology, Department of Odontology, Fukuoka Dental College, [*24]Department of Periodontology, Unit of Translationall Medicine, Graduate School of Biomedical Sciences, Nagasaki University, [*25]Division of Periodontology Department of Oral and Maxillofacial Rehabilitation Course for Developmental Therapeutics Kagoshima University Graduate School of Medical and Dental Sciences, [*26]Department of Dentistry and Oral Surgery, Machida Municipal Hospital and

(Received：November 14, 2011 Accepted：January 28, 2012)

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

Nihon Shishubyo Gakkai Kaishi (J Jpn Soc Periodontol) 54(1)：●●－●●, 2012.

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。

日本歯周病学会会誌（日歯周誌）54(1)：●●－●●, 2012

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

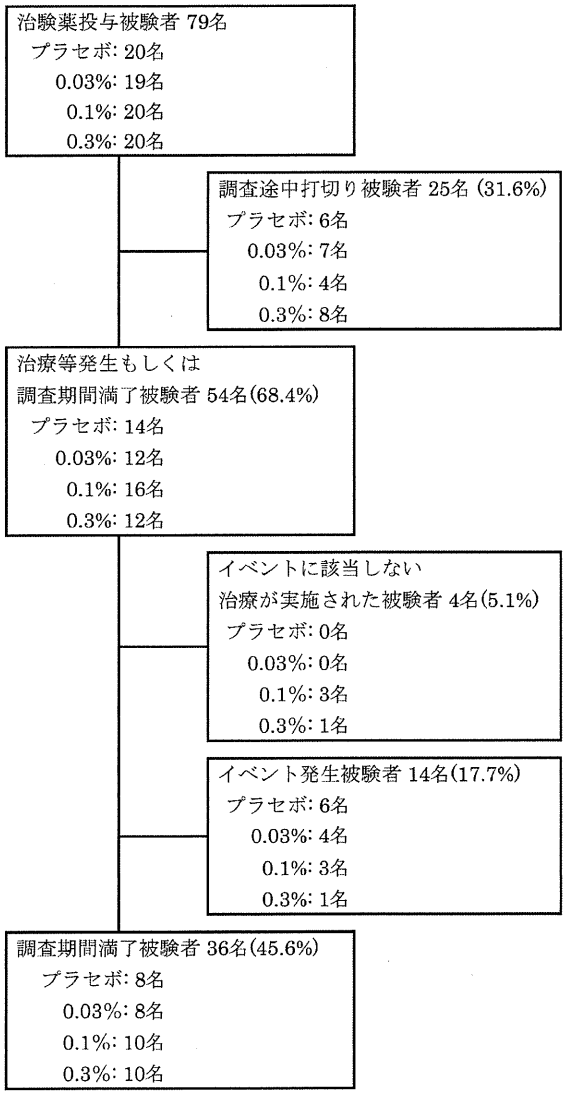

図1　被験者構成

生したか否かを十分に判断できない調査途中打ち切り被験者であった。残りの54名の中で，イベント発生被験者は14名，イベントに該当しない治療等（治療計画上の積極的な抜歯，根尖性歯周炎のための歯根端切除術等）により打ち切りとなった被験者は4名，そして，調査実施日までイベントの発生やイベントに該当しない治療の実施がなかったことが診療情報から確認できた調査期間満了被験者は36名であった（図1）。

2．各群の被験者，被験歯および試験部位の特性と観察期間

KCB-1D探索的試験[5]参加時の被験者の性別や年齢，被験歯の歯種および試験部位の骨吸収等の臨床所見に特記すべき群間の偏りは認められていない。そして，治験薬投与日から最後に被験歯を観察した日までの期間も，全ての群で同程度であった（表1）。

表1　各群の治験薬投与日から最後に被験歯を観察した日までの期間

	中央値(日)	範囲(最小－最大)
プラセボ投与群(20名)	2,887	714－3,143
0.03%FGF－2投与群(19名)	2,879	263－3,093
0.1%FGF－2投与群(20名)	2,886	168－3,150
0.3%FGF－2投与群(20名)	2,868	249－3,122

3．イベント

各群で発生したイベントの内容と発生数を表2に示す。イベントが発生した14名の中で，積極的な介入をした非外科的歯周治療の発生数が7名と最も多かった。続いて，歯周組織再生療法（EMD，GTR等）が3名と多く，抜歯と歯周組織再生療法を除く歯周外科手術がそれぞれ2名であった。その他，歯周炎の進行が原因となって生じた事象は全ての群で発生しなかった。また，各群のイベントの発生数は，プラセボ群6名，0.03%群4名，0.1%群3名，0.3%群1名であった（図1および表2）。

4．生存時間解析

治験薬投与日（0日目）からの経過日数に対するイベント未発生率を表すKaplan-Meier曲線を図2に示す。生存時間解析の結果，0.3% FGF-2投与群は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：$p=0.0345$)。すなわち，0.3%FGF-2投与群では投与後2800日(約7.6年)程度で1名にイベントが発生し，観察期間の最終的なイベント未発生率は約80%であった。これに対してプラセボ群では，投与後2000日(約5.4年)まではイベント未発生率80%を維持していたものの，それ以降にイベント発生が増加し，最終的なイベント未発生率は約60%であった（図2）。

5．安全性

本研究の観察期間中に，被験歯に異常な歯周組織の治癒（歯肉増殖等）が疑われる所見は認められなかった。

表2　発生したイベントと各群における発生数

(1)抜歯	全体 2名 (2.5%)
(治験薬投与部位を有する歯根の抜去を含む)	プラセボ: 1名
	0.03%: 0名
	0.1%: 1名
	0.3%: 0名
(2)歯周組織再生療法	全体 3名 (3.8%)
(EMD, GTR等)	プラセボ: 2名
	0.03%: 1名
	0.1%: 0名
	0.3%: 0名
(3)歯周組織再生療法を除く歯周外科手術	全体 2名 (2.5%)
	プラセボ: 0名
	0.03%: 0名
	0.1%: 2名
	0.3%: 0名
(4)積極的な介入をした非外科的歯周治療	全体 7名 (8.9%)
(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)	プラセボ: 3名
	0.03%: 3名
	0.1%: 0名
	0.3%: 1名
(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)	全体 0名 (0%)
	プラセボ: 0名
	0.03%: 0名
	0.1%: 0名
	0.3%: 0名
合計	全体14名 (17.7%)
	プラセボ: 6名
	0.03%: 4名
	0.1%: 3名
	0.3%: 1名

考　察

現在，進行した歯周炎に対する歯周外科処置としては，組織付着療法に分類されるフラップ手術が標準的治療法として施行されている。フラップ手術は，歯根面および歯周ポケット内部に蓄積した細菌および細菌由来病原物質を汚染セメント質とともに除去することが可能であり，術後には，炎症の軽減，臨床的アタッチメントの獲得，歯周ポケットの減少が認められる[7]。しかしながら，フラップ手術単独では，本来の歯周組織に見られる歯槽骨，歯根膜およびセメント質の新生を伴う線維性付着はわずかしか形成されず，歯周組織の再生はほとんど期待できない。また，臨床的アタッチメントの獲得も長い上皮性付着の形成によることが多く[8]，長期的には再度の付着喪失を伴う歯周炎の再発が懸念される。そのため，フラップ手術等の従来の歯周外科処置では達成できない歯周組織の再生に対する臨床現場の期待は非常に大きく，盛んに研究開発が行われてきた。そして現在，歯周組織再生誘導法（GTR 法）およびエナメルマトリックスタンパク（EMD）を用いた歯周組織再生療法が我国で臨床適応されており，米国では血小板由来増殖因子(PDGF-BB)と骨補填材である多孔性リン酸三カルシウム(β-tricalcium phosphate; β-TCP）との合剤がヒト型リコンビナントサイトカインを用いた歯周組織誘導材料として登場している[9]。しかしながら，これらの方法は，治療成績が術者の技術レベルに大きく左右されたり，動物由来製剤であったりといった問題点から，重度歯周炎に対する標準的治療法となり得る歯周組織再生誘導薬の開発が強く求められている。そこで，我々は，様々な細胞に対する生理活性を有する FGF-2 を，歯周組織再生誘導薬の有力な候補の一つと考え，その歯周組織誘導能の動物実験[2-4]による確認を経て，3，2 壁性歯槽骨欠損を有する歯周炎患者を対象とした第 II 相臨床治験[5,6]を展開してきた。その結果，FGF-2 投与 36 週後において，通常のフラップ手術と同程度の付着を獲得するとともに有意な歯槽骨新生がもたらされたことから，FGF-2 の歯周組織再生誘導薬としての有用性が強く示唆されている。そこで，本研究では，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し，投与 36 週後において歯周組織再生誘導薬としての有用性が確認された FGF-2 のさらに長期的な効果と安全性を検討するため，第 II 相臨床治験の中でも臨床治験終了後の経過期間が約 8 年と長期に及ぶ KCB-1D 探索的試験[5]に参加した被験者を対象として，フラップ手術時に各種濃度の 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た。

歯周組織の再生を歯槽骨，セメント質，歯根膜などの再生と定義[10]すれば，これらの組織の再生を直接かつ明確に確認する唯一の方法は組織学的評価である。しかしながら，ヒトでの組織学的な評価は倫理的な観点から困難を極めるため，一般的に臨床試験では，規格エックス線写真による歯槽骨レベルの評価や臨床的アタッチメントレベル（CAL）の測定が歯周組織再生を評価する代替評価項目として利用される[10]。しかしながら，数年を超える長期の臨床経過の追跡では，その間に咬耗・摩耗や修復処置による歯冠形態の変化や歯の移動が生じるケースが多く，X 線検査の規格化や一定の基準点を設定した CAL の測定は，実際には不可能な場合が多い。そこで，本研究では，一連の動的な歯周治療を受けサポーティブペリオドンタルセラピィを受けている被験者では，歯周炎の治療経過が悪

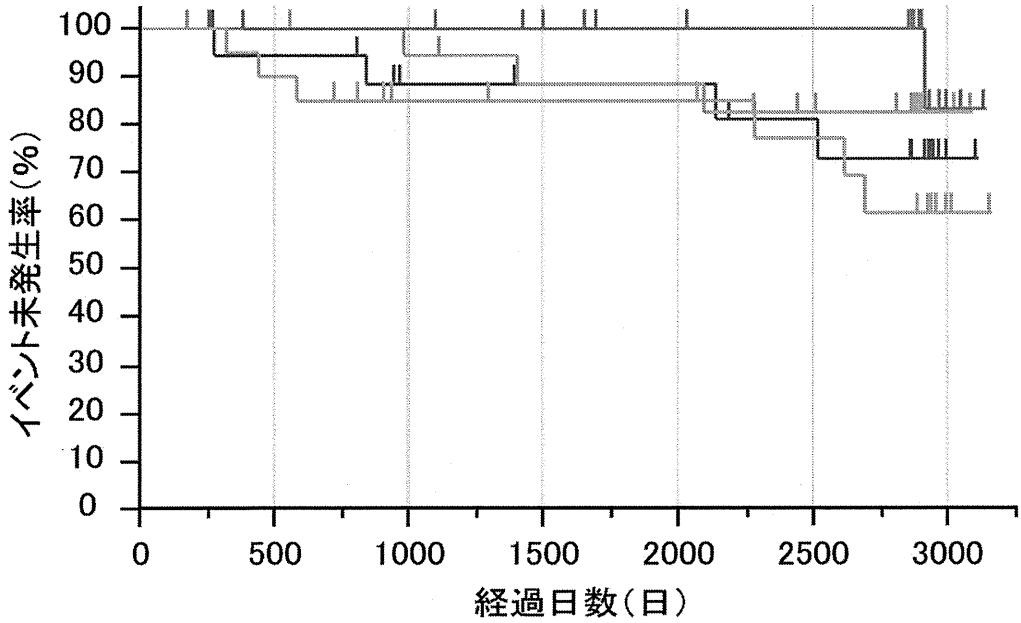

図2　イベント未発生率を表すKaplan-Meier曲線

赤：0.3%FGF-2投与群，緑：0.1%FGF-2投与群，青：0.03%FGF-2投与群，桃：プラセボ投与群。各群のKaplan-Meier曲線上のヒゲは打ち切りを表す。生存時間解析の結果，0.3%FGF-2投与群は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：$p=0.0345$）。

く抜歯に至るケースでも抜歯に至る前に歯周外科的処置や歯周ポケットに対する非外科的な処置が通常行われることにヒントを得て，表2に示した(1) 抜歯，(2) 歯周組織再生療法，(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 (5) その他，歯周炎の進行が原因となって生じた事象をイベントとして，FGF-2投与の長期的効果を判定する評価指標とした。

本研究では，転院等によって診療情報の一部が得らなかったため観察期間に治療等が発生したか否かを十分判断できなかった被験者は全被験者（79名）の約30%（25名）であった（図1）。しかしながら，KCB-1D探索的試験[5]で割り付けられた被験者の背景情報および調査途中打切り被験者数に大きな群間の偏りがなく，本研究における各群の観察期間が各群で同程度であったことから（表1），各群の被験者の一般的特性が結果に大きな影響を与えることはなかったと考えられる。

観察期間における各群のイベントの発生数は，プラセボ群6名，0.03%群4名，0.1%群3名，0.3%群1名と，高濃度のFGF-2を投与した群ほど低かった（図1および表2）。さらに，生存時間解析の結果においても，0.3% FGF-2投与群がフラップ手術単独施行に相当するプラセボ群に対して有意にイベント発生までの時間を延長させることが示され，0.3%FGF-2投与が，歯周炎の再発・悪化のリスクを長期的に低減することが示唆された（図2）。

本研究で被験歯に歯周炎の悪化による抜歯を認めたのは約2.5%(2歯)で，当初予想した通り非常に少なかった。Palcanis[11]はフラップ手術を含む歯周外科処置が歯の長期的な保存に与える影響を総説として報告している。この総説によると，Ramfjordら[12]は，歯周外科処置を行った1800歯の5年間の経過観察によって22歯(約1.2%)が抜去されていたことを報告しているが，その他の多くの長期観察では抜歯はほとんど認められていない。また，5年未満の観察では，フラップ手術後の臨床的アタッチメントの喪失は多くの場合1mm未満であり，5年程度の経過観察ではフラップ手術とFGF-2投与群との術後のイベント発生に大きな違いを認めなかったという本研究の結果と合致する。今回対象とした被験者が参加したKCB-1D探索的試験[5]の結果を受け2005年～2007年に実施されたKCB-1D用量反応試験[6]における同様の観察では，観察期間が約5年間と本研究の観察期間の8年より短く，イベントの発生に統計学的に有意な群間差を認めていない（結果未発表）。以上の結果から，治療に

よる長期的予後の違いを検討するには本研究と同程度か，それ以上の観察期間を要するものと考えられる。今後，より多くの被験者が参加した臨床治験を対象とした同様の観察研究の検討が望まれる。

本研究の結果も含め，これまでの研究からフラップ手術後に認められる長い上皮性の付着による治癒形態は，術後5年程度であれば維持されることが示唆されることから，フラップ手術が患者に与えるメリットは少なくない。しかしながら，それ以降の長期的な歯の予後には，FGF-2投与により歯槽骨の再生および結合性付着の再構築を図ることが有益であることが本研究で示された。世界初の歯周組織再生薬として市場に出たFGF-2が，歯周炎患者の歯の喪失を防ぎ，口腔の働きが支えるQOLの向上に寄与することが期待される。

結　論

FGF-2を用いた歯周組織再生療法臨床治験の施行後約8年間の観察で，0.3% FGF-2投与がフラップ手術単独と比較して再治療等のイベント発生までの期間を延長させることが示された。また，フラップ手術単独群のイベントの発生率は5年以降に増加する傾向が認められ，治療による長期的予後の違いを検討するには本研究と同程度かそれ以上の観察期間を要することが示唆された。また，本研究の観察期間を含めて安全性に関する問題は認めなかった。

本研究の一部は，平成23年度科学研究費補助金（日本学術振興会，基盤研究(B)，課題番号23390452）の補助で実施された。

文　献

1) 財団法人8020推進財団 平成17年永久歯の抜歯原因調査報告書
2) Murakami S, Takayama S, Ikezawa K, Shimabukuro Y, Kitamura M, Nozaki T, Terashima A, Asano T, Okada H. Regeneration of periodontal tissues by basic fibroblast growth factor. J Periodontal Res. 34：425-30, 1999.
3) Murakami S, Takayama S, Kitamura M, Shimabukuro Y, Yanagi K, Ikezawa K, Saho T, Nozaki T, Okada H. Recombinant human basic fibroblast growth factor (bFGF) stimulates periodontal regeneration in class II furcation defects created in beagle dogs. J Periodontal Res. 38：97-103, 2003.
4) Takayama S, Murakami S, Shimabukuro Y, Kitamura M, Okada H. Periodontal regeneration by FGF-2 (bFGF) in primate models. J Dent Res. 80：2075-9 2001.
5) Kitamura M, Nakashima K, Kowashi Y, Fujii T, Shimauchi H, Sasano T, Furuuchi T, Fukuda M, Noguchi T, Shibutani T, Iwayama Y, Takashiba S, Kurihara H, Ninomiya M, Kido J, Nagata T, Hamachi T, Maeda K, Hara Y, Izumi Y, Hirofuji T, Imai E, Omae M, Watanuki M, Murakami S. Periodontal tissue regeneration using fibroblast growth factor-2：randomized controlled phase II clinical trial. PLoS One. 2;3：e2611, 2008.
6) Kitamura M, Akamatsu M, Machigashira M, Hara Y, Sakagami R, Hirofuji T, Hamachi T, Maeda K, Yokota M, Kido J, Nagata T, Kurihara H, Takashiba S, Sibutani T, Fukuda M, Noguchi T, Yamazaki K, Yoshie H, Ioroi K, Arai T, Nakagawa T, Ito K, Oda S, Izumi Y, Ogata Y, Yamada S, Shimauchi H, Kunimatsu K, Kawanami M, Fujii T, Furuichi Y, Furuuchi T, Sasano T, Imai E, Omae M, Yamada S, Watanuki M, Murakami S. FGF-2 stimulates periodontal regeneration：results of a multi-center randomized clinical trial. J Dent Res. 90：35-40, 2011.
7) 特定非営利活動法人 日本歯周病学会編「歯周病の検査・診断・治療計画の指針 2008」
8) Stahl SS, Froum SJ, Kushner L. Periodontal healing following open debridement flap procedures. II. Histologic observations. J Periodontol. 53：15-21, 1982.
9) Nevins M, Giannobile WV, McGuire MK, Kao RT, Mellonig JT, Hinrichs JE, McAllister BS, Murphy KS, McClain PK, Nevins ML, Paquette DW, Han TJ, Reddy MS, Lavin PT, Genco RJ, Lynch SE. Platelet-derived growth factor stimulates bone fill and rate of attachment level gain：results of a large multicenter randomized controlled trial. J Periodontol. 76：2205-15, 2005.
10) Consensus report. Periodontal regeneration around natural teeth. Ann Periodontol. 1：667-70, 1996.
11) Palcanis KG. Surgical pocket therapy. Ann Periodontol. 1：589-617, 1996.
12) Ramfjord SP, Caffesse RG, Morrison EC, Hill RW, Kerry GJ, Appleberry EA, Nissle RR, Stults DL. Four modalities of periodontal treatment compared over five years. J Periodontal Res. 22：222-3, 1987.

Biomedical Research 32 (4) 285-291, 2011

Osteoinductive and anti-inflammatory effect of royal jelly on periodontal ligament cells

Manabu YANAGITA, Yuko KOJIMA, Kenta MORI, Satoru YAMADA, and Shinya MURAKAMI
Department of Periodontology, Division of Oral Biology and Disease Control, Osaka University Graduate School of Dentistry, Osaka 565-0871, Japan

(Received 17 June 2011; and accepted 30 June 2011)

ABSTRACT

Royal jelly (RJ) has been reported to possess several physiological and pharmacological properties such as the ability to prevent osteoporosis in rats and anti-inflammatory effects. We hypothesized that RJ could have beneficial effects on the prevention or treatment of periodontal diseases, which are chronic inflammatory diseases caused by bacterial infection that result in resorption of the tooth-supporting bone. We assessed the effect of RJ on mineralization in mouse periodontal ligament cell clone 22 (MPDL22 cells), which are of an osteogenic and cementogenic lineage. The mRNA expression of osteopontin, osteocalcin and osterix, and mineralized nodule formation were significantly enhanced in RJ-treated MPDL22 cells. In addition, we investigated the effects of RJ on the production of inflammatory cytokines from MPDL22 cells stimulated with lipopolysaccharide (LPS) of *Porphyromonas gingivalis*, a periodontopathic bacterium. RJ suppressed LPS-induced interleukin-6 and CXC chemokine ligand 10 production from MPDL22 cells. Furthermore, RJ suppressed the expression of CD54 in MPDL22 cells: CD54 is the adhesion molecule involved in the accumulation of leukocytes in periodontal lesions. These findings suggest that the osteoinductive and anti-inflammatory effects of RJ can provide benefits for the treatment and prevention of periodontal diseases.

Periodontal disease is a bacterial biofilm-induced chronic inflammatory disease characterized by the destruction of periodontal tissues including the periodontal ligament (PDL), cementum, gingiva and alveolar bone (10, 24). Among these, PDL, which is located between the alveolar bone and cementum of the tooth, plays crucial roles in the homeostasis and repair/regeneration of periodontal tissues. PDL has been revealed to possess multipotential mesenchymal stem cells that can differentiate into osteoblasts and cementoblasts which can create alveolar bone and cementum (21), respectively, and to express bone-related markers (2, 18). Furthermore, several lines of evidence have shown that PDL cells produce inflammatory cytokines and chemokines, such as interleukin (IL)-6 and IL-8 in response to lipopolysaccharide (LPS) of *Porphyromonas gingivalis* (*P. gingivalis*), one of the causative black-pigmented Gram-negative anaerobes, responsible for the development of chronic inflammation in the periodontium (27). Thus, PDL cells are involved in the repair and maintenance of the periodontium, as well as in inflammation.

Royal jelly (RJ) is produced in the hypopharyngeal and mandibular glands of worker honeybees (*Apis mellifera*) and is a necessary food for the growth of the queen honeybee. RJ consists of proteins (18%), sugars (15%), lipids (3–6%) and water (50–60%). RJ also contains vitamins, amino acids and minerals (17). Interestingly, RJ has been shown to exhibit

Address correspondence to: Dr. Shinya Murakami, PhD, D.D.S., Department of Periodontology, Division of Oral Biology and Disease Control, Osaka University Graduate School of Dentistry, Yamadaoka 1-8, Suita, Osaka 565-0871, Japan
Tel: +81-6-6879-2930, Fax: +81-6-6879-2934
E-mail: ipshinya@dent.osaka-u.ac.jp

several biological activities, including anti-hypercholesterolemic (25), anti-tumor (3), anti-microbial (4), anti-allergic (20), anti-inflammatory properties (11), and vasodilative and hypotensive activities (22). Recent studies have shown that RJ has weak estrogenic effects and prevents osteoporosis in ovariectomized rats (7, 15). Like osteoporosis, periodontal diseases are typically accompanied by bone resorption. In fact, the positive association between osteoporosis and periodontitis has been suggested (13). We hypothesized that administration of RJ in the daily diet or local application of RJ to periodontal tissue might prevent periodontal disease.

In the present study, we investigated the effects of RJ on osteogenic mineralized nodule formation using mouse PDL (MPDL) cells and on the secretion of IL-6 as inflammatory cytokine and CXC chemokine ligand 10 (CXCL10), chemokine suggested to be involved in alveolar bone destruction in periodontal diseases lesion (9), from LPS-stimulated MPDL cells.

MATERIALS AND METHODS

Reagent. Dried powders of raw RJ originating from China were supplied by Yamada Apiculture Center, Inc. (Okayama, Japan). LPS of *P. gingivalis* was purchased from InvivoGen (San Diego, CA, USA).

Culture of MPDL. In a previous work, we established an MPDL clone cell line, MPDL22, isolated from the PDL tissue of the molar teeth extracted from 2.5-week-old BALB/c mice (26). MPDL22 cells were maintained in α-MEM (Nikken, Kyoto, Japan) supplemented with 10% fetal calf serum (FCS; JRH Biosciences, Lenexa, KS, USA) and 100 ng/mL fibroblast growth factor-2 (FGF-2; Kaken, Kyoto, Japan) (26). When the cells reached confluence, we replaced the culture medium (α-MEM supplemented with 10% FCS and FGF-2) with the mineralization medium (α-MEM supplemented with 10% FCS, 10 mM β-glycerophosphate, and 50 mg/mL ascorbic acid).

Proliferation assay. MPDL22 cells (5×10^3 cells/well) were incubated in 96-well plates in α-MEM containing 1% FCS in the presence of RJ or FGF-2 (100 ng/mL) for 48 h. Cell proliferation was measured using the nonradioactive colorimetric assay WST-1 assay (Roche Diagnostics GmbH, Penzberg, Germany) according to the manufacturer's instructions. The OD450/650 was measured after 2 h on a microplate reader (Bio-Rad, Hercules, CA).

Real-time PCR for osteopontin, osteocalcin and osterix mRNA. RNA samples were obtained from MPDL22 cells cultured in mineralization medium for 3, 5, and 7 days in the presence or absence of RJ. Total RNA extract (0.4 mg) was reverse-transcribed using a High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems, Foster City, CA, USA) to generate single-stranded cDNA. PCR reactions were carried out using the ABI 7300 Fast Real-Time PCR System (Applied Biosystems) with Power SYBR^R Green PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol. All reactions were run in triplicate. The primer sequences used for real-time PCR were as follows; osteopontin, (sense) 5'-TAC GAC CAT GAG ATT GGC AGT GA-3', (antisense) 5'-TAT AGG ATC TGG GTG CAG GCT GTA A-3'; osteocalcin, (sense) 5'-AGC AGC TTG GCC CAG ACC TA-3', (antisense) 5'-TAG CGC CGG AGT CTG TTC ACT AC-3'; osterix, (sense) 5'-CGC ATC TGA AAG CCC ACT TG-3', (antisense) 5'-CAG CTC GTC AGA GCG AGT GAA-3'.

Mineralization assay. Calcified nodules were stained by an alizarin red staining method. Cell layers were washed twice with phosphate-buffered saline (PBS) and then fixed in dehydrated ethanol. After fixation, the cell layers were stained with 1% alizarin red in 0.1% NH_4OH (pH 6.3–6.5) for 5 min. The culture dishes were then washed with water and observed, digitized and analyzed using WinRoof software (Mitani Corporation, Fukui, Japan).

Measurement of CXCL10 and IL-6 production. MPDL22 cells were seeded in 24-well culture dishes with α-MEM supplemented with 10% FCS and 100 ng/mL FGF-2. When the cells reached 60–70% confluence, quiescent was induced by replacing the medium with serum-free α-MEM for 24 h, and then the medium was replaced with α-MEM supplemented with 1% FCS with or without *P. gingivalis* LPS and RJ. The supernatants from the MPDL22 cultures were collected after 48 h. The concentrations of CXCL10 and IL-6 in the supernatants were measured by ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. In some experiments, MPDL22 cells were cultured for 1, 2, 4, or 6 h in the presence of RJ prior to LPS stimulation.

Flow cytometric acquisition. MPDL22 cells were seeded in 24-well culture dishes with α-MEM supplemented with 10% FCS and 100 ng/mL FGF-2.

When the cells reached 60–70% confluence, quiescent was induced by replacing the medium with serum-free α-MEM for 24 h, and then the medium was replaced with α-MEM supplemented with 1% FCS with or without *P. gingivalis* LPS and RJ. After 48 h, the cells were washed with PBS harvested by incubation with Cell Dissociation Solution (Sigma-Aldrich). Cells were washed with PBS and stained with phycoerythrin-conjugated rat anti-mouse CD54 antibody (eBiosciences, San Diego, CA, USA) for 30 min. The cells were washed twice and data were acquired using FACSCalibur (BD Biosciences, San Jose, CA, USA). Analyses of viable cells were performed using CELLQuestTM software (BD Biosciences).

Statistical analysis. Results were analyzed for statistical significance using analysis of variance with the Bonferroni test. Differences were considered significant at P values less than 0.05. The mRNA expression levels were normalized against the expression levels of hypoxanthine phosphoribosyltransferase 1 (HPRT).

RESULTS

Effect of RJ on MPDL22 proliferation

To investigate the effect of RJ on the proliferation response of MPDL cells, cells were cultured for 48 h in a medium containing 1% FCS with or without RJ. As previous studies have shown that FGF-2 induced the proliferation of PDL cells (28), FGF-2 at a concentration of 100 ng/mL, which was the most optimal concentration for MPDL proliferation in preliminary experiment, was used as a positive control of this assay. Exposure to 0.004–0.5 mg/mL RJ did not induce significant proliferative responses in MPDL22 cells (Fig. 1).

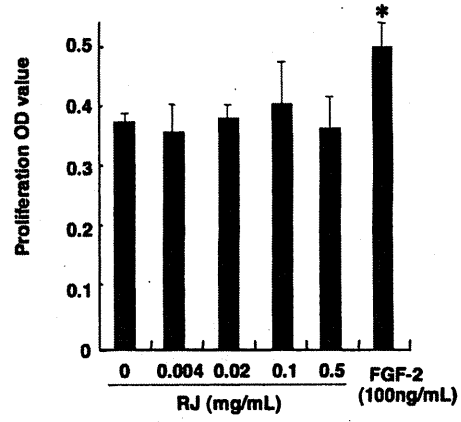

Fig. 1 Effect of RJ on proliferation of MPDL22 cells. MPDL22 cells were cultured with various doses of RJ or FGF-2 (100 ng/mL) for two days. RJ did not induce significant proliferative responses in MPDL22 cells. Values are the means ± SD of four assays. $^*P < 0.05$ compared with medium only.

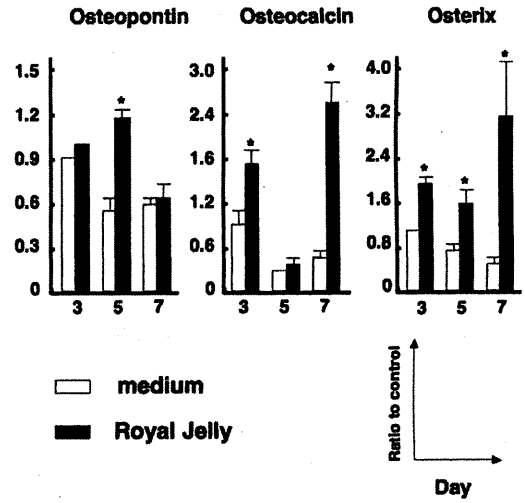

Fig. 2 Analysis of mRNA expression of osteopontin, osteocalcin and osterix in MPDL22 cells. RNA samples were obtained from MPDL22 cells at 3, 5 and 7 days after RJ treatment (0.5 mg/mL). The relative expression of each gene was normalized to the expression levels of HPRT, and the expression of osteopontin, osteocalcin and osterix in MPDL22 cells without RJ was taken as 1.0. RJ significantly enhanced mRNA expression of osteopontin, osteocalcin and osteorix in MPDL22 cells. Values are the means ± SD of three or four assays. $^*P < 0.05$ compared with non-treated cells.

Effects of RJ on osteopontin, osteocalcin and osterix gene expression in MPDL22 cells

We examined the effects of RJ (0.5 mg/mL) on gene expression of extracellular matrices such as osteopontin and osteocalcin, and osteoblastic transcription factor, osterix (Fig. 2). RJ-treated-MPDL22 cells showed significantly enhanced mRNA expression of osteopontin (day 5), osteocalcin (day 3, 7) and osterix (day 3, 5, 7). Although RJ enhanced osterix gene expression at all points we investigated, there was the difference of gene expression pattern between osteopontin and osteocalcin. Whereas osteopontin expression was upregulated with peak at day 5, osteocalcin had two peaks of mRNA expression at day 3 and day 7. The mechanism of suppression of osteocalcin expression at day 5 was unclear, however enhanced mRNA expression of osteocalcin at day 7 suggested emerging mature osteoblastic cells in the presence of RJ.

Effects of RJ on mineralization in MPDL22 cells
We cultured MPDL22 cells with or without RJ in mineralization medium and then examined mineralized nodule formation on day 12. We exposed confluent MPDL22 cells to RJ at various concentrations. As shown in Fig. 3, RJ significantly enhanced alizarin red staining intensity, and in particular, addition of 0.1 mg/mL RJ was most effective.

Effects of RJ on anti-inflammatory responses in MPDL22 cells
To investigate the effects of RJ on the LPS-stimulated CXCL10 production, IL-6 secretion and CD54 expression, MPDL22 cells were pretreated with the indicated concentration of RJ for 1 h and stimulated with LPS for 48 h. As shown in Fig. 4A, a dose-dependent suppressive effect of RJ on CXCL10 production by MPDL22 cells was observed. Although more than half of reduction of CXCL10 production in the prersence of RJ was shown, an approximately 30% reduction in IL-6 production was observed at each concentration of RJ investigated (Fig. 4B). The most effective dose of RJ for inhibition of IL-6 production was 0.1 mg/mL. CXCL10 and IL-6 inhibition was most efficient when RJ treatment occurred 6 h before LPS stimulation (Fig. 4C and D).

A previous study demonstrated that bacterial components induced a cell surface molecule CD54 in human PDL cells (12). As shown in Fig. 5A, MPDL22 cells without RJ and LPS stimulation expressed low level of CD54 (sample ID. 1), and treatment with only RJ had no effect on CD54 expression (sample ID. 2). MPDL22 cells with LPS strongly enhanced expression of CD54 (sample ID. 3). When we evaluated the expression of CD54 in MPDL22 cells pretreated with RJ 1h prior to LPS stimulation, pretreatment with RJ reversed the LPS-induced upregulation of CD54 (sample ID. 4).

DISCUSSION

In the present study, we observed that RJ increased the formation of mineralized nodules in mouse PDL cells by enhancing osteoblastic differentiation. Additionally, RJ significantly suppressed LPS-induced inflammatory cytokine and chemokine production by MPDL22 cells.

Real-time PCR revealed that RJ enhanced the mRNA expression of osteopontin, osteocalcin, and osterix. Osteopontin is an indicator of bone turnover, and osteocalcin is involved in extracellular matrix mineralization. Osterix is an osteoblast-specific transcription factor that plays an important role in modulating bone formation and osteoblastic differentiation. Upregulation of the above-mentioned mRNAs by RJ correlated with the mineralization of MPDL22 cells. A previous study showed that RJ has estrogenic effects including the prevention of osteoporosis in ovariectomized rats (7, 15). Additionally, RJ has been reported to activate bone metabolism-related genes in the mouse osteoblast-like cell line, MC3T3-E1 (19). Because RJ comprises many components, the effects of RJ on promoting the expression of osteoblastic markers and mineralization in MPDL22 cells may be independent of its estrogenic activity. Isolation and characterization of

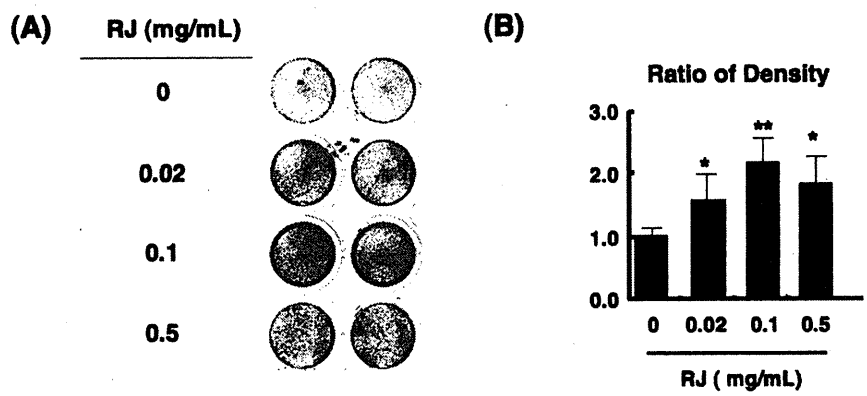

Fig. 3 Effect of RJ on mineralization of MPDL22 cells. (**A**) Effect of RJ (0.02, 0.1, 0.5 mg/mL) on mineralization in MPDL22 cells was examined by alizarin red staining after 12 days of culture in mineralization medium. Results show a representative experiment out of four identical assays. (**B**) The relative intensity of alizarin red staining was determined by image analysis. The data shown were quantified and normalized to those of alizarin red staining without RJ. RJ significantly enhanced mineralized nodule formation in MPDL22 cells. The values are the means ± SD of four different experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with MPDL22 cells cultured with mineralization medium in the absence of RJ.

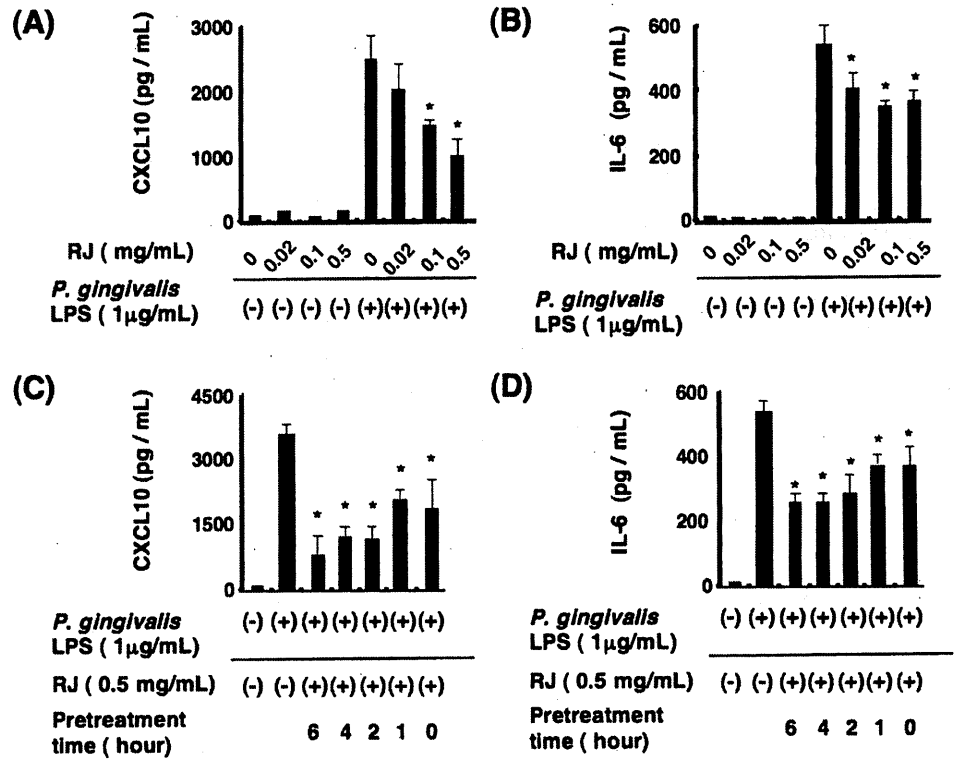

Fig. 4 Effect of RJ on CXCL10 and interleukin-6 (IL-6) production in MPDL22 cells. The effect of RJ on CXCL10 (**A**) and IL-6 (**B**) production in MPDL22 cells. MPDL22 cells were pretreated with RJ prior to LPS stimulation, and supernatants were collected after 24 h. CXCL10 and IL-6 concentrations were determined by ELISA. The results represent the mean values ± SD obtained from triplicate cultures. Next, MPDL22 cells were exposed to 0.5 mg/mL RJ either simultaneously, or up to 6 h before activation with LPS, and supernatants were collected after 24 h. CXCL10 (**C**) and IL-6 (**D**) concentrations were determined by ELISA. The values represent the means ± SD obtained from triplicate cultures. RJ suppressed CXCL10 and IL-6 production in MPDL22 cells. $^*P < 0.05$ compared with LPS-stimulated MPDL22 cells in the absence of RJ.

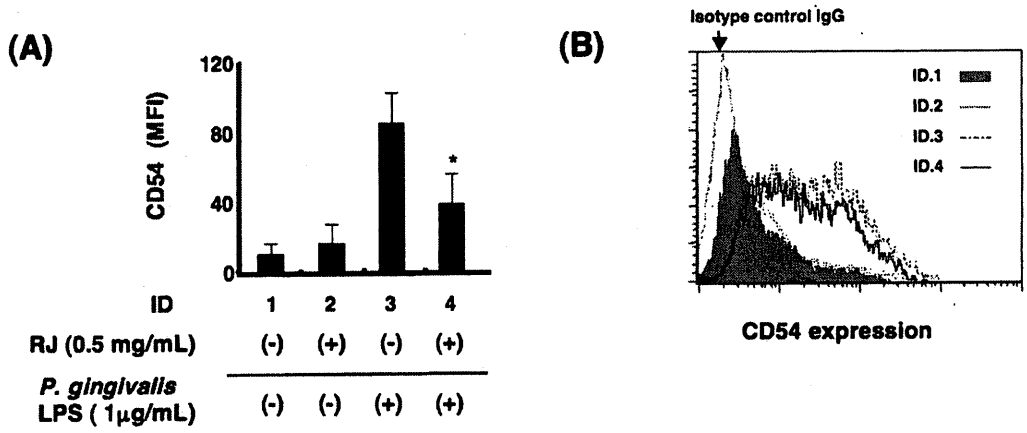

Fig. 5 Effect of RJ on CD54 expression in MPDL22 cells. MPDL22 cells were treated with RJ (0.5 mg/mL) and/or LPS (1 μg/mL). MPDL22 cells were collected after 24 h. The expression levels of CD54 in MPDL22 cells were determined by flow cytometry. (**A**) The results are expressed as mean fluorescent intensity (MFI). The data represent the mean ± SD obtained from three different experiments. $^*P < 0.05$ compared with LPS-stimulated MPDL22 cells in the absence of RJ. (**B**) Fluorescence histogram of CD54 on surface of MPDL22 cells. RJ suppressed CD54 expression in MPDL22 cells stimulated with LPS. The istgramdata is representative of three different experiments. ID. 1: non-treated MPDL22 cells, ID. 2: RJ-treated MPDL22 cells, ID. 3: LPS-stimulated MPDL22 cells, ID. 4: LPS-stimulated MPDL22 cells after RJ-treatment.

the estrogenic compounds in RJ should aid in the understanding of its involvement in bone metabolism.

P. gingivalis is a black-pigmented Gram-negative anaerobe and one of the causative microorganisms for the development of chronic inflammation in the periodontium (14). This is the first report that LPS of *P. gingivalis* can induce secretion of IL-6 and CXCL10 from mouse PDL cells. In human PDL cells, some reports have documented that stimulation by LPS derived from *P. gingivalis* (27) or *Escherichia coli* (23) induced IL-6, IL-8, IL-1β and tumor necrosis factor (TNF)-α production. In this study, we also revealed that RJ possesses inhibitory effects on LPS-stimulated IL-6 and CXCL10 production. IL-6 is a pleiotropic cytokine with a wide range of biological functions including immune responses and bone metabolism (1). CXCL10 is a chemoattractant for T helper1 cells, which are suggested to be involved in alveolar bone destruction in periodontal diseases (9). A previous study reported that RJ suppressed IL-6 and TNF-α production by LPS and interferon-γ stimulated mouse macrophages (11), which is consistent with our observation.

CD54 is a cell surface adhesion molecule whose expression is increased at inflammatory sites such as in the periodontal tissues of periodontitis patients (6). Bacterial components or IL-1β have been reported to upregulate CD54 expression in human PDL cells (8, 12). Consistently, we found that LPS of *P. gingivalis* induced CD54 expression in MPDL22 cells. Furthermore, we revealed that RJ reduced CD54 expression in LPS-stimulated MPDL22 cells (Fig. 5). As anti-inflammatory compounds in RJ, we speculate that adenosine and adenosine derivatives, which have been identified as active components in RJ (5), could be candidates for suppressing CD54 expression in MPDL22 cells. Supporting this, our previous study showed that IL-1β-induced CD54 expression in human gingival fibroblasts was abrogated by adenosine (16). Adenosine and/or other anti-inflammatory compounds in RJ may contribute to the reduction of inflammatory responses in periodontal lesions.

In summary, our present results showed that RJ enhanced osteoblastic metabolism and exerted anti-inflammatory effects. This work suggests that several constituents in RJ may be of benefit to oral and periodontal health. Elucidation of the mechanisms of the effects of RJ in mouse and human PDL cells requires further investigation.

Acknowledgments

This work was supported in part by Grants-in-Aid for Scientific Research (No. 20390529, 20390530, 20592427) from the Ministry of Education, Culture, Sports, Science and Technology, Japan. This work was also supported by Yamada Bee Farm Grant for Honeybee Research (to M.Y.). This funding source had no involvement in the study design, collection, analysis and interpretation of data, writing of the report, and the decision to submit the paper for publication. The other authors have declared that they have no conflict of interest.

REFERENCES

1. Akira S, Taga T and Kishimoto T (1993) Interleukin-6 in biology and medicine. *Adv Immunol* **54**, 1–78.
2. Arceo N, Sauk J, Moehring J, Foster R and Somerman M (1991) Human periodontal cells initiate mineral-like nodules in vitro. *J Periodontol* **62**, 499–503.
3. Bincoletto C, Eberlin S, Figueiredo C, Luengo M and Queiroz M (2005) Effects produced by Royal Jelly on haematopoiesis: relation with host resistance against Ehrlich ascites tumour challenge. *Int Immunopharmacol* **5**, 679–688.
4. Fujiwara S, Imai J, Fujiwara M, Yaeshima T, Kawashima T and Kobayashi K (1990) A potent antibacterial protein in royal jelly. Purification and determination of the primary structure of royalisin. *J Biol Chem* **265**, 11333–11337.
5. Hattori N, Nomoto H, Mishima S, Inagaki S, Goto M, Sako M and Furukawa S (2006) Identification of AMP N1-oxide in royal jelly as a component neurotrophic toward cultured rat pheochromocytoma PC12 cells. *Biosci Biotechnol Biochem* **70**, 897–906.
6. Hayashi J, Saito I, Ishikawa I and Miyasaka N (1994) Effects of cytokines and periodontopathic bacteria on the leukocyte function-associated antigen 1/intercellular adhesion molecule 1 pathway in gingival fibroblasts in adult periodontitis. *Infect Immun* **62**, 5205–5212.
7. Hidaka S, Okamoto Y, Uchiyama S, Nakatsuma A, Hashimoto K, Ohnishi S and Yamaguchi M (2006) Royal jelly prevents osteoporosis in rats: beneficial effects in ovariectomy model and in bone tissue culture model. *Evid Based Complement Alternat Med* **3**, 339–348.
8. Joe BH, Borke JL, Keskintepe M, Hanes PJ, Mailhot JM and Singh BB (2001) Interleukin-1beta regulation of adhesion molecules on human gingival and periodontal ligament fibroblasts. *J Periodontol* **72**, 865–870.
9. Kawai T, Eisen-Lev R, Seki M, Eastcott JW, Wilson ME and Taubman MA (2000) Requirement of B7 costimulation for Th1-mediated inflammatory bone resorption in experimental periodontal disease. *J Immunol* **164**, 2102–2109.
10. Kinane D (2001) Causation and pathogenesis of periodontal disease. *Periodontol 2000* **25**, 8–20.
11. Kohno K, Okamoto I, Sano O, Arai N, Iwaki K, Ikeda M and Kurimoto M (2004) Royal jelly inhibits the production of proinflammatory cytokines by activated macrophages. *Biosci Biotechnol Biochem* **68**, 138–145.
12. Lee S, Kim K and Choi B (2005) Upregulation of intercellular adhesion molecule 1 and proinflammatory cytokines by the major surface proteins of *Treponema maltophilum* and

Treponema lecithinolyticum, the phylogenetic group IV oral spirochetes associated with periodontitis and endodontic infections. *Infect Immun* **73**, 268–276.
13. Lerner U (2006) Inflammation-induced bone remodeling in periodontal disease and the influence of post-menopausal osteoporosis. *J Dent Res* **85**, 596–607.
14. Mayrand D and Holt SC (1988) Biology of asaccharolytic black-pigmented Bacteroides species. *Microbiol Rev* **52**, 134–152.
15. Mishima S, Suzuki K, Isohama Y, Kuratsu N, Araki Y, Inoue M and Miyata T (2005) Royal jelly has estrogenic effects in vitro and in vivo. *J Ethnopharmacol* **101**, 215–220.
16. Murakami S, Hashikawa T, Saho T, Takedachi M, Nozaki T, Shimabukuro Y and Okada H (2001) Adenosine regulates the IL-1 beta-induced cellular functions of human gingival fibroblasts. *Int Immunol* **13**, 1533–1540.
17. Nagai T, Nagashima T, Myoda T and Inoue R (2004) Preparation and functional properties of extracts from bee bread. *Nahrung* **48**, 226–229.
18. Nagatomo K, Komaki M, Sekiya I, Sakaguchi Y, Noguchi K, Oda S, Muneta T and Ishikawa I (2006) Stem cell properties of human periodontal ligament cells. *J Periodontal Res* **41**, 303–310.
19. Narita Y, Nomura J, Ohta S, Inoh Y, Suzuki KM, Araki Y, Okada S, Matsumoto I, Isohama Y, Abe K, Miyata T and Mishima S (2006) Royal jelly stimulates bone formation: physiologic and nutrigenomic studies with mice and cell lines. *Biosci Biotechnol Biochem* **70**, 2508–2514.
20. Okamoto I, Taniguchi Y, Kunikata T, Kohno K, Iwaki K, Ikeda M and Kurimoto M (2003) Major royal jelly protein 3 modulates immune responses in vitro and in vivo. *Life Sci* **73**, 2029–2045.
21. Seo B, Miura M, Gronthos S, Bartold P, Batouli S, Brahim J, Young M, Robey P, Wang C and Shi S (2004) Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet* **364**, 149–155.
22. Shinoda M, Nakajin S, Oikawa T, Sato K, Kamogawa A and Akiyama Y (1978) [Biochemical studies on vasodilative factor in royal jelly (author's transl)]. *Yakugaku Zasshi* **98**, 139–145. (In Japanese)
23. Shu L, Guan SM, Fu SM, Guo T, Cao M and Ding Y (2008) Estrogen modulates cytokine expression in human periodontal ligament cells. *J Dent Res* **87**, 142–147.
24. Socransky S, Smith C and Haffajee A (2002) Subgingival microbial profiles in refractory periodontal disease. *J Clin Periodontol* **29**, 260–268.
25. Vittek J (1995) Effect of royal jelly on serum lipids in experimental animals and humans with atherosclerosis. *Experientia* **51**, 927–935.
26. Yamada S, Tomoeda M, Ozawa Y, Yoneda S, Terashima Y, Ikezawa K, Ikegawa S, Saito M, Toyosawa S and Murakami S (2007) PLAP-1/asporin, a novel negative regulator of periodontal ligament mineralization. *J Biol Chem* **282**, 23070–23080.
27. Yamaji Y, Kubota T, Sasaguri K, Sato S, Suzuki Y, Kumada H and Umemoto T (1995) Inflammatory cytokine gene expression in human periodontal ligament fibroblasts stimulated with bacterial lipopolysaccharides. *Infect Immun* **63**, 3576–3581.
28. Yanagita M, Kojima Y, Kawahara T, Kajikawa T, Oohara H, Takedachi M, Yamada S and Murakami S (2010) Suppressive effects of nicotine on the cytodifferentiation of murine periodontal ligament cells. *Oral Diseases* **16**, 812–817.

ARCHIVES OF ORAL BIOLOGY XXX (2011) XXX–XXX

Available online at www.sciencedirect.com
SciVerse ScienceDirect
journal homepage: http://www.elsevier.com/locate/aob

Nicotine up-regulates IL-8 expression in human gingival epithelial cells following stimulation with IL-1β or *P. gingivalis* lipopolysaccharide via nicotinic acetylcholine receptor signalling

*Yoichiro Kashiwagi, Manabu Yanagita *, Yuko Kojima, Yoshio Shimabukuro, 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

ARTICLE INFO

Article history:
Accepted 7 October 2011

Keywords:
Nicotine
Gingival epithelial cells
IL-8
Innate immunity

ABSTRACT

Objective: Cigarette smoking is an important risk factor for periodontal disease. The aim of this study is to evaluate the effect of nicotine, a major component of cigarette smoke, on interleukin-8 (IL-8) production and cellular signalling via nicotinic acetylcholine receptors (nAChRs) in human gingival epithelial cells (HGECs).

Design: Messenger RNA (mRNA) expression of nAChR subunits in three different HGEC lines (epi 4, Tfx and E6E7) was assessed using reverse transcription-polymerase chain reaction (RT-PCR). HGECs were stimulated by 1×10^{-3} M nicotine in the presence or absence of IL-1β or *Porphyromonas gingivalis* lipopolysaccharide (LPS). IL-8 production was then examined using real-time PCR and enzyme-linked immunosorbent assay. Nicotine-mediated signalling in the epi 4 cell line was also evaluated by Western blotting.

Results: HGECs expressed several nAChR subunits. Nicotine increased the secretion of IL-8 from HGECs that were cultured in the presence of IL-1β or *P. gingivalis* LPS and also induced the phosphorylation of extracellular signal-regulated kinase (ERK) in epi 4. Pretreatment with non-selective nAChR antagonist or intracellular calcium chelator reduced the nicotine-induced phosphorylation of ERK. Furthermore, nicotine-induced IL-8 secretion was decreased by pretreatment with non-selective nAChR antagonist, ERK1/2 inhibitor or intracellular calcium chelator.

Conclusion: These findings indicate that nicotine increases IL-8 production in gingival epithelial cells via ERK phosphorylation following Ca^{2+} signalling after nAChR activation.

1. Introduction

Gingival epithelial cells play an important role in homeostasis of periodontal tissue. They prevent entry of periodontopathogen into periodontal tissue by forming a physical barrier and provide nonspecific, rapid host defence reaction resulting in recruitment of professional immune cells such as macrophages, dendritic cells and lymphocytes.[1–3] Several lines of evidence have indicated that bacterial stimulation induces pro-inflammatory cytokines (interleukin (IL)-1β, IL-6, IL-8 and tumour necrosis factor-α) in oral and gingival epithelial cells.[4,5] In addition, we have previously revealed that human gingival epithelial cells (HGECs) and oral epithelial cell line (KB

* *Corresponding author.* Tel.: +81 6 6879 2932; fax: +81 6 6879 2934.
E-mail address: yanagita@dent.osaka-u.ac.jp (M. Yanagita).
0003–9969/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.archoralbio.2011.10.007