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分担研究報告書

in vivoモデルマウスを用いた早期発症型侵襲性歯周炎病態解析

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研究要旨「早期発症型侵襲性歯周炎（遺伝性急性進行型歯槽膿漏症候群）の診断基準の更なる推進に関する研究」を開発するために、マルファン病モデル動物を用いて歯周組織の創傷治癒機構を解析した。昨年度までに、同モデル動物マウスにおいて歯周組織を構成する歯根膜が機能不全を起こし、創傷治癒不全を引き起こすことを見いだしている。マルファン病は微細線維形成不全による発症するため、本年度は微細線維形成を促進するADAMTSL6βにより症状が改善されるかを検証した。その結果、ADAMTSL6βをマルファン症候群モデル動物の歯根膜に局所投与すると、微細線維形成不全のみならず創傷治癒不全も改善することが判明した。
ADAMTSL6β局所投与による治療効果を解析したところ、マルファン病における組織崩壊の原因である病的なTGF-βシグナルの活性化に伴うリン酸化SMAD2/3とMMP-9の発現が抑制されていることが判明した。以上の結果より、ADAMTSL6βは早期発症型侵襲性歯周炎の新規治療薬として発展する可能性が示された。

A. 研究目的

歯周病は通常中高年以上で発症する慢性炎症性疾患であるが、歯周病患者数の0.1%である5万人の患者が若年層で発症し、早期に歯を喪失してしまう早期発症型歯周病を発症している。これまで、大動脈瘤、肺気胸を引き起こすマルファン症候群で重度の歯周病を引き起こすことが報告されており、同疾患の原因となる弾性線維の早期発症型歯周病の原因になり得ることが示唆されている。マルファン症候群とは、機械的圧力の負担の大きい大動脈や、肺、歯根膜で、体の弾力を調節する微細線維の形成不全により結合組織疾患を発症する。これまで研究分担者は、マルファン病では歯周病の標的組織である歯根膜の形成不全が生じることから、早期発症型歯周病の要因になることを報告してきた。その一方で、微細線維の成分であるADAMTSL6βが微細線維形成の誘導能を有することが見出され、微細線維修復を介してマルファン病に早期発症型歯周病を改善する可能性が考えられた。そこで本研究では、マルファン病モデル動物を用いてADAMTSL6βの早期発症型歯周病の治療効果を検証することを目標とする。

B. 研究方法

(1)歯根膜損傷モデルを用いた創傷治癒能力の判定試験

マルファン病モデル動物の歯根膜の損傷治癒効果を判定するために、歯根膜の損傷モデルを用いて判定した。生後１ヶ月の下顎の歯根膜を脱臼による損傷を与え、整復した後に１週間、３週間後に顎を摘出し創傷治癒過程を組織学的に評価した。組織評価はと

同様の手法で解析した。

(2) ADAMTSL6βの局所投与実験

組み換え ADAMTSL6βを293□free styleにて産生させて精製した後に、コラーゲンゲル内に混入しゲルを作製した。このADAMTSL6β配合型コラーゲンゲルを（１）のモデルにて歯根膜脱臼部位に挿入した。（１）と同様の方法にて組織解析を行った。

(3)微細線維形成の確認

(2)で ADAMTSL6β配合型コラーゲンゲルの局所投与効果による微細線維の修復効果は、微細線維のマーカーである抗 fibrillin-1 抗体、抗ADAMTSL6βを用いた免疫組織化学的手法を用いて解析を行った。

(4)TGF-beta シグナルの評価

ADAMTSL6βの歯根膜損傷モデルによる TGF-beta シグナルに及ぼす影響を解析する目的に、抗リン酸化 SMAD2/3 抗体および抗マトリックスメタロプロテアーゼ（MMP）-9 抗体を用いた免疫染色にて評価した。

（倫理面への配慮）

本動物実験は、東京理科大学動物実験委員会の承認を得た上で行われた。

C．研究結果

(1) マルファン病モデル動物の歯根膜創傷治癒に及ぼす影響

歯根膜損傷モデルでマルファンモデル動物の歯根膜にでは創傷治癒不全が引き起こされることが観察された。また抗 fibrillin-1 抗体陽性の微細線維は断裂化し、これに伴い歯根膜の構造の崩壊が観察された。

(2) ADAMTSL6βの局所投与実験による歯根膜創傷治癒改善効果の解析

歯根膜損傷部位に ADAMTSL6β配合型コラーゲンゲルを局所投与した結果、マルファン病モデル動物の歯根膜における創傷治癒不全の改善が観察された。HE染色にて歯根膜の構造は回復が確認され、また抗 fibrillin-1 抗体および抗 ADAMTSL6β抗体陽性の微細線維の修復が観察された。

(3) TGF-βシグナルに及ぼす影響

ADAMTSL6β配合型コラーゲンゲルの局所投与による創傷治癒改善機構を調べるため、TGF-βシグナルに及ぼす影響を調べた。免疫染色の結果、ADAMTSL6β配合型コラーゲンゲルの局所投与によりリン酸化 SMAD2/3 の活性化と MMP-9 の発現の低下が認められた。一方、コントロールとしてコラーゲンゲルを投与した部位においては、リン酸化 SMAD2/3、MMP-9 の発現は維持されており、歯根膜の組織破壊の亢進が確認された。

D．考察

本研究成果により、ADAMTSL6β配合型コラーゲンゲルの局所投与により、マルファンモデル動物における歯根膜の創傷治癒能力を回復できることが判明した。この結果より、微細線

維の回復は早期発症型歯周病の治療において、創傷治癒の促進に重要な役割を果たしていること示された。

今後のマルファンモデル動物が、早期発症型侵襲性歯周炎の原因になるか否かを明らかにするためには、同マウスを用いて歯周病を発症させるモデルの開発が必要になる。また、このような早期発症型侵襲性歯周炎モデルマウスにおける組織崩壊に対してADAMTSL6β局所投与の有効性を検証する必要性が考えられた。

E．結論

マルファンモデル動物を用いて、微細線維低下が早期発症型侵襲性歯周炎の原因になる可能性が示された。また ADAMTSL6βを補充する新たな治療技術が、早期発症型歯周炎の有効な治療技術として発展する可能性が示された。

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H．知的財産権の出願・登録状況（予定を含む。）

1.　特許取得

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[III]　研究成果の刊行に関する一覧表

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[IV]　研究成果の刊行物・別刷り

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Chapter 15

Periodontal regeneration by FGF-2: Present status and future outlook

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Introduction

At present a variety of regenerative therapies are available in the field of periodontal therapy, such as bone grafts, guided tissue regeneration (GTR) and application of enamel matrix derivatives, all of which have achieved a measure of success. However, a number of issues with these techniques remain to be solved, including technique sensitivity, limitation of indications, predictability, and the longevity of outcomes.

In the 1990s, Langer and Vacanti (1993) developed the concept of tissue engineering, consisting three key elements: signaling molecules, scaffolds and stem cells (Figure 1). They proposed that the active introduction of one or more of the triad enables the induction of desirable tissue regeneration. In relation to periodontal regenerative therapy, the use of somatic tissue stem cells and/or progenitor cells within periodontal ligaments to act as "stem cells" has been demonstrated (Seo *et al* 2004). In order to enhance the outcomes of tissue regenerative therapy, it is crucial to stimulate the biological activities of these cells, and a physiologically efficient method for doing so is through the use of cytokines or growth factors. The ability of various recombinant cytokines to enhance periodontal tissue regeneration has been investigated in preclinical and clinical studies (Table 1). This chapter reviews the potential use of basic fibroblast growth factor (bFGF, FGF-2) to promote periodontal tissue regeneration, with a discussion of the current status and prospects of FGF-2 therapy.

In vivo analyses of effects of FGF-2 on periodontal regeneration

Fibroblast growth factor (FGF) was discovered in 1974 as a protein from bovine pituitary glands that strongly induces proliferative activity in fibroblasts (Gospodarowicz 1974). In 1984, two distinct proteins with different isoelectric points were fractionated from the pituitary extract using acidic and basic pHs, which became known as acidic FGF (aFGF, FGF-1) and basic FGF (bFGF, FGF-2), respectively (Bohlen *et al* 1984, Thomas *et al* 1984). A year later the entire amino acid sequence of bovine FGF-2 was determined, and the cDNA of human FGF-2 was cloned in 1986 (Abraham *et al* 1986, Esch *et al* 1985). FGF-2 has received particular attention in the field of regenerative therapy, as it stimulates various stem cells to proliferate while maintaining their multipotency, and is a strong inducer of angiogenesis.

In order to evaluate the effectiveness of

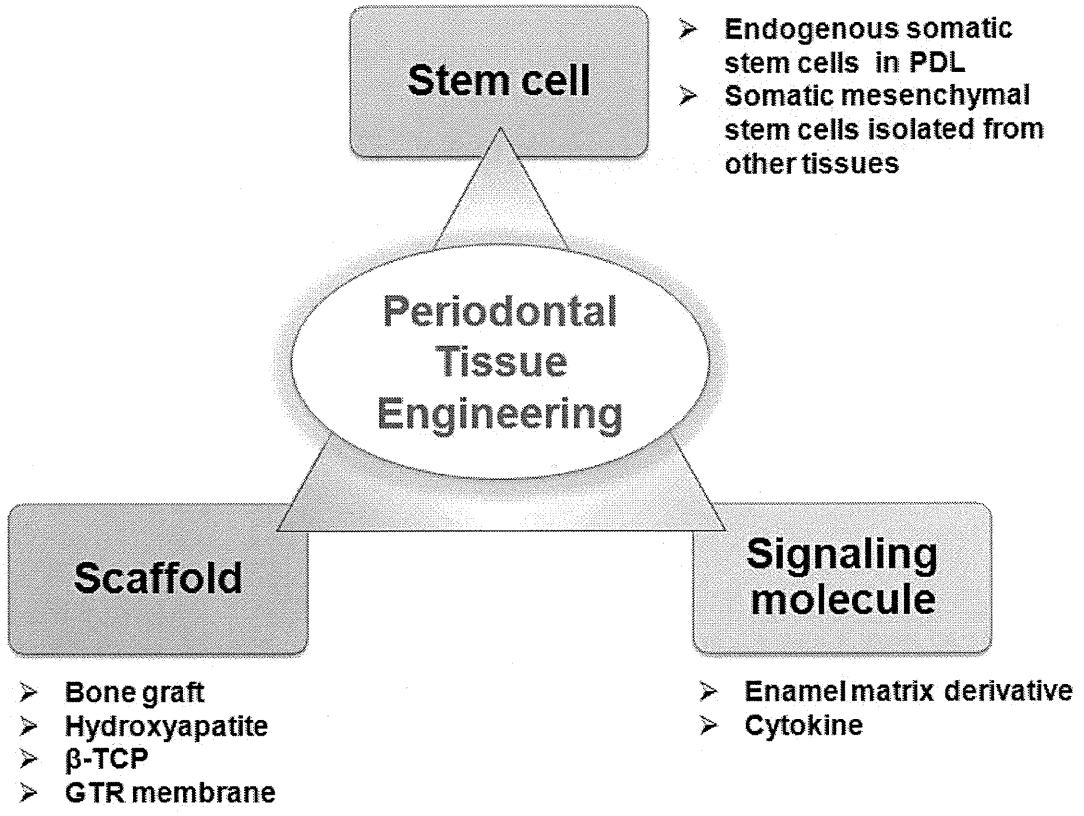

Figure 1. Triad of periodontal tissue engineering. The concept of tissue engineering consists of stem cells, a signaling molecule and a scaffold. In the case of periodontal tissue engineering, the above-indicated stem cells, signaling molecules and scaffold materials have been examined pre-clinically, with some having already been introduced into clinics.

1. PDGF-BB (platelet-derived growth factor) plus IGF-I (insulin-like growth factor-I)
2. BMP-2 (bone morphogenetic protein-2)
3. TGF-β (transforming growth factor-β)
4. OP-1 (BMP-7) (osteogenic protein-1)
5. BDNF (brain-derived neurotrophic factor)
6. FGF-2 (bFGF) (basic fibroblast growth factor
7. PDGF-BB (platelet-derived growth factor) plus β-TCP (GEM21S™) (β-tricalcium phosphate)
8. GDF-5 (growth and differentiation factor-5)

Table 1. Periodontal regeneration by recombinant cytokines

applying topical FGF-2 to induce periodontal tissue regeneration, a series of animal studies using beagle dogs and non-human primates was performed (Murakami *et al* 2003c, Takayama *et al* 2001c). The mandibular molars of beagle dogs, and the first and second molars of non-human primates, were utilized for experimentation. After elevation of mucoperiosteal flaps, class II furcation defects were surgically created and the exposed cementum removed by curettage, before vinyl polysiloxane impression material was placed in the defects to induce inflammation. Four weeks after the first surgery, a flap was raised to expose the inflamed furcation, granulation tissues were removed and the root surfaces curetted. A small round bur was used to make a horizontal groove on each root in order to indicate the base of the defect. Furcation defects were filled with a gelatinous carrier without or with FGF-2 and the wound was surgically closed. Periodontal tissue regeneration at the test sites of beagle dogs and non-human primates was examined at 6 and 8 weeks respectively, after FGF-2 application to the defects.

As shown in Tables 2 and 3, topical application of FGF-2 significantly stimulated periodontal regeneration in both the beagle and the non-human primate models when compared to control sites (Figure 2). Histological observation revealed new cementum with Sharpey's fibers, new functionally-oriented periodontal ligament fibers and new alveolar bone (Murakami *et al* 2003a, Takayama *et al* 2001). Interestingly, enhancement of angiogenesis and regeneration of peripheral nerve fibers at the FGF-2-treated sites were also observed one week after FGF-2 application (Murakami 2011a).

More importantly, no epithelial

	Control site (n=6)	**0.1% FGF-2-applied site (n=6)**
NBF (%)	35.4 ± 8.9	83.6 ± 14.3*
NTBF (%)	16.6 ± 6.2	44.1 ± 9.5*
NCF (%)	37.2 ± 15.1	97.0 ± 7.5*

*: $p<0.01$, Control site - gelatinous carrier alone was applied.

Table 2. Efficacy of FGF-2 for periodontal tissue regeneration in animal models - Furcation class II model in beagle dogs (6-week follow up) (modified from Murakami *et al* 2003)

	Control site (n=6)	**0.4% FGF-2-applied site (n=6)**
NBF (%)	54.3 ± 8.0	71.3 ± 13.5*
NTBF (%)	31.6 ± 3.5	48.7 ± 8.9**
NCF (%)	38.8 ± 8.6	72.2 ± 14.4**

*: $p<0.05$, **: $p<0.01$, Control site - gelatinous carrier alone was applied.

Table 3. Efficacy of FGF-2 for periodontal tissue regeneration in animal models - Furcation class II model in non-human primates (8-week follow up) (modified from Takayama *et al* 2001)

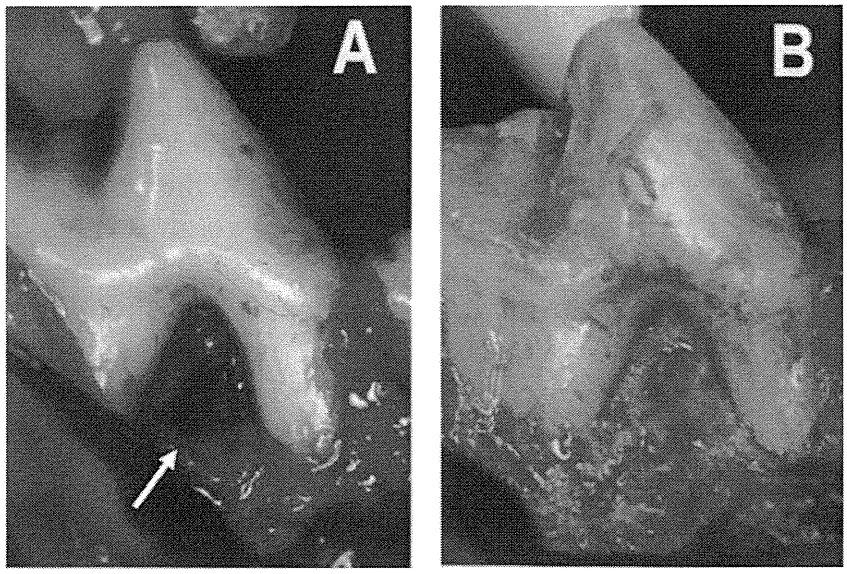

Figure 2. Periodontal tissue regeneration by FGF-2 (furcation class II beagle model).
FGF-2 (0.1%) plus gelatinous carrier was topically applied to surgically-created class II furcation defects in the mandibular molars of beagle dogs. Representative images at (A) baseline and (B) 6 weeks after FGF-2 application are shown. Arrow indicates furcation. (from Murakami *et al* 2003)

downgrowth, ankylosis or root resorption was observed at the FGF-2 sites in any of the *in vivo* experiments, nor was any severe gingival inflammation or swelling observed at any of the sites examined throughout the experimental periods.

In vitro analyses of effects of FGF-2

It has already been demonstrated that FGF-2 promotes proliferation of fibroblasts and osteoblasts, and enhances angiogenesis. These activities are crucial in the process of periodontal tissue regeneration. However, periodontal ligament (PDL) cells also play an important role during periodontal tissue regeneration (Seo *et al* 2004, Lekic *et al* 2001, Murakami *et al* 2003b, Shimono *et al* 2003). To reveal the molecular and cellular mechanisms by which FGF-2 enhances periodontal tissue regeneration, a series of *in vitro* experiments using PDL cells were carried out.

RT-PCR experiments demonstrated that PDL cells express FGF receptor (FGFR) 1 and FGFR2 mRNA (Takayama *et al* 2002), and *in vitro* experiments revealed that FGF-2 regulates the proliferation, differentiation, migration and extracellular matrix (ECM) production of PDL cells (Takayama *et al* 1997, Shimabukuro *et al* 2005, Shimabukuro *et al* 2008, Shimabukuro *et al* 2010, Terashima *et al* 2008). FGF-2 also enhances the proliferative responses of PDL cells, and does so *via* the extracellular signal-regulated kinase (ERK) 1/2 signaling pathway, an important second messenger system downstream of FGFRs. Interestingly it was found that FGF-2 significantly decreased both ALPase activity and the formation of calcified nodules in PDL cells in a dose-dependent manner. However, the suppressive effect of FGF-2 on PDL cell differentiation into hard-tissue-forming cells such as osteoblasts and cementoblasts was reversible. Thus, when FGF-2-stimulated PDL cells were re-cultured in the absence of FGF-2, calcified nodule formation resumed. By temporarily inhibiting the differentiation of PDL cells, FGF-2 facilitates their proliferation while maintaining their multipotency, but once the influence of FGF-2 is biologically diminished immature PDL cells begin to

differentiate into osteoblasts and cementoblasts.

FGF-2 also stimulated significant migration of PDL cells, even when their proliferation was completely inhibited by mitomycin-C. Furthermore, it was shown that FGF-2 stimulates the biosynthesis of hyaluronan (HA) and the cell surface expression of CD44, and that the interaction between these molecules plays a crucial role in PDL cell migration (Shimabukuro *et al* 2010).

This series of *in vitro* studies has facilitated the development of a hypothesis on the mode of action of FGF-2. Thus, during the early stages of periodontal tissue regeneration, FGF-2 stimulates proliferation of PDL cells while suppressing their differentiation (Figure 3). Then, during the subsequent healing process, when FGF-2 is no longer present at the administration site, PDL cells begin to differentiate into hard-tissue-forming cells such as osteoblasts and cementoblasts resulting in marked periodontal tissue regeneration at sites of FGF-2 application. In addition, FGF-2 induces the angiogenesis that is indispensable in the regeneration of tissue, and regulates the production of osteopontin, heparan sulfate and HA from PDL cells (Takayama *et al* 1997, Shimabukuro *et al* 2005, Shimabukuro *et al* 2008, Terashima *et al* 2008). Notably, FGF-2 specifically promotes the production of high molecular weight HA, which plays an important role in cell migration and the early stages of wound healing (Shimabukuro *et al* 2005). Based on the results described above, we concluded that FGF-2 contributes to the overall regeneration

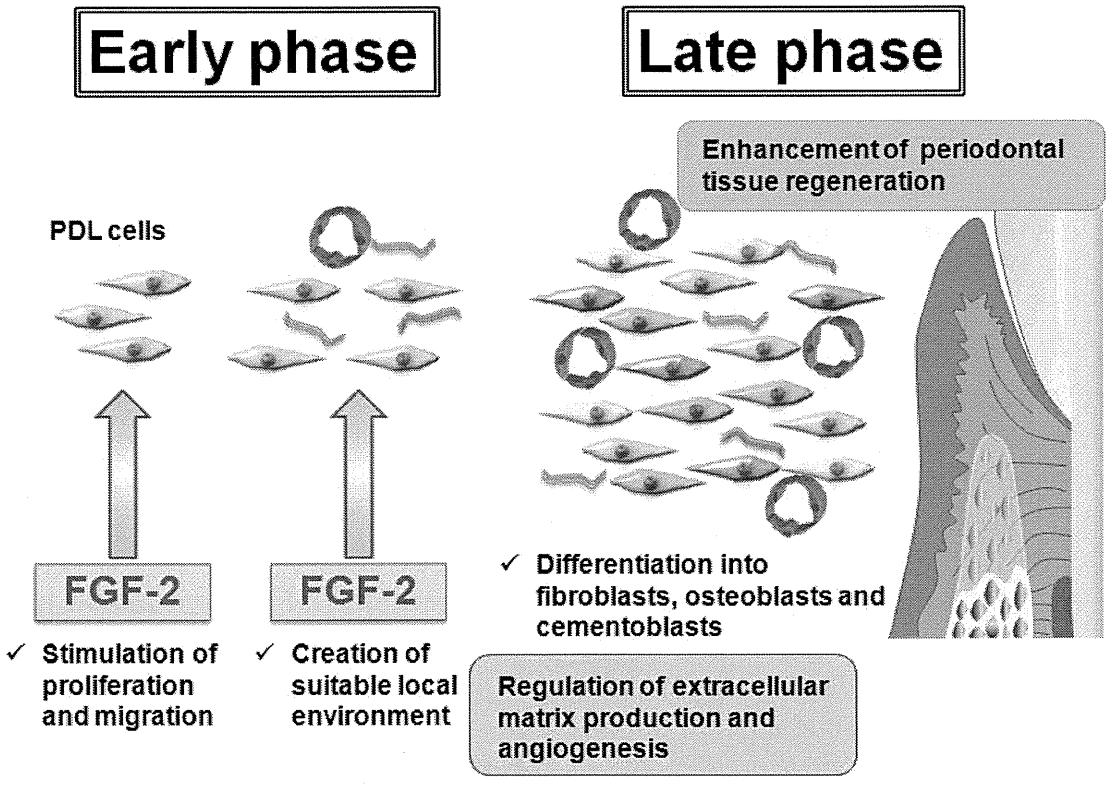

Figure 3. Possible mode of action of FGF-2 in induction of periodontal regeneration.
During the early stages of periodontal tissue regeneration, FGF-2 stimulates the proliferation and migration of PDL cells while maintaining their multipotent nature, and in later stages induces their differentiation into hard-tissue-forming cells such as osteoblasts and cementoblasts. Furthermore, FGF-2 induces angiogenesis and increases the production of osteopontin, HS and macromolecular HA from PDL cells, creating a local environment suitable for the regeneration of periodontal tissue.

of periodontal tissue by creating a local environment that facilitates the function of this mechanism (Murakami 2011b).

Clinical trial of FGF-2 for periodontal tissue regeneration

Phase IIA clinical trial

Given the promise shown by FGF2 as a periodontal regeneration agent, we performed a Phase II clinical trial. Using data from animal trials, we estimated that an effective FGF-2 concentration for periodontal tissue regeneration is 0.03 to 0.3%. This concentration range was therefore applied in the Phase IIA trial.

We prepared gel-like investigational drugs using 3% hydroxypropylcellulose (HPC) as a vehicle. We then designed a double-blinded clinical trial with approximately 80 periodontitis patients from 13 dental facilities in Japan. Patients displaying a two- or three-walled vertical bone defect ≥3 mm from the top of the alveolar bone were registered for this clinical trial and randomly divided into four groups: Group P (Placebo), Group L (0.03% FGF-2), Group M (0.1% FGF-2) and Group H (0.3% FGF-2). Patients underwent flap surgery during which we administered 200 μl of the appropriate investigational drug to periodontal tissue defects. For efficacy analysis, standardized radiographs of the region of investigation were taken before and 36 weeks after administration of the investigational drug. The rate of increase in alveolar bone height was independently measured by five specialist dental radiologists who were blinded to the treatment each patient had received. The median of five measurements taken from the same image was then selected for efficacy analysis.

We observed that the mean alveolar bone height in Group H (0.3% FGF-2) gradually increased for 36 weeks after application (Figure 4). After 36 weeks, a significant increase (p=0.021) in alveolar bone height was seen on standardized radiographs between Group P (23.92%) and Group H (58.62%) (Figure 4) (Kitamura *et al* 2008). No serious adverse effects were seen during the course of this clinical trial. The data obtained from this clinical trial suggest that topical application of FGF-2 is efficacious in regenerating periodontal tissue in patients with two- or three-walled intrabony defects.

Phase II B clinical trial

Having obtained positive results from the Phase IIA trial, we progressed to a Phase IIB trial (Kitamura *et al* 2011). In this large clinical trial, approximately 260 periodontitis patients from 25 dental facilities in Japan were registered, and were randomly divided into four groups comprising a placebo group and three FGF-2 groups (0.2, 0.3 and 0.4%). Results, in terms of efficacy and safety, were similar to the Phase IIA trial (Kitamura *et al* 2011, Murakami *et al* 2011a).

However, in both the Phase IIA and IIB trials, no significant differences in the regain of clinical attachment loss (CAL) between Group P and the FGF-2 groups were found. This is in agreement with observations reported in a clinical trial showing the efficacy of PDGF-BB plus β-TCP for periodontal regeneration (Nevins *et al* 2005). We speculate that differences may exist between Group P and the three FGF-2 groups in the histological ratio of fibrous and epithelial attachments achieving CAL acquisition.

Future Outlook of FGF-2 therapy

"Tissue engineering" is a fundamental concept in tissue regeneration. As mentioned above, we observed that topical application of FGF-2 significantly induces periodontal tissue regeneration, including fibrous attachment

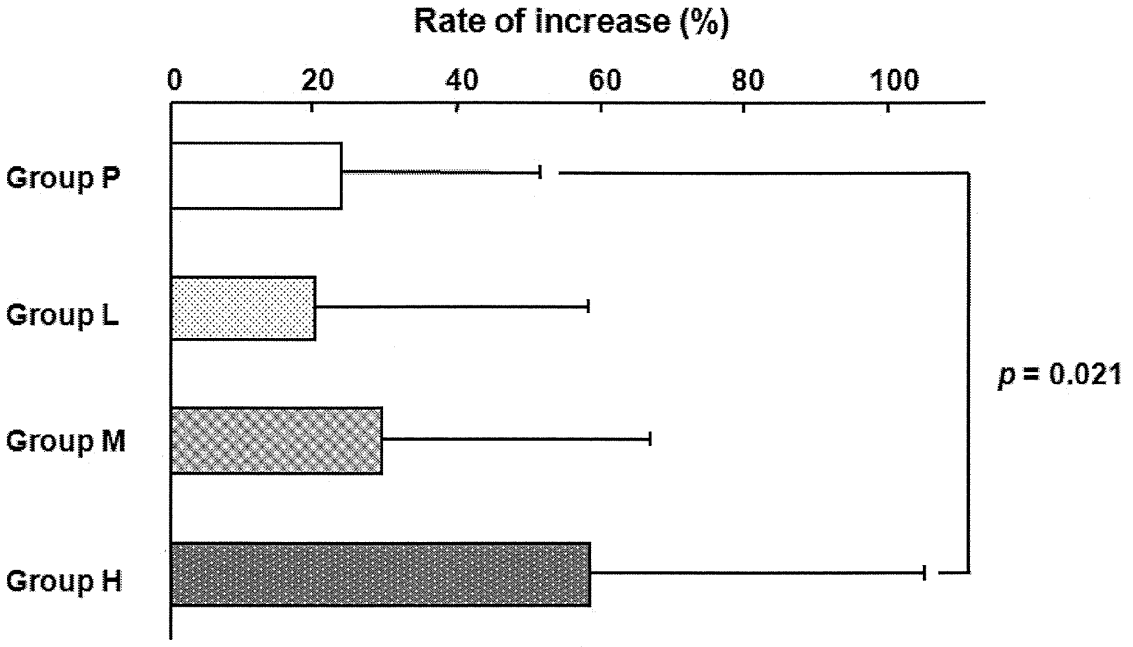

Figure 4. Rates of increase in alveolar bone height in cases of 2- and 3-walled intrabony defects.
We compared rates of increase in alveolar bone height at 36 weeks after FGF-2 administration among Group P (Placebo; n=19), Group L (0.03% FGF-2; n=19), Group M (0.1% FGF-2; n=19) and Group H (0.3% FGF-2; n=17). Graph shows mean rates of increase in alveolar bone height (%) ± standard deviation. While no significant difference was observed between Groups L, M and P, Group H showed significantly (p = 0.021) increased alveolar bone height in the bone defect region compared to Group P. (Modified from Kitamura *et al* 2008)

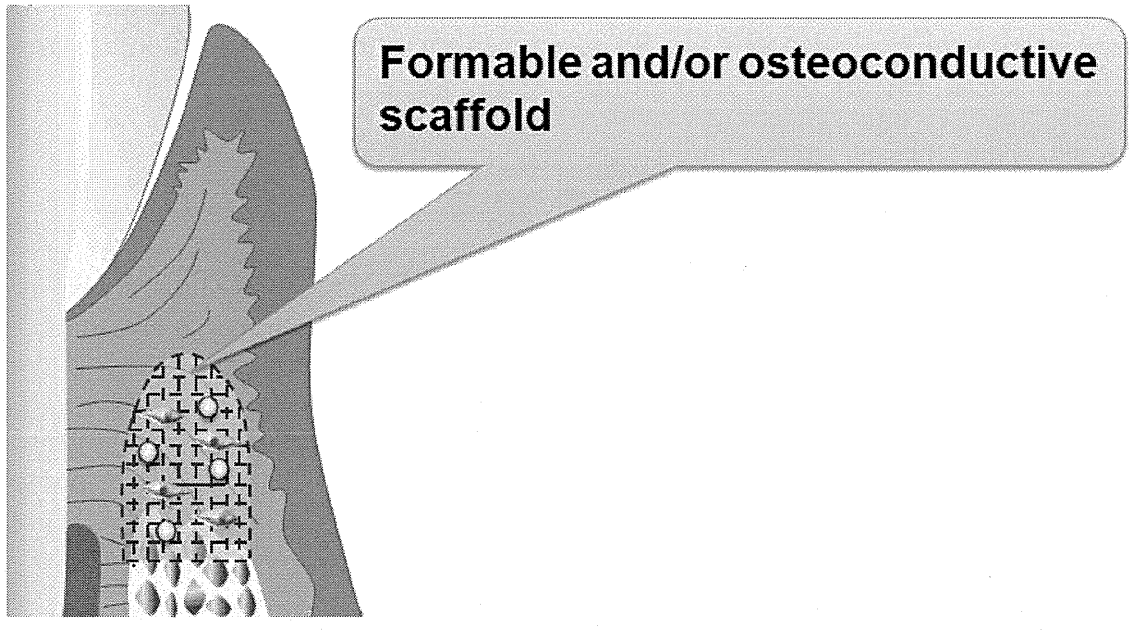

Figure 5. Ideal FGF-2 carrier for periodontal tissue regeneration.
An FGF-2 carrier that could provide a formable and osteoconductive scaffold for undifferentiated cell types within periodontal ligament would dramatically increase the indications of an FGF-2-based drug.

and neogenesis of alveolar bone and cementum in animal models. It is also noteworthy that no gingival epithelial downgrowth was observed at sites to which FGF-2 was applied. In the clinical trials of 0.3% FGF-2, we observed significant differences in the rate of increase in alveolar bone height between the placebo group and the FGF-2 group (Kitamura *et a*l 2008, Kitamura *et al* 2011). This suggests that FGF-2 is efficacious for periodontal regeneration of intraosseous bone defects such as 2- or 3-walled bone defects and probably furcation involvements. However, to treat severe bony defects such as 1-wall or horizontal bone defects with FGF-2, the FGF-2 carrier may require the function of a "scaffold" to reinforce/direct its actions. HPC, which was used in our clinical trials as a carrier, does not function as a scaffold. Development of an FGF-2 carrier that provides a formable and osteoconductive scaffold for undifferentiated cell types would dramatically increase the indications of FGF-2 drugs beyond dental applications and into the craniofacial field (Figure 5). We recently examined the combined effects of FGF-2 and β-TCP on periodontal regeneration in 1-wall bony defects in beagle models and found that the combination induced significant periodontal tissue regeneration, compared with β-TCP alone (Anzai *et al* 2010). This suggests that the combination of scaffold material(s) and bioactive molecule(s) such as FGF-2 could be useful for the treatment of severe cases.

The efficacy of "cytokine therapy" in periodontal tissue regeneration was first reported in the 1990s. Since then, various recombinant cytokines have been investigated for their efficacy (and safety) in stimulating periodontal tissue regeneration, however few have been approved for use in the dental field. Therefore, we need to evaluate carefully the usefulness and safety of cytokine therapy in stimulating periodontal tissue regeneration. We hope that our work, together with future investigations, will provide a framework within which to understand "cytokine therapy" and its application to periodontal regeneration and oral reconstruction. Furthermore, "stem cell therapy" may also assist in improving periodontal regenerative therapy. It has already been reported that transplantation of bone marrow-derived cells or adipose-tissue derived stem cells can enhance periodontal regeneration (Murakami 2011b). The combined effects of "cytokine therapy" and "stem cell therapy" still require investigation.

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