and differentiation of cementoblasts, osteoblasts and periodontal ligament cells, as well as induction of angiogenesis (7, 11, 27, 104, 124). An animal study showed that topical application of enamel matrix derivative induced acellular cementum formation, resulting in statistically significant periodontal tissue regeneration (36). In the Cochrane database, it is reported that enamel matrix derivative is able to significantly improve probing attachment levels (1.1 mm) and probing pocket depth reduction (0.9 mm) compared to flap surgery, but these results may not have a great clinical impact, as was not shown that more periodontally compromised teeth could be saved (21). The database also indicates that there is no evidence of clinically important differences between guided tissue regeneration and enamel matrix derivative treatments (21).

Recombinant human growth factors (cytokines) as signaling molecules

Topical application of recombinant cytokines is one of the most effective methods to stimulate endogenous stem cells and/or progenitor cells. Clinical application of cytokines has been enabled by the availability of recombinant products. A variety of recombinant cytokines have already been investigated in terms of their ability to enhance periodontal tissue regeneration, and those whose potential to induce periodontal regeneration has been shown in animal studies are listed in Table 1. Direct local application of platelet-derived growth factor and insulin-like growth factor-I (64), bone morphogenetic protein-2 (54, 114), transforming growth factor-β (74), osteogenic protein-1 (28), brain-derived neurotrophic factor (118) or growth and differentiation factor-5 (52) to artificial bone defects created in laboratory animals reportedly stimulates and promotes periodontal regeneration. Unfortunately, however, few double-blinded clinical trials involving multiple facilities and complying with good clinical practice guidelines have been performed to confirm the efficacy of cytokines as a signaling molecule for periodontal tissue regeneration. To date, only cocktails of platelet-derived growth factor plus insulin-like growth factor-1 (41) or platelet-derived growth factor plus β-tricalcium phosphate as osteoconductive scaffolds (85), and basic fibroblast growth factor (FGF-2) (55) have been evaluated for their efficacy in inducing periodontal tissue regeneration in multicenter clinical trials.

Table 1. Recombinant cytokines used in studies of periodontal regeneration

Studies	Recombinant cytokines
Lynch et al. (64)	PDGF-BB plus IGF-I (platelet-derived growth factor plus insulin-like growth factor-I)
Kinoshita et al. (54) Sigurdsson et al. (114)	BMP-2 (bone morphogenetic protein-2)
Mohammed et al. (74)	TGF-β (transforming growth factor-β)
Giannobile et al. (28)	Osteogenic protein-1 (BMP-7)
Takeda et al. (118)	BDNF (brain-derived neurotrophic factor)
Kitamura et al. (55)	FGF-2 (bFGF) (basic fibroblast growth factor
Nevins et al. (85)	PDGF-BB plus β-TCP (GEM21S) (platelet-derived growth factor plus β-tricalcium phosphate)
Kim et al. (52)	GDF-5 (growth and differentiation factor-5)

Platelet-derived growth factor

Platelet-derived growth factor is a dimeric glycoprotein consisting of two A chains (AA), two B chains (BB), or a combination of A and B chains (AB). The possible clinical applications of platelet-derived growth factor BB have been intensively examined. Platelet-derived growth factor BB is already commercially available as a prescription drug for the treatment of deep neuropathic diabetic foot ulcers (135). *In vitro* analysis showed that platelet-derived growth factor BB is a potent mitogen and chemotactic protein for periodontal ligament fibroblasts and alveolar bone cells, and that it improves angiogenesis (40).

The efficacy of platelet-derived growth factor BB in inducing periodontal tissue regeneration has been demonstrated in animal studies (45, 64, 84). The first human clinical trial of platelet-derived growth factor BB in the field of periodontal therapy was performed in combination with recombinant human insulin-like growth factor-I (40). Thirty-eight patients with moderate to severe periodontitis were entered into the trial at two test centers. Utilizing a split-mouth design, defects were treated with either a low dose (50 μg/ml) or high dose (150 μg/ml) of

platelet-derived growth factor BB/insulin-like growth factor-I. Nine months after application, 150 μg/ml of platelet-derived growth factor-BB/insulin-like growth factor-I had induced 2.08 mm of vertical new alveolar bone formation and 42.3% filling of defects, compared to 0.75 mm and 18.5% at control sites, respectively. Subsequently, a primate study revealed that platelet-derived growth factor alone was as effective as the combination of platelet-derived growth factor-BB/insulin-like growth factor-I in producing new attachment 3 months after application (64), although the osseous defect filling induced by platelet-derived growth factor alone was not significantly greater than that in controls. It was subsequently reported that, at sites treated with platelet-derived growth factor-BB plus allograft, the gain in clinical attachment level and the radiographic bone fill were 4.8 ± 2.5 and 2.1 ± 0.9 mm, respectively ($P < 0.001$ and $P < 0.002$ compared to baseline) (84). This suggests that topical application of platelet-derived growth factor-BB plus bone graft results in robust periodontal regeneration.

Subsequently, a randomized double-blind clinical trial was performed in order to evaluate the effectiveness of 0.03% (0.3 mg/ml) and 0.1% (1.0 mg/ml) recombinant human platelet-derived growth factor-BB plus β-tricalcium phosphate (85). A total of 180 patients from 11 dental facilities were registered in this clinical trial. It was found that 0.03% recombinant human platelet-derived growth factor-BB plus β-tricalcium phosphate induced significantly more new alveolar bone formation (2.6 mm new bone and 57% defect filling) compared to β-tricalcium phosphate alone (0.9 mm new bone and 18% defect filling) 6 months after application ($P < 0.001$) (85). The gain in clinical attachment level was not significantly greater at 6 months after application, although significant differences between 0.03% platelet-derived growth factor-BB plus β-tricalcium phosphate and β-tricalcium phosphate alone were observed 3 months after application. Thus, platelet-derived growth factor-BB apparently offers an early benefit with regard to gain in clinical attachment level. Based on these data, the combination of 0.03% platelet-derived growth factor-BB plus β-tricalcium phosphate was approved by the US Food and Drug Administration, and is now commercially available as a medical device for periodontal regeneration (GEM21S). This is the first example of a human recombinant cytokine being used in periodontal tissue regeneration, and a very important first step into the future for periodontal tissue bioregeneration.

Basic fibroblast growth factor (bFGF, FGF-2)

The efficacy of a human recombinant basic fibroblast growth factor (FGF-2) for periodontal tissue regeneration has also been being evaluated in a multi-center clinical trial. At present, a drug containing FGF-2 for periodontal tissue regeneration is not commercially available. However, a series of *in vitro* and preclinical studies, and the results of multi-center clinical trials discussed below, have indicated the efficacy of FGF-2 in periodontal tissue regeneration (45).

Background information on the FGF family and biological activities of FGF-2

Fibroblast growth factor (FGF) was discovered in 1974 as a protein that strongly induced proliferation activity in fibroblasts from cow pituitary glands (30). In 1984, two proteins with different basic and acidic isoelectric points were identified as acidic FGF (aFGF, FGF-1) and basic FGF (bFGF, FGF-2) (6, 121). In 1985, the entire amino acid sequence of FGF-2 from cow pituitary gland was determined, and the cDNA of human FGF-2 was cloned in 1986 (1, 20).

Subsequently, attempts were made to identify new *FGF* genes based on sequence similarities of proteins to FGF, and the FGF family is currently known to include 22 genes. The proteins have been classified into seven subfamilies, FGF-1 (FGF-1,2), FGF-4 (FGF-4,5,6), FGF-7 (FGF-3,7,10,22), FGF-8 (FGF-8,17,18), FGF-9 (FGF-9,16,20), FGF-11 (FGF-11,12,13,14) and FGF-19 (FGF-19,21,23), based on structural similarities, with each subfamily consisting of two to four types of FGF. Each FGF is a polypeptide of approximately 150–200 amino acid residues, with core regions that show high levels of homology (30–70%) in amino acid sequence (44, 92).

Nearly all FGFs transmit signals via receptor-type tyrosine kinase. When FGF binds to a receptor, tyrosine kinase is activated as a result of receptor dimerization and autophosphorylation. Genes belonging to the FGF family have heparan-binding sites called 'glycine boxes', and the dimerized receptors are stabilized by binding of heparan sulfate. Following activation of tyrosine kinase, various tyrosine residues on the receptor are phosphorylated, and signal transmission is triggered by the binding of effector proteins to these sites (1, 20).

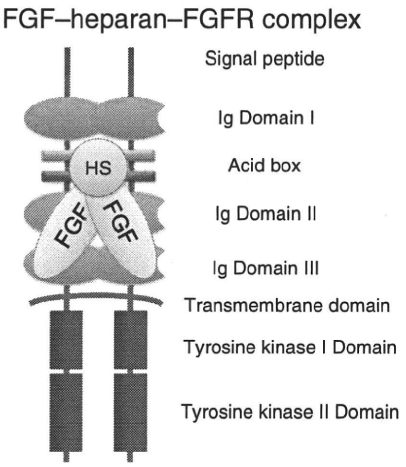

Fig. 2. Schematic illustration of FGF receptors. The extracellular ligand-binding region of FGF receptors (FGFRs) is composed of immunoglobulin (Ig)-like domains, which are required for FGF binding, and the acidic box domain, heparan-binding domain and cell-adhesion homology domain, which are required for interaction with the extracellular matrix, particularly heparan sulfate (HS). The binding of the FGF-2-HS complex to an FGFR is illustrated. Modified from Böttcher RT, Niehrs C. Fibroblast growth factor signaling during early vertibrate development. *Endocrine Reviews* 2005: 26: 63–77. Copyright 2005, The Endocrine Society (8).

FGF receptors have extracellular immunoglobulin-like domains, and the transmembrane region consists of tyrosine kinase and C-terminal domains (Fig. 2). Four types of FGF receptor have been identified (FGFR1–FGFR4), and, because of the existence of b and c types resulting from selective splicing in the immunoglobulin-like domains of FGFR1, 2 and 3, there are seven types of receptor with varying ligand activities (FGFR1b, FGFR1c, FGFR2b, FGFR2c, FGFR3b, FGFR3c and FGFR4). Thus, FGF receptor signaling is diverse and fulfils various roles in the body (44, 92).

Many FGF knockout mice have been created and used to analyse these functions. FGF-2 knockout mice have been found to have mild cardiovascular, skeletal and nerve abnormalities (141), whereas FGF-1 knockout mice do not show any conspicuous abnormalities (73). Inner ear, caudal vertebrae and central nervous system abnormalities are seen in FGF-3 knockout mice (95), and longer hair is seen in FGF-5 knockout mice (38). Impaired skeletal muscle regeneration is seen in FGF-6 knockout mice (24), abnormal hair follicle and ureter formation are seen in FGF-7 knockout mice (34, 98), and abnormal cerebellar formation is seen in FGF-17 knockout mice (136). Embryonic death is observed in FGF-4 knockout mice (embryoblast proliferative disorder) (23), FGF-8 knockout mice (no gastrulation and impaired development of central nervous system and limbs) (72), FGF-9 knockout mice (abnormal formation of gonads, heart, gastrointestinal tract, skeleton and lungs) (12), FGF-10 knockout mice (impaired development of multiple organs, including limbs and lungs) (87) and FGF-18 knockout mice (impaired development of lungs and skeleton) (86, 128).

In terms of human disease, excessive activity of FGF-3–6, which were originally identified as oncogene products, is thought to be a trigger causing cells to become cancerous. Delayed growth accompanying bone abnormalities is also seen in FGF-23 knockout mice. FGF-23 is recognized as a phosphate metabolism regulator, and, because the gene encoding FGF-23 has been identified as a common causative gene of hypophosphatemic rickets and osteomalacia, these diseases are thought to be induced by over-activity of FGF-23 (2, 132). Human diseases known to be caused by over-activity of FGF due to mutations in the genes encoding FGF receptors FGFR1–3 include bone and cartilage diseases, including craniosynostosis, achondroplasia and hypochondroplasia (70).

FGF signaling also plays an important role in tissue repair and regeneration. FGF-2 induces particularly strong angiogenic activity and proliferative capacity in undifferentiated mesenchymal cells. Recombinant human FGF-2 has been developed and subjected to various investigations with the aim of developing pharmaceutical products. FGF-2 is widely expressed in various tissues from fetal stages through to adulthood, and, because it binds to all FGFR, with the exception of FGFR2b and 3b (94), it is thought to show its effects in various cell types. In 2001, FGF-2 was first applied clinically in Japan as a decubitus ulcer medication (Fiblast spray; Kaken Pharmaceutical, Tokyo, Japan). In addition, clinical trials are currently underway to investigate the use of FGF-2 in the treatment of bone fractures and in periodontal tissue regeneration. In the field of regenerative medicine, the most advanced research among FGFs is being performed on FGF-2.

Medical uses of FGF-2

Treatment of intractable ulcers

FGF-2 facilitates all major reactions necessary for revascularization, including lysis of the vascular basement membrane mediated by activation of

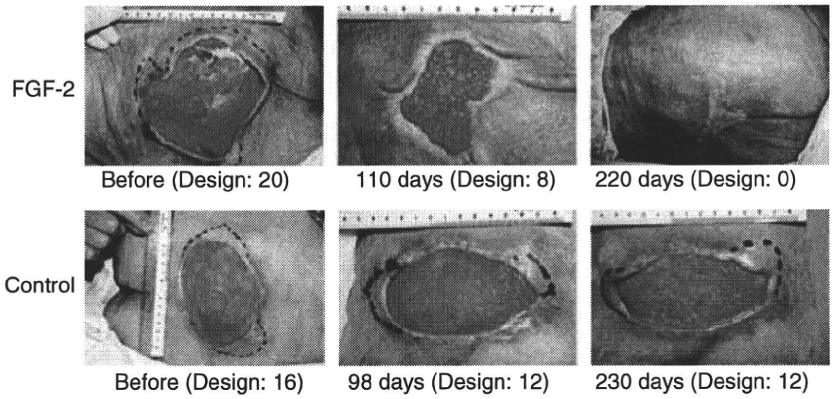

Fig. 3. Efficacy of FGF-2 for treating intractable pressure ulcers. In the patients treated with FGF-2 (Fiblast Spray), the ulcers were coated in covering material after application of the medication, and ulcers in control patients were only treated with the covering material. Wound sites were evaluated using the DESIGN criteria devised by the Japanese Pressure Ulcer Society (http://www.jspu.org/). This image was kindly provided by Dr Toshio Nakajou at Aoba Hospital, Tokyo, Japan. Reproduced, with permission, from Nakajou T. The potential of Fiblast spray to lead to medical cost abatement: analysis of the improvement rate of pressure ulcer. *Geriat Med* 2002: 40: 1803–1824.

plasminogen activator (97), migration and proliferation of endothelial cells (31), and tubule formation, and regenerates capillary blood vessels *in vivo* (25, 103). Fibroblasts not only produce collagen, but develop into myofibroblasts and induce so-called wound constriction (126). It has been reported that, when anti-FGF-2 antibodies are administered to normal animals that show normal wound healing, both granulation and wound healing are inhibited (9).

A full-thickness skin defect model was developed in genetically diabetic mice in 1990, and it was reported that FGF-2 promoted the migration of inflammatory cells, and the wound healing processes of angiogenesis, granulation and skin formation (33, 125). Further investigation of the action of FGF-2 in various animal models of wounds found that local injection of FGF-2 increased the number of cases of complete healing in a murine decubitus model, reduced the wound area and shortened the number of days until full healing in a burn wound model in malnourished rats, and inhibited wound deterioration resulting from infection and promoted healing in a diabetic mouse bacterial infection wound model (90).

The dose–response effect was investigated using single and continuous administrations of FGF-2 in a diabetic mouse model to assess the possibility of clinical use of FGF-2 for wound healing promotion. It was found that daily administration of 0.1–1 $\mu g/cm^2$ was most effective (89). The treatment effects of continuous administration of FGF-2 were dose-dependent, and it was reported that induction of granulation and angiogenesis by FGF-2 produced a bell-shaped response in the wound area. With respect to this phenomenon, it has been noted that continuous administration of FGF-2 at greater than optimal doses extends the period during which granulation and angiogenesis are promoted, which may result in a delay in skin formation.

The dose–response of FGF-2 was investigated in a double-blind comparative study for patients with various types of skin ulcers (42). FGF-2 preparations were produced at four dose levels (0.001–0.1%), and the amounts administered to the wound area were 0.1, 1, 5 and 10 $\mu g/cm^2$. The rate of improvement in the ulcers after once daily administration for 4 weeks was evaluated. The improvement rates were 68.6% (0.001%), 81.1% (0.01%), 70.1% (0.05%) and 60.3% (0.1%), and a bell-shaped dose-responsiveness curve was seen. In a comparative study with an existing drug (sugar and povidone iodine ointment), FGF-2 showed equivalent or better efficacy, safety and usefulness in 218 patients (76 institutions) with decubitus or skin ulcers (burn ulcers and leg ulcers) (43), and approval for manufacture (Fiblast Spray) was received from the Ministry of Health, Labour and Welfare of Japan in April 2001 (Fig. 3).

Treatment of intractable bone fractures

The role of FGFs in bone formation has been closely studied in recent years. FGFR3 knockout mice showed overgrowth of growth plate cartilage (13), and mice with constantly activated FGFR3 are known to have poor growth (131). FGFR abnormalities also appear in human pathologies, and constant activation of FGFR3 leads to achondroplasia (100, 108) and

thanatophoric dysplasia (101, 119). FGFR1 and FGFR2 abnormalities also lead to craniosynostosis syndromes, including Crouzon syndrome, Pfeiffer syndrome and Apert syndrome (91). Based on analysis of systemic bone disease caused by mutation of FGFR, it is apparent that FGFR-mediated signaling plays an important role in osteogenesis.

In 1994, Kawaguchi et al. (49) reported that local administration of FGF-2 at the site of fibula fractures in healthy rats and those with streptozotocin-induced diabetes significantly increased callus bone mineral content, breaking strength and breaking energy compared to the control group. Similarly, callus formation was reported to be promoted by local administration of FGF-2 in tibia fractures in rabbits and dogs (10, 46, 79). Administration of 200 μg of FGF-2 to the site of ulna fractures in *Macaca fascicularis* using cross-linked gelatin as a carrier also promoted fracture healing. In the 10th week after bone fracture, union was seen in all 10 monkeys that received FGF-2, but union was not seen in four control monkeys (50). The results of such preclinical studies indicate that clinical application of FGF-2 as an agent to treat fractures is possible (Fig. 4).

One of the mechanisms by which FGF-2 facilitates fracture healing is thought to be by promoting the proliferation of marrow-derived mesenchymal cells, and inducing differentiation into osteoblasts. In a mineralized nodule formation experiment in the presence of β-glycerophosphate, ascorbic acid and dexamethasone using rat bone marrow-derived stem cells, Pitaru et al. (93) found that, after FGF-2 stimulation, cell proliferation and increased amounts of hydroxyproline and proteins were present in the initial stage, followed by alkaline phosphatase (ALP) activity and increased osteocalcin and calcification in later stages. It has also been shown that bone morphogenetic protein-2 and dexamethasone-sensitive cell populations in bone marrow-derived mesenchymal cells increase in response to stimulation with FGF-2 (37, 106). It is thought that FGF-2 promotes the healing of fractures by stimulating both the growth and the biochemical functions of mesenchymal stem cells (50, 81).

In an investigation of continuous intravenous administration of FGF-2 in rats, it was shown that FGF-2 promotes the proliferation of endosteal osteoblasts and accelerates bone formation (69, 78). It was also observed that administration of FGF-2 to rats and rabbits in which osteoporosis had been induced by ovariectomy caused trabecular bone formation and increased bone mineral content (81, 82).

Based on these results, clinical trials using FGF-2 have been performed. An FGF-2 preparation with gelatin gel as a carrier was used to investigate the bone union effect of FGF-2 during high tibial osteotomy for osteoarthritis of the knee. Single local administrations of 200, 400 or 800 μg FGF-2 were given immediately after the bone was cut in 57 patients (20, 18 and 19 patients for the respective doses), and bone union was assessed by a third party who was unaware of the treatment used using X-ray images taken over time for up to 16 weeks postoperatively. These assessments indicated that FGF-2 increased the number of patients with bone union in a dose-dependent manner. The times until 50% of patients with each dose showed bone union were 11.5 weeks (200 μg), 10.1 weeks (400 μg) and 8.1 weeks (800 μg). No adverse events correlating with dosage were seen (48).

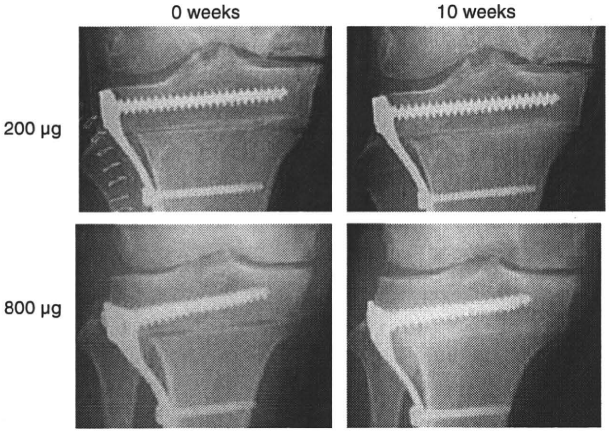

Fig. 4. Efficacy of FGF-2 for treatment of bone fractures. Anteroposterior radiographs of tibial osteotomy sites of representative patients in the two FGF-2 groups at 0 and 10 weeks after surgery. This image was kindly provided by Kaken Pharmaceutical Co. Ltd.

Others

Based on the fact that FGF-2 has a powerful angiogenic action, clinical trials are being performed on intermittent claudication in peripheral arterial disease and atherosclerotic peripheral vascular disease (59). In the TRAFFIC study (60), a single intravenous administration of 30 μg/kg resulted in a significant increase in peak walking time after 90 days. Clinical studies are also being performed on peripheral occlusive arterial disease with FGF-2 gene transfer, and the powerful angiogenesis action of FGF-2 is thought to be one of the most promising treatment factors in therapeutic angiogenesis.

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

In order to evaluate the activity of topical application of FGF-2 in inducing significant periodontal tissue regeneration, a series of preclinical animal studies was performed. Female beagle dogs and male *Macaca fascicularis* were used (76, 116). In beagle dogs, the mandibular molars (M1) were utilized. After elevation of the mucoperiosteal flaps, furcation class II bone defects were surgically created, and the exposed cementum of the teeth was removed by curettage. Vinyl polysiloxane impression material was placed in the created defects to induce inflammation. Four weeks after the first surgery, a flap was raised to expose the inflamed furcation, granulation tissue was removed and the root surfaces were curettaged. A small round bur was used to produce a horizontal groove on each root in order to indicate the base of the defect. Furcation defects were filled with either gelatinous carrier alone or 0.3% FGF-2 plus gelatinous carrier, and then surgical closure was performed. Periodontal tissue regeneration at the test sites was examined 6 weeks after application of FGF-2 to the defects.

In *Macaca fascicularis,* the first and second molars (M1 and M2) were utilized. Inflamed furcation class II defects were created as described above. Furcation defects were filled with either gelatinous carrier alone, or either 0.1 or 0.4% FGF-2 plus gelatinous carrier, and periodontal regeneration at the test sites was evaluated 8 weeks after FGF-2 application. As shown in Table 2, local application of FGF-2 significantly enhanced periodontal regeneration in both the beagle (Fig. 5) and non-human primate models compared to control sites. Histological observation showed new cementum with Sharpey's fibers, new functionally oriented periodontal ligament fibers and new alveolar bone (76, 116). Naturally occurring periodontitis in beagle dogs has also been treated with FGF-2. Performing conventional flap surgery, we applied 0.4% FGF-2 plus gelatinous carrier to the naturally occurring furcations. As shown in Fig. 6, significant regeneration was observed at these bony defects, with newly formed cementum and alveolar bone 7 weeks after topical application of FGF-2 (Table 2). Importantly, enhancement of angiogenesis and regeneration of peripheral nerve fibers at the FGF-2-treated sites were also observed (Fig. 7).

These data suggest that topical application of FGF-2 is efficacious in the regeneration of human periodontal tissue that has been destroyed by periodontitis. More importantly, there were no instances

Table 2. Efficacy of FGF-2 in periodontal tissue regeneration in animal models

	Control	FGF-2
Furcation class II model in beagle dogs*		
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**
Furcation class II model in non-human primates†		
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**
Naturally occurring periodontitis (furcation class II) in beagle dogs‡		
NBF	6.2 ± 10.62	54.7 ± 36.87***
NTBF	24.0 ± 35.89	68.3 ± 11.23***
NCF	31.7 ± 22.14	69.2 ± 20.89***

The mesio-distal plane of the created bone defect was stained with Azan and was subjected to histometric analysis. The percentage new bone formation rate (NBR), new trabecular bone formation rate (NTBR) and new cementum formation rate (NCR) were histomorphometrically calculated as follows: NBR = newly formed bone area/bone defect area × 100; NTBR = newly formed trabecular bone area/bone defect area × 100; NCR = length of newly formed cementum/length of root from fornix of furcation to bottom of defect × 100. $^{**}P < 0.01$; $^{***}P < 0.05$ compared with control.

*Modified from (98). Gelatinous carrier alone was applied at the control sites. $n = 6$ for both groups. The dose of FGF-2 was 0.1%. Six-week follow-up.

†Modified from (99). Gelatinous carrier alone was applied at the control sites. $n = 6$ for both groups. The dose of FGF-2 was 0.4%. Eight-week follow-up.

‡Fibrin gel alone was applied at the control site. $n = 4$ for both groups. The dose of FGF-2 was 0.4%. Seven-week follow-up.

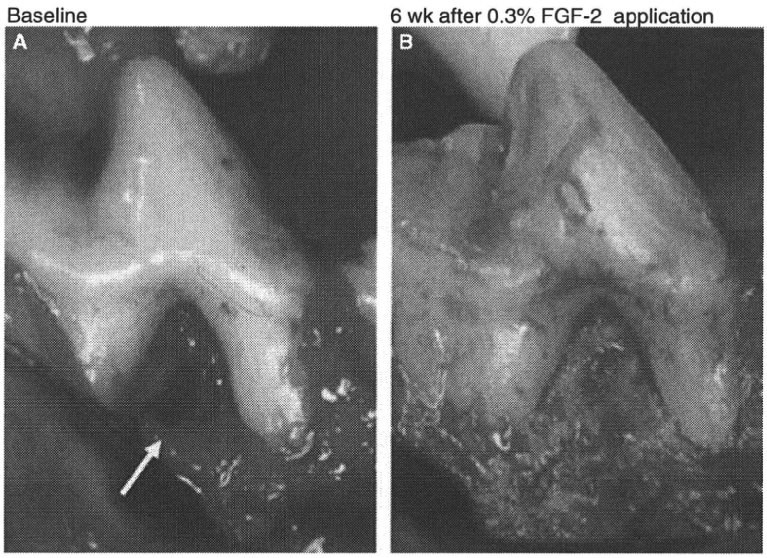

Fig. 5. Periodontal tissue regeneration by FGF-2 (furcation class II beagle model). FGF-2 (0.1%) plus gelatinous carrier was topically applied to surgically created furcation class II defects in the mandibular molars of beagle dogs. Representative images at (A) baseline and (B) 6 weeks after FGF-2 application are shown. Arrow indicates furcation. Reproduced from (105) with permission from Wiley-Blackwell.

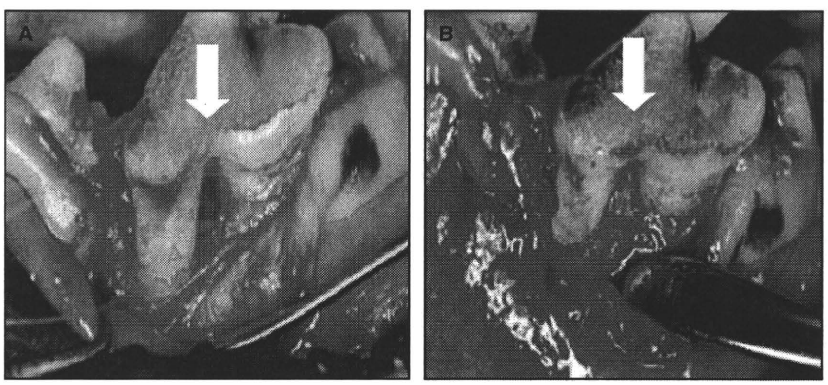

Fig. 6. Periodontal tissue regeneration by FGF-2 (naturally occurring periodontitis beagle model). Naturally occurring furcation involvement (class II) in beagle dogs was treated with 0.4% FGF-2. Representative images at (A) baseline and (B) 4 weeks after topical application of FGF-2 are shown. Arrows indicate furcation.

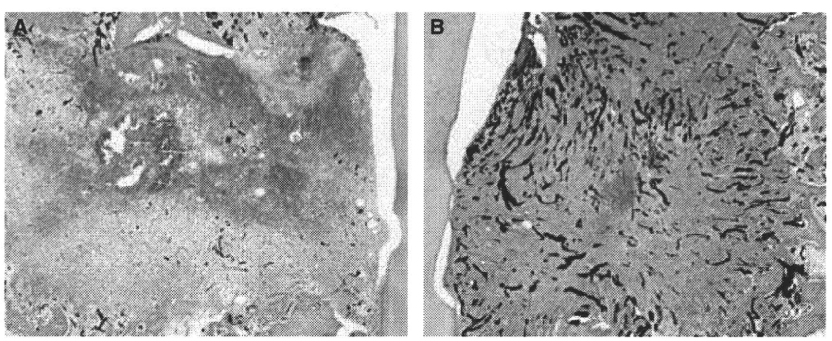

Fig. 7. Angiogenesis at periodontal tissue defect sites to which FGF-2 was applied. FGF-2 (0.3%) was topically applied to artificially prepared three-walled bony defects in beagle dogs. One week after application, newly formed blood vessels were observed by perfusion with Indian ink at the defect sites. Representative histological overviews of (A) control (no treatment) and (B) FGF-2-treated sites are shown.

of epithelial downgrowth, ankylosis or root resorption at the FGF-2 sites in any of the *in vivo* experiments described above. Furthermore, no severe inflammation or swelling was observed at any of the sites examined throughout the experimental periods.

In vitro analyses of effects of FGF-2

As described previously, FGF-2 acts on various cell types. FGF-2 promotes proliferation of fibroblasts and osteoblasts, and enhances angiogenesis. These activities are directly associated with periodontal tissue regeneration. However, it is agreed that periodontal ligament cells are key players during periodontal tissue regeneration (61, 75, 107, 113). To reveal the molecular and cellular mechanisms by which FGF-2 induces periodontal tissue regeneration, we have performed various *in vitro* experiments in which the effects of FGF-2 on periodontal ligament cells were examined.

When considering the responsiveness of periodontal ligament cells to FGF-2, it is very important to clarify expression of the FGF receptors on periodontal ligament cells. RT-PCR experiments showed that periodontal ligament cells express FGFR1 and FGFR2 mRNA (117). In contrast, gingival epithelial cells express mRNA of FGFR1, 2, 3 and 4 (117). Scatchard analysis revealed the presence of approximately 1.0×10^5 FGF-2 binding sites per periodontal ligament cell, with an apparent K_d of 1.2×10^{-10} M. Interestingly, binding of ^{125}I-FGF-2 to periodontal ligament cells reached its maximum on day 6 of culture, before gradually decreasing. Scatchard analysis also demonstrated that the number of FGFRs on periodontal ligament cells changed during the course of culture, but the affinity between FGF-2 and its receptor did not. This suggests that the responsiveness to FGF-2 is higher in undifferentiated peridontal ligament cells than in mature periodontal ligament cells.

Focusing on proliferation, migration, differentiation and extracellular matrix production of periodontal ligament cells, we review the biological activities of FGF-2 below.

Proliferation and differentiation

It has been demonstrated that FGF-2 enhances the proliferative responses of periodontal ligament cells in a dose-dependent manner, and that the extracellular signal-regulated kinase (ERK) 1/2 signaling molecule, which is located in an important pathway among downstream effectors of FGF receptors, mediates the proliferative responses (unpublished data). We also found that FGF-2 weakly induces the proliferation of gingival epithelial cells in a dose-dependent manner. Interestingly, however, co-stimulation with fetal calf serum inhibited FGF-2-induced proliferation of gingival epithelial cells, but synergistically enhanced FGF-2-induced periodontal ligament cell proliferation (117). This suggests that the biological effects on periodontal ligament cells may be synergistically increased *in vivo*, as serum components are present, and that FGF-2 acts differently on periodontal ligament cells and gingival epithelial cells *in vivo* in terms of the proliferative response. Similar to the *in vitro* observations described here, the animal studies described above showed that epithelial downgrowth at FGF-2-treated sites was significantly inhibited compared to control sites.

ALPase activity and calcified nodule formation of FGF-2-stimulated periodontal ligament cells were then assayed. We found that addition of FGF-2 significantly decreased both ALPase activity and calcified nodule formation in periodontal ligament cells in a dose-dependent manner. However, the suppressive effects of FGF-2 on cytodifferentiation of periodontal ligament cells into hard-tissue forming cells was reversible, and calcified nodules were formed when FGF-2-stimulated periodontal ligament cells were re-cultured in the absence of FGF-2. This shows that, by temporarily inhibiting the cytodifferentiation of periodontal ligament cells, FGF-2 facilitates their proliferation while maintaining their multi-potent nature.

Migration

Cell migration is essential for a wide range of biological events, including embryogenesis, tissue development, wound healing and tissue regeneration. Given the influence of FGF-2 on periodontal tissue regeneration, it is important to investigate the effects of FGF-2 on the migration of periodontal ligament cells. FGF-2 activated significant migration of periodontal ligament cells, even when proliferation of the periodontal ligament cells was completely inhibited by treatment with mitomycin C. In addition, we found that FGF-2 activated the growth and migration of human dental pulp cells (112). Experiments using metabolic inhibitors suggested that the phosphatidylinositol 3-kinase/akt pathway is involved in the FGF-2-stimulated migration of periodontal ligament cells. (akt is a serine/threonine kinase and a critical enzyme in signal

transduction pathways.) It is known that cell migration requires multiple coordinated cellular and molecular processes. Furthermore, we confirmed that FGF-2 positively regulates the biosynthesis of hyaluronan and cell-surface expression of CD44, and that the interaction between these molecules plays a key role in migration of periodontal ligament cells (Shimabukuro et al., unpublished data).

Extracellular matrix production

The extracellular matrix provides structural support and anchorage for cells, and is known to act as glue between cells and to regulate a wide range of cellular functions and behaviors. Furthermore, it sequesters growth factors and acts as a local reservoir for them, for example in the interaction between heparan sulfate, FGF-2 and heparan-binding growth factor. To create suitable environments for subsequent tissue regeneration, orchestrated regulation of various extracellular matrix products is essential.

Collagen

Collagen is the main component of the periodontal ligament, and plays an important role in supporting the tissue and cells (4, 83). Collagen also functions as the main bone matrix component. Interestingly, it was demonstrated that FGF-2 down-regulates expression of type I collagen mRNA and production of total collagen (115). This inhibitory effect is reversible, and is apparently correlated with the temporal inhibition of cytodifferentiation of periodontal ligament cells into hard-tissue forming cells, such as osteoblasts and cementoblasts.

Osteopontin

Osteopontin is a protein found in the bone-related matrix, and plays multiple regulatory roles in mineralizing and non-mineralizing tissue (14, 16). In osteogenic cell lines, the expression of osteopontin increases with the progression of differentiation (15). Osteopontin is detected within the cementum surface and periodontal ligament cells, and is implicated in cementogenesis and homeostasis of periodontal tissues (58, 66, 67, 71). We confirmed that FGF-2 up-regulated the expression of osteopontin in periodontal ligament cells at both the mRNA and protein levels, and enhanced the concentration of osteopontin in the culture supernatant (120), but decreased transcription of genes encoding almost all the bone-related proteins, including osteocalcin, osteonectin and bone sialoprotein. In addition, we found that FGF-2-induced osteopontin in the culture supernatant is involved in the survival activity of periodontal ligament cells (22).

Furthermore, we found that stimulation with inorganic phosphate, which induces late-stage osteogenic cytodifferentiation of periodontal ligament cells, also induces osteopontin, and that the osteopontin was associated with the network of extracellular matrices around the surfaces of periodontal ligament cells (120). This demonstrates that osteopontin produced in the late stages of cytodifferentiation plays a role as a bone matrix protein. In contrast, FGF-2 induces expression of osteopontin, which plays a different role from other bone-related proteins during the process of periodontal tissue regeneration by FGF-2.

Heparan sulfate

The extracellular matrix is composed of collagens, non-collagenous proteins such as fibronectin and laminin, and proteoglycans. Heparan sulfate proteoglycans are widely distributed biological molecules that mediate a variety of physiological responses in development, cell growth, cell migration and wound healing (3, 22). We examined the effects of FGF-2 on the production of heparan sulfate proteoglycan in periodontal ligament cells, and on the expression of syndecans, a major family of membrane-bound heparan sulfate proteoglycans. Treatment of periodontal ligament cells with FGF-2 for 72 h resulted in a pronounced increase in heparan sulfate levels in the culture supernatant in a dose-dependent manner (110). However, FGF-2 had no effect on transcription of genes encoding enzymes associated with heparan sulfate biosynthesis. On the other hand, FGF-2 marginally reduced expression of the genes encoding syndecans 1, 2 and 4, and did not alter the levels of syndecan 3 mRNA. Interestingly, FGF-2 did not alter the levels of syndecans 1 and 2, but enhanced the levels of syndecan 4 in culture supernatants from FGF-2-stimulated periodontal ligament cells. These results suggest that the FGF-2-activated increase in the levels of heparan sulfate in conditioned medium may be due to shedding of syndecan 4 from the periodontal ligament cell surface. Taken together, this indicates that FGF-2 may differentially regulate the expression of heparan sulfate proteoglycans in an heparan sulfate proteoglycan subtype-dependent manner. The shed heparan sulfate may be involved in the migration of periodontal ligament cells and the enhancement of local wound healing, resulting

in periodontal tissue regeneration. In contrast, the production of chondroitin sulfate was barely influenced by FGF-2 (109).

Hyaluronan

Hyaluronan is a non-sulfated glycosaminoglycan consisting of alternate residues of D-glucuronic acid and *N*-acetylglucosamine, and may be very large, with a molecular weight often reaching millions. Hyaluronan is known to be translocated out of the cell during biosynthesis by hyaluronan synthase, and plays important roles in homeostasis, cell migration and inflammatory/wound healing responses as one of the chief components of the extracellular matrix (88, 129, 134). We confirmed that FGF-2 significantly increases hyaluronan production in periodontal ligament cells in a dose-dependent manner, and that hyaluronan has a higher molecular mass in conditioned medium of FGF-2-treated periodontal ligament cells compared to untreated periodontal ligament cells (109). RT-PCR analysis revealed enhancement of transcription of the genes encoding hyaluronan synthases 1 and 2, both of which contribute to the production of hyaluronan with a high molecular mass in FGF-2-treated periodontal ligament cells. Taken together, these results suggest that FGF-2 promotes the production of hyaluronan with high molecular mass by up-regulating hyaluronan synthase expression, resulting in increased biosynthesis. Platelet-derived growth factor-BB also prominently enhanced hyaluronan production by periodontal ligament cells, consistent with previous observations (18, 39, 122). However, platelet-derived growth factor-BB only increased transcription of the genes encoding hyaluronan synthase 2 (unpublished data), with slightly increased levels of high-molecular-mass hyaluronan (109). Hyaluronan production increases in the early stages of wound healing (56), and exogenous application of high-molecular-mass hyaluronan has been reported to accelerate tissue repair (53, 80). In addition, an hyaluronan-rich environment and high levels of hyaluronan in the wound have been suggested to provide a favorable environment for repair and minimal inflammation in fetal wound healing (62, 63). Thus, local production of hyaluronan by FGF-2 stimulation plays important roles in cell migration and the early stages of wound healing, resulting in enhanced periodontal tissue regeneration. We also found that FGF-2 activates hyaluronan production in human dental pulp cells (111).

Possible mode of action of FGF-2 to induce periodontal tissue regeneration

A series of *in vitro* analyses revealed that FGF-2 regulates proliferation, differentiation, migration and extracellular matrix production of periodontal ligament cells. However, calcified nodule formation and alkaline phosphatase activity in periodontal ligament cells were inhibited in the presence of FGF-2. Furthermore, the suppressive effects of FGF-2 on differentiation are reversible; when FGF-2 is eliminated, the suppressive effects of FGF-2 also dissipate, thus initiating periodontal ligament differentiation. Tracer experiments using radiolabeled FGF-2 showed that topically applied FGF-2 disappeared from the sites in approximately 1 week. This suggests that the effects of topically applied FGF-2 will disappear within 1 week. We also found that the effects of FGF-2 on various cellular functions in periodontal ligament cells decreased gradually over the course of periodontal ligament cell culture. Taken together, these data suggest that FGF-2 acts effectively on immature periodontal ligament cells at the early stages of wound healing. Based on these findings, we have deduced that, during the early stages of periodontal tissue regeneration, FGF-2 increases the number of periodontal ligament cells while suppressing differentiation into hard tissue-forming cells such as osteoblasts and cementoblasts (Fig. 8). During the subsequent healing processes, probably 1 week after application, when FGF-2 activity disappears at the administration site, periodontal ligament cells begin to differentiate, inducing marked periodontal tissue regeneration. In addition, we now know that FGF-2 does not simply induce angiogenesis, an action that is indispensable in the regeneration of tissue, but also increases the production of osteopontin, heparan sulfate and macromolecular hyaluronan from periodontal ligament cells (109, 110, 120). Thus, FGF-2 creates a local environment suitable for regeneration of periodontal tissue through the activities described above (Fig. 8).

Clinical trial of FGF-2 for periodontal tissue regeneration

As described above, numerous animal studies have been performed, and have demonstrated the efficacy of human recombinant cytokines in periodontal

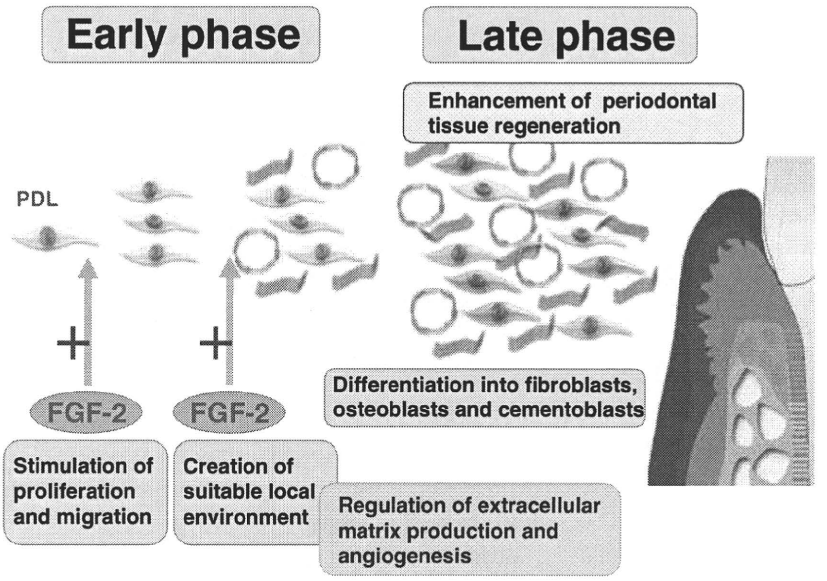

Fig. 8. Possible mode of action of FGF-2 to induce periodontal regeneration. During the early stages of periodontal tissue regeneration, FGF-2 stimulates the proliferation and migration of periodontal ligament cells while maintaining their multi-potent nature, to differentiate into hard tissue-forming cells such as osteoblasts and cementoblasts. Furthermore, FGF-2 induces angiogenesis and increases the production of osteopontin, heparan sulfate and macromolecular hyaluronan from periodontal ligament cells, thus leading to a local environment suitable for the regeneration of periodontal tissue.

tissue regeneration (Table 1). However, reports on clinical trials, particularly randomized controlled clinical trials, are very limited (41, 85). Thus, performing well-designed clinical trials to evaluate the effectiveness and safety of cytokine therapy is currently very important.

Phase IIA clinical trial

To assess the possibility of clinically applying FGF-2 for periodontal tissue regeneration, we designed a phase II clinical trial as follows. We chose the concentrations administered to periodontal regions of patients in the phase IIA clinical trial based on effective concentrations of FGF-2 used for periodontal tissue regeneration in animal trials, and the results of the phase I trial in which FGF-2 was administered intravenously to healthy adult humans. The results of testing with artificial defect models of periodontal tissue in beagles (76) led us to estimate that an effective FGF-2 concentration for induction of periodontal tissue regeneration is 0.03–0.3%. This range of concentrations was therefore used in the phase IIA clinical trial. We selected 200 μl as a sufficient volume to act on the defect region of periodontal tissue. In addition, results of the preclinical study suggested that the maximum quantity of administered FGF-2 that would enter the blood would be approximately 0.08 μg/kg, less than the 0.48 μg/kg for which safety was confirmed in our phase I clinical trial. Howell et al. (41) reported that, in platelet-derived growth factor-BB/insulin-like growth factor-I-treated subjects (n = 16), mean bone fill was 18.5 ± 7% for control sites and 42.3 ± 9% for platelet-derived growth factor-BB/insulin-like growth factor-I-treated sites, a mean difference of 23.8% (41). Assuming a rate of increase in alveolar bone of the defect region of 20% for placebo controls, the planned sample size of 20 patients in each group would provide 90% power to detect a clinically relevant treatment difference of 30% at a two-tailed significance level of 0.05.

We prepared gel-like drugs for this clinical trial containing human recombinant FGF-2 and 3% hydroxypropylcellulose as vehicle, and performed a double-blind clinical trial using 74 periodontitis patients from 13 dental facilities in Japan. In our preclinical study, we confirmed that topical application of 3% hydroxypropylcellulose into the intraosseous alveolar bone defects had no influence on periodontal tissue regeneration. The subjects comprised 74 patients who displayed a two- or three-walled vertical bone defect ≥ 3 mm from the top of the alveolar bone. Patients were randomly divided into four groups: group P, given vehicle only with no FGF-2; group L, given hydroxypropylcellulose containing 0.03% FGF-2; group M, given hydroxypropylcellulose containing 0.1% FGF-2; group H, given hydroxypropylcellulose containing 0.3% FGF-2. Patients underwent periodontal surgical treatment, during which we

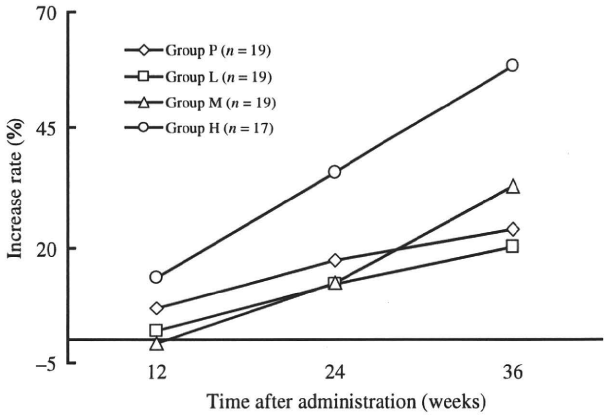

Fig. 9. Changes in alveolar bone height over time (phase II clinical trial). The study comprised a total of 74 cases involving two- or three-walled periodontal tissue loss: group P, placebo; group L, 0.03% FGF-2; group M, 0.1% FGF-2; group H, 0.3% FGF-2. Standardized radiographs were compared before and after treatment (3, 6 and 9 months), and bone heights were assessed by five individual oral radiologists. Rates of increase for alveolar bone height were obtained and compared between the groups. The rates of increase after administration of FGF-2 tended to be higher in all groups. After 9 months, a significant difference ($P = 0.021$) in alveolar bone height was seen between group P (23.9%) and group H (58.6%).

administered 200 μl of the appropriate concentration of drug or vehicle to sites displaying periodontal tissue defects. To evaluate the efficacy of the drug, all patients underwent standardized radiography of the region of investigation before and at 12, 24 and 36 weeks after administration of the drug. Five doctors specializing in dental radiology but unaware of the group to which the patient had been assigned independently measured the rate of increase in alveolar bone height. The median of the five measurements taken from the same image was used for efficacy analysis.

The mean alveolar bone height in patients in group H (0.3% FGF-2) gradually increased for 9 months after application (Fig. 9). After 9 months, a significant increase ($P = 0.021$) in alveolar bone height was seen on standardized radiographs in patients of group H (58.6%) compared with those in group P (23.9%) (Figs 9 & 10) (55). When we retrospectively analyzed the standardized dental radiographs of patients in group H, we noticed new vertical and horizontal bone growth from the remaining alveolar bone surface. No adverse effects were seen during the course of this multi-center trial. The data obtained suggest that topical application of FGF-2 is efficacious in regenerating periodontal tissue in patients with two-walled or three-walled intrabony defects. Regain of clinical attachment level was also evaluated in this clinical trial. The mean percentage clinical attachment level regained in groups P, L, M and H was 29.7, 24.0, 24.2 and 29.7, respectively. Statistical analysis confirmed that there weree no significant differences in clinical attachment level regain between group P and the three FGF-2 groups. Interestingly, similar observations were reported in the clinical trial that showed the efficacy of platelet-derived growth factor-BB plus β-tricalcium phosphate (85). We thus speculate that differences may exist between group P and the three FGF-2 groups in terms of the histological ratio of fibrous and epithelial attachments in clinical attachment gain.

Phase IIB clinical trial

The data obtained in the above-mentioned phase IIA clinical trial led to a subsequent larger clinical trial being performed. This has recently been completed. Approximately 260 periodontitis patients from 25 dental facilities in Japan were included in this clinical trial, and were randomly divided into four groups comprising a placebo group and three FGF-2 groups (0.2, 0.3 and 0.4%). Similar results were obtained in terms of efficacy and safety, and thorough analyses are now underway. Briefly, a significant increase in alveolar bone height was seen in patients of 0.3% FGF-2 group. These trials will provide crucial information regarding the safety and efficacy of utilizing FGF-2 in periodontal regeneration.

Future outlook for FGF-2 therapy

Numerous studies have been performed in order to examine the safety, efficacy and mechanism of FGF-2-induced periodontal tissue regeneration. With regard to the clinical application of FGF-2, a large phase III trial must be performed in order to confirm the efficacy and safety of FGF-2-based drugs. The following preclinical studies should also be performed in order to expand the indications of FGF-2 therapy in the dental field.

Intelligent scaffold containing FGF-2 for periodontal tissue regeneration

For ideal tissue regeneration, it is very important to fully introduce the concept of 'tissue engineering'. As mentioned above, we observed that topical application of FGF-2 significantly induces periodontal tissue regeneration, including osteogenesis and cemento-

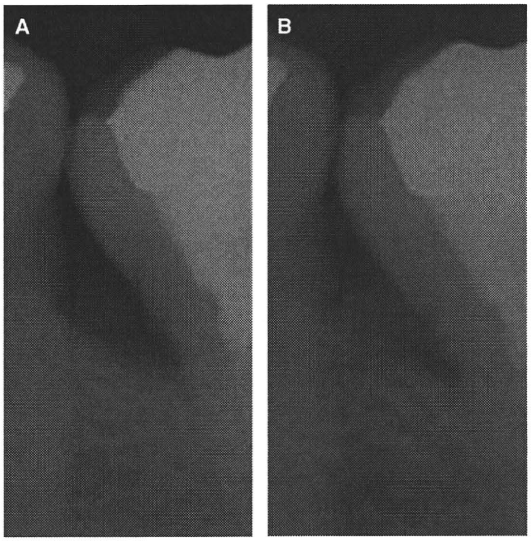

Fig. 10. Representative radiographic images from the group treated with 0.3% FGF-2 at (A) baseline and (B) after 9 months, showing improvement of the defect in terms of bone fill. These images were kindly provided by Dr Masahiro Kitamura of Osaka University Graduate School of Dentistry, Japan, and Kaken Pharmaceutical Co. Ltd.

genesis in animal models. It is also noteworthy that no gingival epithelial downgrowth was observed at FGF-2-treated sites without barrier membranes. In the clinical trial, we observed significant differences in the rate of increase in alveolar bone height between the placebo group and the FGF-2 group in which 0.3% FGF-2 plus 3% hydroxypropylcellulose was applied (55). This suggests that FGF-2 is efficacious for periodontal regeneration of intraosseous bone defects. However, in order to treat severe bony defects or horizontal bone resorption with FGF-2, it is essential to introduce the concept of a 'scaffold' into the FGF-2 carrier. An FGF-2 carrier that could provide a moldable and osteoconductive scaffold for undifferentiated cell types would dramatically increase both the dental and craniofacial medicine applications. We recently examined the combined effects of FGF-2 and β-tricalcium phosphate on periodontal regeneration in one-walled bony defects, and found that the combination enhanced periodontal tissue regeneration (Anzai et al., unpublished data). In particular, the influence on neogenesis of cementum and periodontal ligaments was very clear, suggesting that the combination of scaffold material(s) and bioactive molecule(s) such as FGF-2 is useful for the treatment of severe cases.

Application of FGF-2 for implant therapy

The utility and longevity of dental implants have improved markedly, and they have become a vitally important treatment option. Many clinicians are therefore seeking effective treatment procedures that enable resolution of bone volume deficits where implants will be placed. Cytokines or growth factors have attracted particular attention in this field. An example is the application of bone morphogenetic protein-2, an osteoinductive cytokine. The combination of recombinant human bone morphogenetic protein-2 and bovine type I collagen sponge has been approved by the US Food and Drug Administration as a medical device indicated for use in the dental field in sinus and ridge augmentation. There are also reports of animal experiments and clinical applications in humans showing that sufficient volumes of bone can be successfully regenerated in previously deficient alveolar ridges by topical application of recombinant human platelet-derived growth factor-BB (65). Similarly, topical application of FGF-2 may induce bone augmentation and/or promote osseointegration of implants. Studies to investigate its efficacy are now in progress.

Stem cell therapy

The majority of the current periodontal regeneration therapies, such as bone grafting, guided tissue regeneration, and enamel matrix derivative and cytokine therapies, depend on endogenous tissue stem cells that exist within periodontal ligament tissue. However, as humans age, the proliferation and mineralized nodule formation of periodontal ligament cells are negatively influenced, and the total number of stem cells within periodontal ligaments tends to decrease (5, 140). Thus, it is important to utilize stem cells isolated from other tissues, which can be transplanted into the alveolar bony defects where periodontal tissue regeneration is desired. Embryonic stem cells or induced pluripotent stem cells will probably be available in this field in the future. However, numerous issues remain to be overcome, not only with regard to efficacy but also with regard to safety and ethics. Many researchers are attempting to utilize somatic mesenchymal stem cells isolated from other tissues such as bone marrow and adipose tissue in order to enhance periodontal regeneration. It has already been reported that transplantation of bone marrow-derived cells into alveolar bone defects is efficacious for periodontal tissue regeneration, ridge augmentation and sinus lift (47, 127, 137). In such cases, cells were transplanted together with carriers or scaffolds, such as platelet-rich plasma, atelocollagen or hydroxyapatite. Furthermore, researchers, including our group, have

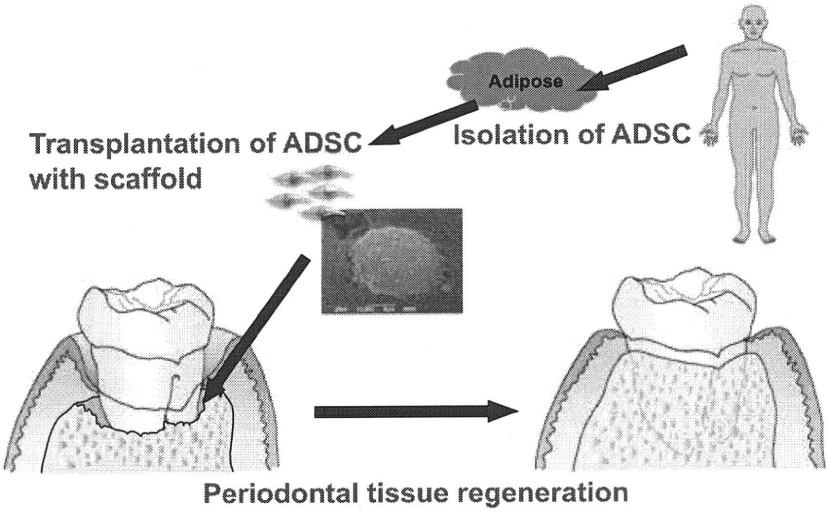

Fig. 11. Cell therapy using adipose tissue-derived mesenchymal stem cells (ADSC) for periodontal tissue regeneration. Mesenchymal stem cells are obtained from adipose tissue. Transplanting cells with scaffold materials into periodontal tissue defects enhances periodontal tissue regeneration at the transplanted sites. This type of cell therapy is a cutting-edge procedure that may be used to treat severe periodontitis patients in the future.

demonstrated that mesenchymal stem cells can be obtained from adipose tissues, and that transplantation of adipose-tissue derived stem cells enhances periodontal regeneration at applied sites (123, T. Hashikawa, unpublished data) (Fig. 11). The above-mentioned cell therapy will also assist in improving periodontal treatment. The combined effects of 'cell therapy' and 'cytokine therapy' need to be assessed in the near future, to allow the establishment of 'periodontal tissue engineering'.

Conclusions

The ultimate goal of periodontal therapy is to achieve complete regeneration of periodontal tissue destroyed by the progress of periodontal diseases. The chain of events involved in regeneration of periodontal tissue comprises stimulation of the differentiation of cementoblasts and osteoblasts on the dental root and alveolar bone surfaces facing the periodontal tissue defects, followed by regeneration of the cementum and alveolar bone. Collagen fibers produced by the periodontal ligament fibroblasts are then embedded into the regenerated hard tissues in order to rebuild new tissue and support teeth. Since the 1990s, the knowledge associated with stem cells has advanced rapidly. We now understand that undifferentiated somatic tissue stem cells exist within the periodontal ligament, and this allows periodontal regeneration to be stimulated in clinical settings. The important questions relate to the most suitable and practical methods for activating the latent abilities of these cells. We believe that cytokine therapy may provide a possible solution. The effectiveness of cytokines in periodontal tissue regeneration was first reported in the 1990s (114). Since then, numerous cytokines have been investigated in terms of their efficacy to enhance periodontal tissue regeneration (Table 1). However, few cytokines have been approved for use in the dental field. Therefore, we need to carefully evaluate the usefulness and safety of cytokine therapy to stimulate periodontal tissue regeneration. We hope that further investigations will provide crucial information to better understand cytokine therapy for periodontal regeneration and oral reconstruction.

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