

Review

Regeneration of the periodontium for preservation of the damaged tooth

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Summary. The population of the world grows every year, and life expectancy tends to increase. Thus, long-term preservation of teeth in aged individuals is an urgent issue. The main causes of tooth loss are well known to be periodontitis, caries, fractures, and orthodontic conditions. Although implant placement is a widely accepted treatment for tooth loss, most patients desire to preserve their own teeth. Many clinicians and researchers are therefore challenged to treat and preserve teeth that are irreversibly affected by deep caries, periodontitis, fractures, and trauma. Tissue engineering techniques are beneficial in addressing this issue; stem cells, signal molecules, and scaffolds are the main elements of such techniques. In this review, we describe these three elements with respect to their validation for regeneration of the periodontium and focus particularly on the potency of diverse scaffolds. In addition, we provide a short overview of the ongoing studies of 4-methacryloxyethyl trimellitate anhydride/methyl methacrylate-tri-n-butyl-borane resin including calcium chloride or hydroxyapatite for periodontium regeneration.

Key words: Biomaterials, 4-META-MMA-TBB, Periodontal ligament, Tooth preservation

Overview of the tooth and its surrounding structures

The tooth is composed of three hard tissues, namely the enamel, dentin, and cementum, while the periodontium is composed of four major tissues, namely

the periodontal ligament (PDL), bone, cementum, and gingiva (Fig. 1). The surface of the tooth root is covered with cementum, the constituents of which are similar to those of bone. The tooth root is located in the alveolar bone socket, and the PDL is a specialized connective tissue that plays a central role in connecting the tooth root to this surrounding bone. It also has additional nutritive and sensory functions. The PDL is a very thin tissue with a width range of 150-380 μm (Nanci and Somerman, 2006), and damage to this tissue results in increased tooth mobility and bone defects. Deep caries, severe periodontitis, and trauma trigger unrecoverable changes to the PDL, leading to tooth loss.

The PDL is a very complicated tissue because it includes heterogeneous cell populations comprising fibroblasts, which are the primary cells in the PDL (Berkovitz and Maden, 1995; Beertsen et al., 1997); PDL stem cells (PDLSCs); epithelial cell rests of Malassez, endothelial cells, and others. Seo et al. (2004) demonstrated the localization of PDLSCs in PDL tissue and their potential to differentiate into PDL fibroblasts, osteoblasts, and cementoblasts *in vivo*, suggesting their functional contribution to the generation, maintenance, and regeneration of the PDL (Seo et al., 2004).

Tooth loss and its consequences

Periodontitis, caries, fractures, and trauma are the primary causes of tooth loss. Tooth loss not only provokes oral malfunction, such as eating and speech difficulties and aesthetic problems, but also compromises systemic health and welfare (Holm-Pedersen et al., 2008; Hugo et al., 2009; Ansai et al., 2010), resulting in decreased quality of life. Recent studies suggested an association between tooth loss and carcinogenesis (Wang et al., 2013) or memory

impairment independent of the amyloid cascade (Oue et al., 2013). Thus, tooth loss is a serious concern, especially in elderly people (Chen et al., 2012). However, downward trends in edentulism among middle-aged and elderly populations have been noted worldwide (Wu et al., 2012).

A recent report demonstrated that life expectancy worldwide has shown a tendency to increase over the past two decades secondary to progress in medicine and public hygiene, while the number of years lost because of disability has also increased (Salomon et al., 2012). In this context, while it is important to prevent oral diseases, treatment of diseased teeth is also inevitable. The treatment modalities for periodontal defects have undergone multidirectional developments by many researchers, but few studies have addressed the treatment for deep caries with infrabony defects and root fracture. Because tooth extraction is applicable to most of these severe cases, subsequent implant placement is a current trend. However, a recent study reported that even implant-treated patients desire to save their natural teeth to the maximum extent possible (Gatten et al., 2011). Thus, we are challenged to develop new treatment modalities for such severely diseased teeth from the perspective of tissue engineering.

Three elements indispensable to periodontal regeneration

It is well known that stem cells, scaffolds, and signal molecules are requisites for tissue engineering (Langer and Vacanti, 1993). It has been argued that cells on and

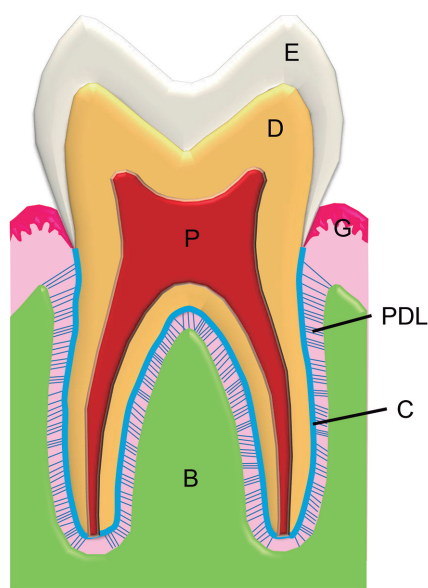


Fig. 1. Schema of tooth and its surrounding periodontium. E, enamel; D, dentin; P, pulp; G, gingiva; C, cementum; B, bone; PDL, periodontal ligament.

within scaffolds and matrices controlling the release of signal molecules over the long term are important in this field. In addition, angiogenesis and neurogenesis are indispensable for PDL regeneration. The precise alignment of these elements would provide ideal regeneration of the periodontium and largely contribute to prolonged tooth retention (Fig. 2).

Stem cells

The characteristics of stem cells include self-renewal and multi- or pluripotency. These cells hold great promise in the field of regenerative medicine with respect to their ability to fabricate the desired tissues. The candidate stem cells efficient in periodontal regeneration are embryonic stem cells; induced pluripotent stem cells (iPSCs); somatic stem cells such as bone marrow-derived mesenchymal stem cells (BMMSCs) and adipose-derived stem cells; and dental-derived mesenchymal stem cells such as PDL stem cells (PDLSCs), dental pulp stem cells, stem cells from exfoliated deciduous teeth, stem cells from the apical papilla, and dental follicle progenitor cells (DFPCs) (Seo et al., 2004; Lin et al., 2008; Huang et al., 2009; Maeda et al., 2013a; Tobita and Mizuno, 2013). The multipotency of these various somatic stem cells is almost identical *in vitro*, but their ectopic tissue formation differs *in vivo*, indicating that their differentiation lineages are predetermined to some extent depending on their origin (Huang et al., 2009). PDL fibroblasts, osteoblasts, and cementoblasts are critical cells in the periodontium, and PDLSCs are required to construct and reconstruct the periodontal structure by differentiating into these cell types. Therefore, PDLSCs, DFPCs, or more immature stem cells might be leading candidates for the regeneration of lost periodontium.

Cranial neural crest cells fabricate many tissues and organs in craniofacial regions, including teeth, bones, muscles, and neurons (Couly and Le Douarin, 1990).

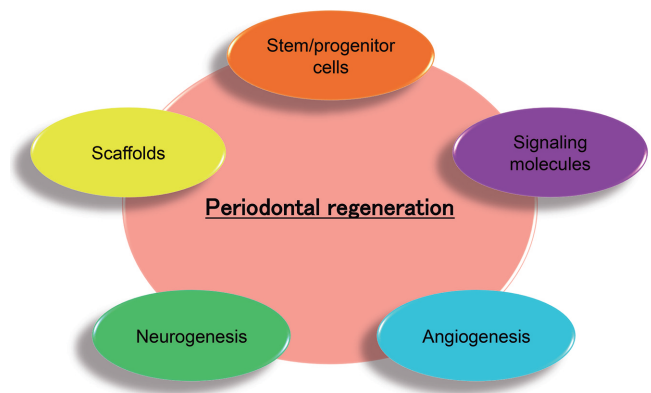


Fig. 2. Elements indispensable for periodontal regeneration. Combined application among five elements will create the ideal therapeutic methods.

During tooth germ development, PDL tissue grows from the dental follicle that originates from cranial neural crest-derived ectomesenchymal cells (Chai et al., 2000). Therefore, the cells in this lineage are promising candidates for periodontium reconstruction. However, it is difficult to secure an adequate number of such promising cells for clinical and research use because the number of STRO-1/CD146-double-positive PDLSCs in human PDL tissue is reportedly only 0.07% (Hidaka et al., 2012). Thus, acquisition of these cells with ease and with minimal invasion remains problematic.

Resolution of these issues will require efficient expansion of PDLSCs with maintenance of stemness and the induction of differentiation of stem cells of other lineages into the periodontal lineage. By definition, beneficial scaffolds and signaling molecules are required to achieve this. Some research groups have developed sheets or pellets of PDL cells that can be applied to periodontal defect areas (Guo et al., 2014; Iwata et al., 2014). This technique does not require the use of a specific matrix, but instead uses the extracellular matrices produced by the cell itself, thus maintaining the structural relationship between the cells. Pandula et al. (2014) developed a three-dimensional cell sheet, including PDLSCs and human umbilical vein endothelial cells for vascular support (Pandula et al., 2014).

We have developed human clonal PDLSC-like cell lines by gene transfer to analyze such PDLSCs (Fujii et al., 2006, 2008; Tomokiyo et al., 2008). The features of these cell lines match those of PDLSCs with respect to their surface markers, multipotency, and PDLSC-like differentiation after transplantation into rat PDL tissue (Tomokiyo et al., 2012; Maeda et al., 2013a).

Signaling molecules

Signal molecule multifunctionality is needed to promote osteo/cementogenic and fibroblastic differentiation, and furthermore, angiogenesis is needed for wound healing and regeneration of PDL tissue. Accumulating studies have demonstrated that various signaling molecules are involved in cell growth,

differentiation, adhesion, migration, and immunoregulation of PDLSCs and PDL cells during periodontal generation, regeneration, and healing. In the past decade or so, the inherent properties of many factors have been discovered (Table 1), including basic fibroblast growth factor (bFGF or FGF2) (Murakami, 2011), transforming growth factor- β (TGF- β) (Wikesjo et al., 1998; Teare et al., 2008; Maeda et al., 2013b), enamel matrix derivative (EMD) (Zetterstrom et al., 1997; Grandin et al., 2012), bone morphogenetic proteins (BMPs) (King et al., 1997; Sorensen et al., 2004; Yang et al., 2010; Chiu et al., 2013; Hakki et al., 2013), brain-derived neurotrophic factor (BDNF) (Takeda et al., 2005), connective tissue growth factor (CTGF/CCN2) (Asano et al., 2005), glial cell-line derived neurotrophic factor (GDNF) (Yamamoto et al., 2012), epidermal growth factor (EGF) (Nishimura and Terranova, 1996; Teramatsu et al., 2014), platelet-derived growth factor (PDGF) (Lynch et al., 1989; Thakare et al., 2013), insulin-like growth factor-1 (IGF-1) (Chen et al., 2006; Yang et al., 2010), growth/differentiation factor-5 (GDF-5/BMP-14) (Kwon et al., 2010), and BMP-2 + nerve growth factor (NGF) (Yan et al., 2010). In particular, bFGF, EMD, and PDGF + beta-tricalcium phosphate (β -TCP) have been demonstrated to be dominant and efficient agents in clinical studies of periodontal disease (Kitamura et al., 2011; Thakare et al., 2013).

In our recent study, we revealed that the combined application of bFGF and TGF- β 1 to human PDL stem/progenitor cell lines accelerated fibroblastic differentiation of these cells (Kono et al., 2013). Thus, developments of various application methods of the above-mentioned molecules will broaden therapeutic options.

Table 1. Candidate factors efficient for periodontal regeneration.

FGF-2	Takayama et al., 2001
BDNF	Takeda et al., 2005
BMP-2 + NGF	Yan et al., 2010
BMP-6	Chiu et al., 2013
CTGF/CCN2	Dangaria et al., 2009
EGF	Teramatsu et al., 2014
GDF-5 /BMP-14	Kim et al., 2002, 2009a,b
GDF-7/BMP-12	Wikesjöet al., 1998
GDNF	Yamamoto et al., 2012
HGF	Sakuraba et al., 2012
IGF-1	Chen et al., 2006
OP-1/BMP-7	Giannobile et al., 1998
PDGF + IGF-1	Lynch et al., 1989
TGF- β	Teare et al., 2013

Table 2. Scaffolds efficient for periodontal regeneration.

1. Synthetic materials	2. Natural materials
1) Degradable	Alginate
PEG	Agarose
PLA	Chitosan
PLLA	Collagen
PLGA	Gelatin
PLDLA	Fibrin
PLC	Hyaluronicacid
PVA	
PHEMA	3. Ceramic and titanium
PMMA + PHEMA	HA
	β -TCP
2) Nondegradable	Titanium
PMMA	
PTFE	4. Composite materials
4-META/MMA	HA/Col
	HA/Col/CS
	HA/ β -TCP
	HA/Col/PLGA
	HA/Chitosan
	HA/Chitosan/genipin
	β -TCP/Chitosan
	η β -TCP/Col
	β -TCP/gelatinhydrogel

Scaffolds

Two types of scaffolds are used for tissue regeneration: synthetic and natural materials. These scaffolds are used as substrates or encapsulating substances. These materials include degradable or non-degradable properties (Table 2).

Synthetic materials

Degradable materials

Representative degradable materials available for periodontal regeneration include polymers such as polyethylene glycol, poly-lactic acid (PLA), poly-L-lactic acid (PLLA), poly-glycolic acid (PGA), poly(lactic-co-glycolic acid) (PLGA), poly(L-lactide-co-D,L-lactide) (PLDLA), polycaprolactone (PLC), polyvinyl alcohol (PVA), poly(2-hydroxyethyl methacrylate) (PHEMA), and polymethylmethacrylate (PMMA) and PHEMA composites. Such polymers form a porous structure that is ideal for cell engraftment and survival.

Polyethylene glycol used in combination with fibrin and dental stem cells from the human PDL and pulp has been shown to allow for the growth and differentiation of these cells as well as three-dimensional fabrication *in vivo* implantation (Galler et al., 2011). Porous PLLA membrane permitted the growth and osteogenic differentiation of the periosteum *in vitro* (Kawase et al., 2011). When loaded with tetracycline, PLLA membrane enhanced new bone formation in rat calvarial bone defects (Park et al., 2000). PLA/PLGA membranes with PGA were effective as space makers in periodontal-guided tissue regeneration (Kim et al., 2009a). However, the single use of PLA for rat calvarial bone defects had detrimental effects such as bone resorption and foreign body reaction (Polimeni et al., 2008). PLGA is a copolymer of PLA and PGA and possesses the ability to encapsulate osteogenic factors for mineralization (Jayasuriya and Shah, 2008; Shi et al., 2010; Park et al., 2012b). Biodegradation of PLGA microparticles can be modified to occur from a few weeks to several months by varying the ratio of PGA to PLA (Dreifke et al., 2013). PLGA containing the same proportion of PLA and PGA was reportedly effective in the regeneration of infrabony bone defects of patients with periodontitis (Chhabra et al., 2011). A recent study demonstrated the efficacy of electrospun PLC fibers coated with calcium phosphate as cell carriers for the treatment of rat periodontal defects (Dan et al., 2014). Another group reported the regeneration of a PDL-bone structure using a novel three-dimensionally fabricated PLC scaffold under biomechanical loading conditions (Park et al., 2012a). PVA has been used in tissue engineering because of its biocompatibility, biodegradability, and nontoxicity. PVA and HEMA have both exhibited effectiveness in drug delivery systems (Wang et al., 2007; Li et al., 2013) and bone substitutes (Linh et al., 2013; Bolgen et al.,

2014), suggesting their potential in use in periodontal disease (Kong et al., 2006). Synthetic material comprising PMMA, PHEMA, and calcium hydroxide is a US Food and Drug Administration-approved bioabsorbable bone substitute used as a bone void-filler. Building on this, Hasturk et al. (2011) developed a composite material comprising PMMA, PHEMA, calcium hydroxide, and polyanhydride and demonstrated its potential as bone-replacement graft material (Hasturk et al., 2011).

Thus, recent polymeric materials have made remarkable improvements in their potency in PDL tissue engineering. Such improvements include the promotion of bone regeneration and repair of periodontal bone defects through the release of osteogenic factors, as well as securing a space for regenerating bone, the cell carrier, and degradation at the appropriate time points. In addition, because the combination of certain polymers or the addition of certain crosslinkers or compounds to these polymers can result in the creation of degradable and nondegradable forms, they are capable of being modified in accordance with the patient's requirements.

Nondegradable materials

PMMA, polytetrafluoroethylene (PTFE), and 4-methacryloxyethyl trimellitate anhydride/methyl methacrylate (4-META/MMA) are useful scaffolds in tissue engineering.

PMMA is widely used as bone cement for fixation of joint replacements in the bone during orthopedic surgery (Pneumatics et al., 2013). Badr (2010) demonstrated the efficacy of PMMA as bone cement in endodontic surgery (Badr, 2010). Expanded PTFE, a nonresorbable material, has been used as a barrier membrane in periodontal surgery. This material works for guided tissue regeneration by blocking the penetration of epithelial tissue into areas of periodontal healing that interrupts the regeneration of PDL tissue and hard tissue (Cortellini et al., 1990).

We have reported the biocompatibility of 4-META/MMA-TBB (4MMT) resin in bone implantation and for PDL cell growth (Maeda et al., 1999, 2011). In addition, when used for endodontic surgery, 4MMT resin led to good outcomes of bone healing involving periradicular tissue (Otani et al., 2011).

Natural materials

Scaffolds derived from natural materials such as alginate, agarose, chitosan, collagen, atelocollagen, gelatin, fibrin, and hyaluronic acid have been investigated for application to bone and periodontal regeneration.

Alginate, a natural polysaccharide extracted from seaweed, has been applied in biomedical science and tissue engineering because of its favorable properties, including biocompatibility, drug delivery, and ease of gelation (Lee and Mooney, 2011). BMMSCs, PDLSCs,

Regenerative of the periodontium

and gingival mesenchymal stem cells combined with alginate hydrogels formed extensive mineralized tissue after subcutaneous injection (Moshaverinia et al., 2014).

Agarose, a saccharide polymer originating from sea algae, can be dissolved in water to form a gel with a rigid net, resulting in the creation of a three-dimensional plastic, porous reticulum. Agarose/apatite composite gels have been shown to induce new attachments by the apposition of new cementum and well-oriented fibers in infrabony periodontal defects (Tabata et al., 2003).

Chitosan, an aminopolysaccharide derived from chitin, is a nontoxic, antibacterial, biodegradable, and biocompatible biopolymer (Panos et al., 2008). Because of these properties, it has been widely used for biomedical applications such as the creation of membranes, gels, nanofibers, beads, nanoparticles, and sponge forms. Chitosan also reportedly possesses the ability to promote osteogenic progenitor cell recruitment and attachment, facilitating bone regeneration (Kim et al., 2002). The use of chitosan membranes in periodontal surgical treatment was shown to induce new bone and cementum formation (Yeo et al., 2005).

Collagen is the most widely distributed class of proteins in vertebrates, constituting more than one-third by body weight of body protein tissue. Because of its abundance, ubiquity, versatility, biodegradability, and biocompatibility, collagen scaffolds are very common within the field of tissue engineering. Collagen barrier membranes for regeneration of periodontal defects have been widely used because they induce significantly high alkaline phosphatase (ALP) activity in PDL cells (Takata et al., 2001). Collagen sponges containing PDL cells revealed high immunoreactivity for bone- and cementum-related protein markers, including ALP, osteopontin (OPN), parathyroid hormone 1 receptor, osteocalcin (OCN), RUNX2, and CEMP1 (Wolf et al., 2013). In addition, collagen sponges combined with BMSCs (Li et al., 2009), PDL cells, and cementum-derived cells (Nunez et al., 2011) induced periodontal regeneration with newly formed alveolar bone and cementum with Sharpey's fibers in infrabony periodontal defects. While collagen has many advantageous features, the major antigenic determinant is located in the telopeptides of the collagen molecule. Because atelocollagen is produced by pepsin, which removes these telopeptides, it is much less antigenic than normal collagen. Atelocollagen membranes for guided tissue regeneration successfully blocked epithelial invasion, induced the formation of perpendicular fiber bundles on the root surface (Kodama et al., 1989), and promoted high ALP activity in PDL cells (Takata et al., 2001). Application of atelocollagen scaffolds with BMSCs (Kawaguchi et al., 2004) and dedifferentiated fat cells (Sugawara and Sato, 2014) to experimental models of periodontal tissue loss resulted in significant amounts of new bone, cementum, and PDL tissue formation.

Gelatin, a substance obtained by partial hydrolysis of collagen, is present in skin, white connective tissue, and bones. Because it is a mechanically robust protein but is

degraded relatively quickly, it has been widely used in clinical treatments (Akhyari et al., 2002). In one study, periodontal defects treated with a gelatin carrier were filled with fibrous tissue containing newly formed mineralized tissue (Han et al., 2014). The animals into which the gelatin scaffold was transplanted with BMSCs (Yu et al., 2013) and PDL cells (Akhyari et al., 2002) showed significantly greater regeneration of bone, cementum, and PDL tissue than did the group transplanted with the gelatin scaffold alone. Gelatin hydrogel is reportedly a promising material used as a carrier for growth factors or for drug delivery in various forms such as sponges, sheets, and liquids. However, this carrier is mechanically too weak to maintain its structure when implanted into hard tissue (Tabata, 2003).

Fibrin, a critical blood component, is responsible for hemostasis because the fibrin network is the first scaffold that cells encounter during the wound healing process. The application of platelet-rich fibrin to periodontal infrabony defects resulted in reduction of periodontal pockets and enhancement of clinical restoration (Naik et al., 2013). In addition, implantation of fibrin scaffolds into periodontal defects with iPSC-derived mesenchymal stem cells led to the greater formation of new bone, cementum, and PDL tissue than did fibrin scaffolds alone (Hynes et al., 2013).

Hyaluronic acid is a natural nonsulfated glycosaminoglycan with high molecular weight. It is a critical component of the extracellular matrix and possesses many structural and physiological functions. Hyaluronic acid has been identified in PDL tissue, gingiva, alveolar bone, and cementum in varying quantities. It has also shown anti-inflammatory, anti-edematous, and antibacterial effects in the treatment of periodontal disease (Pirnazar et al., 1999). The application of hyaluronic acid to periodontal wound sites could have significantly beneficial effects on clinical outcomes (Vanden Bogaerde, 2009; Jimbo et al., 2014).

Ceramics and titanium

Ceramics and titanium, which are rigid and brittle, have also been used in the fabrication of scaffolds for tissue engineering.

Hydroxyapatite (HA) and β -TCP, classified as calcium phosphate ceramics, have been popular implant materials in the fields of dentistry, orthopedics, and plastic surgery because of their osteoconductive properties. HA is the main mineral phase of mammalian tooth enamel and bone and is one of the most widely used calcium phosphate graft biomaterials. HA has been shown to promote PDL cell migration and adhesion by activation of ERK1/2 and Akt (Kasaj et al., 2011), as well as activation of BMP-2 expression via activation of p38 MAPK (Suto et al., 2013). In infrabony defects, new PDL formation was induced between the HA scaffold and the denuded root surface with no infiltration of inflammatory cells (Lee et al., 2012). In addition, HA

has the potential to act as a carrier of therapeutic agents, enabling controlled drug release either extracellularly or intracellularly; treatment with a combination of HA and platelet-rich plasma induced clinical improvement, including periodontal pocket reduction, clinical attachment gain, and defect filling in intraosseous periodontal defects (Menezes and Rao, 2012).

β -TCP is a synthetic ceramic material that demonstrates clinical efficiency because it is a completely resorbable graft material, which is a useful property in conjunction with bone formation (Liu et al., 2008). An *in vivo* study revealed that in a bone defect rabbit model, 85.0% of β -TCP but only 5.4% of HA had been resorbed 3 months after implantation (Eggli et al., 1988). PDL cells cultured in β -TCP extract showed increased ALP activity and *OPN*, *DMP-1*, and *OCN*

mRNA expression (Xia et al., 2011). Barney et al. (1986) reported perpendicular PDL tissue regeneration between new bone and cementum after treatment of infrabony periodontal defects involving implantation of β -TCP (Barney et al., 1986). Moreover, β -TCP in combination with GDF-5 resulted in significantly greater stimulation of the formation of new bone and cementum, along with functionally oriented PDL tissues in infrabony periodontal defects than did β -TCP alone (Kim et al., 2009b).

Titanium has been widely used as a suitable material for dental implants because of its corrosion resistance and elasticity, similar to that of bone. Impact forces are greater with dental implants than with natural teeth because implants lack the cushioning provided by the PDL. Therefore, efforts have been made to form PDL

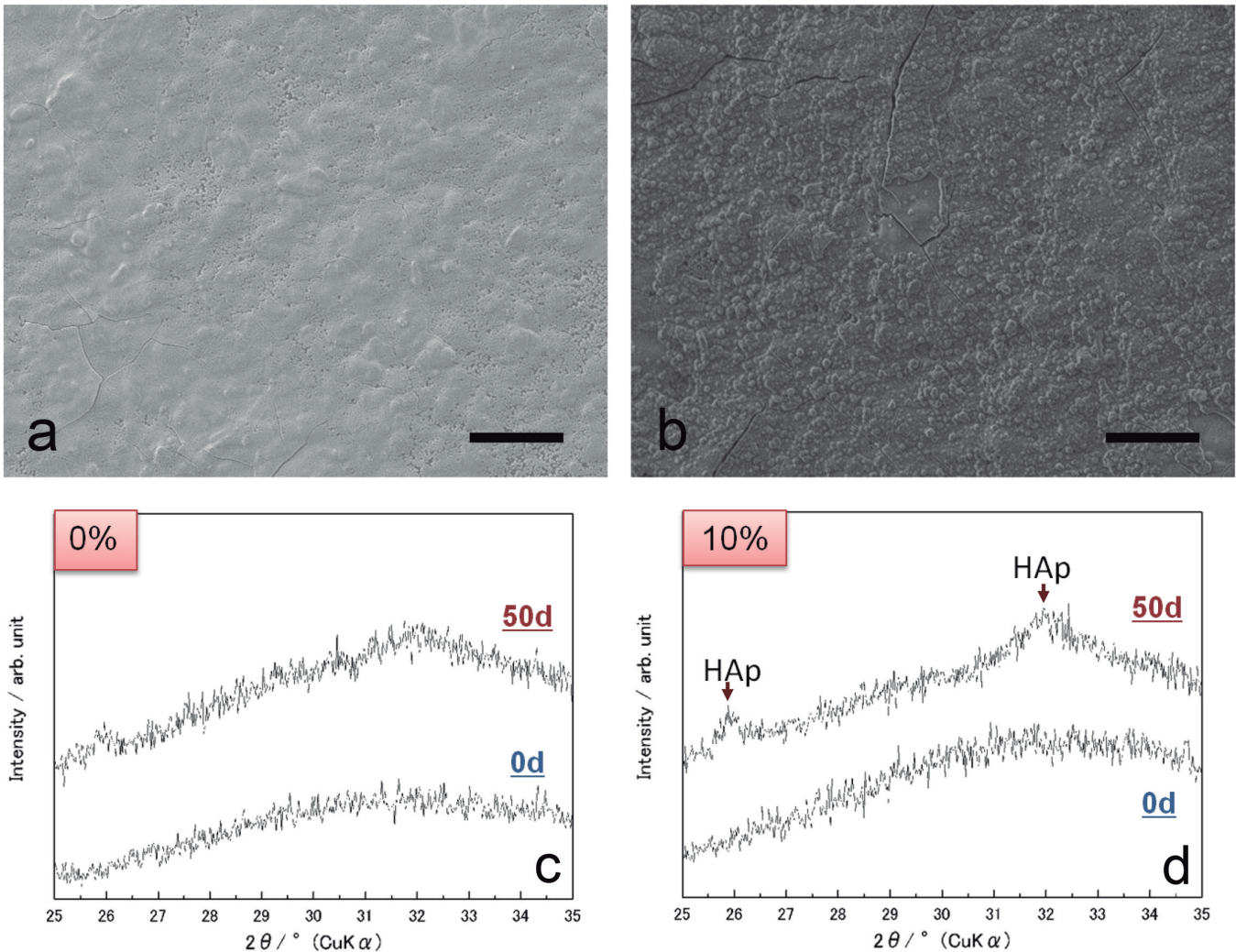


Fig. 3. Induced HA deposition on the surface of Ca/4MMT. 4MMT including 0% or 10% calcium chloride [0%Ca/4MMT (a, c) or 10%Ca/4MMT (b, d), respectively] was immersed in α -MEM for 50 days. Its surface was then investigated with a scanning electron microscope (S-3400N; Hitachi High-Technologies Co., Tokyo, Japan) (a, b) and X-ray diffraction analysis (D8 Advance; Bruker AXS, Inc., Madison, WI, USA) (c, d). The substance that formed on the 10%Ca/4MMT was determined to be HA. Bar: 100 μ m.

tissue around implant surfaces using titanium as a scaffold. PDL cells were attached to the surface of various titanium implants, mainly using integrin subunit $\beta 1$ (Kramer et al., 2009), and showed high proliferative activity (Docheva et al., 2010). In addition, PDL progenitor cell-seeded implants achieved the formation of cementum-like tissue on the surface and PDL tissue with Sharpey's fibers inserted perpendicularly to the implant (Lin et al., 2011).

Composites of bioactive ceramics and polymers

Composites of bioactive ceramics and polymers, termed biomimetic scaffolds, are currently being developed by taking advantage of each of their characteristics to increase the mechanical stability of the scaffold and improve tissue interaction and degradation (Wang, 2003). The fabrication of composites of bioactive ceramics and polymers has generally been accomplished by simple techniques in which bioactive ceramics are immersed in polymer solution or, alternatively, in which polymers are mineralized in saturated bioactive ceramic matrix solutions or simulated body fluids, followed by drying or freeze drying. However, more secure techniques have since been introduced, such as electrospinning and thermally induced phase separation (Takahashi et al., 2005; Danilchenko et al., 2011; Holzwarth and Ma, 2011). A number of studies using composites of the aforementioned materials have reported favorable results of periodontal tissue regeneration.

The combination of HA and collagen (HA/Col) has frequently been used in bone tissue engineering because its structure is similar to that of bone tissue. HA/Col has also been studied with respect to its potential as a

scaffold to regenerate periodontal tissue. Initial *in vivo* studies showed that HA/Col implantation into artificial infrabony defects in dogs resulted in new cementum formation and reinforced interdigitation between the root surface and gingival connective tissue, whereas bone formation was not observed (Minabe et al., 1988; Sugaya et al., 1989).

However, other HA/Col scaffolds with the addition of various components have reportedly shown better outcomes in terms of osteoconductivity. In one clinical study, periodontal surgery with the use of HA/Col containing chondroitin sulfate showed statistically significant improvement of periodontal healing, including bone formation (Scabbia and Trombelli, 2004). Nanosized HA/Col containing a small amount of PLA (nHA/Col/PLA) also showed high osteoinductivity by canine PDLSCs both *in vitro* and *in vivo*. The proliferation rate of cells on the nHA/Col/PLA was significantly higher than that on HA/ β -TCP, and subcutaneous transplantation of dog PDLSCs with nHA/Col/PLA showed greater bone formation than in the HA/ β -TCP group (He et al., 2010). Furthermore, HA/Col with PLGA, which was developed to obtain greater pH stability, is also expected to be a useful scaffold for bone tissue engineering because of its greater strength, stability, and tissue affinity than those of PLGA alone (Takechi et al., 2012).

Studies using human PDLSCs or PDL cells to examine the effects of HA/chitosan scaffolds on osteogenic cell differentiation have been reported (Inanc et al., 2007; Zhang et al., 2007; Ge et al., 2012). PDL cells encapsulated in HA/chitosan microspheres were observed to increase their expression of bone sialoprotein, OCN, OPN, and osteonectin, demonstrating differentiation into an osteoblastic cell lineage (Inanc et

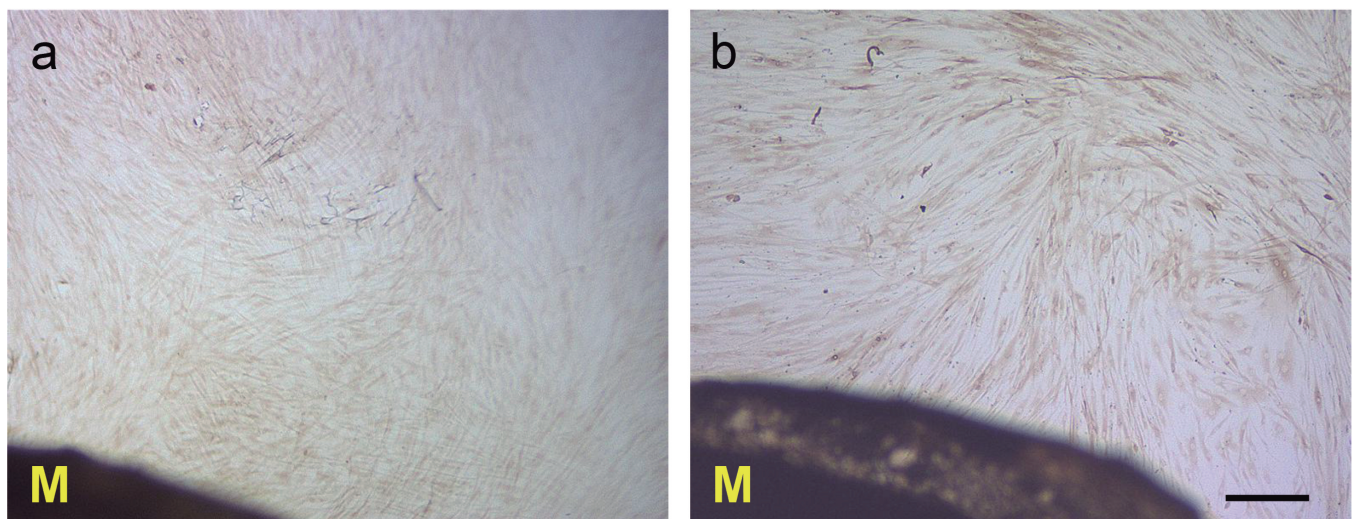


Fig. 4. Expression of BMP-2 in human PDL cells cultured with 0%Ca/4MMT (a) and 10%Ca/4MMT (b). After culturing for 14 days, BMP-2 expression was immunocytochemically examined using the ABC-DAB method. M, cultured material (0%Ca/4MMT or 10%Ca/4MMT). Bar: 200 μ m.

al., 2007). Another report indicated that PDLSCs seeded onto HA/chitosan containing genipin, a cross-linker (HA/chitosan/genipin), induced viability, ALP activity, and bone-related marker expression (Ge et al., 2012). Furthermore, in a rat calvaria bone defect model, PDLSC-seeded HA/chitosan/genipin promoted the formation of new bone tissue with abundant osteoblast and blood infiltration after implantation into bone defects (Ge et al., 2012). bFGF-loaded HA/chitosan reportedly maintained the release of bFGF for up to 7 days, and this property enhanced the proliferation rate, cell attachment to scaffolds, ALP activity, and osteogenic cell differentiation of human PDL cells and

cementoblast cell lines (Akman et al., 2010).

When complexes of β -TCP/chitosan and PDL cells were subcutaneously implanted into athymic mice, intense expression of ALP and OPN was detected around the scaffold. A recent study revealed that a scaffold comprised of nanosized β -TCP and collagen also showed biocompatibility and osteoconductivity both *in vitro* and *in vivo* (Ibara et al., 2013).

Gelatin hydrogel combined with β -TCP (β -TCP/gelatin) was fabricated to compensate for this weakness (Takahashi et al., 2005) and showed a good ability to deliver growth factors and drugs (Tsuzuki et al., 2012). Animal studies have demonstrated that the

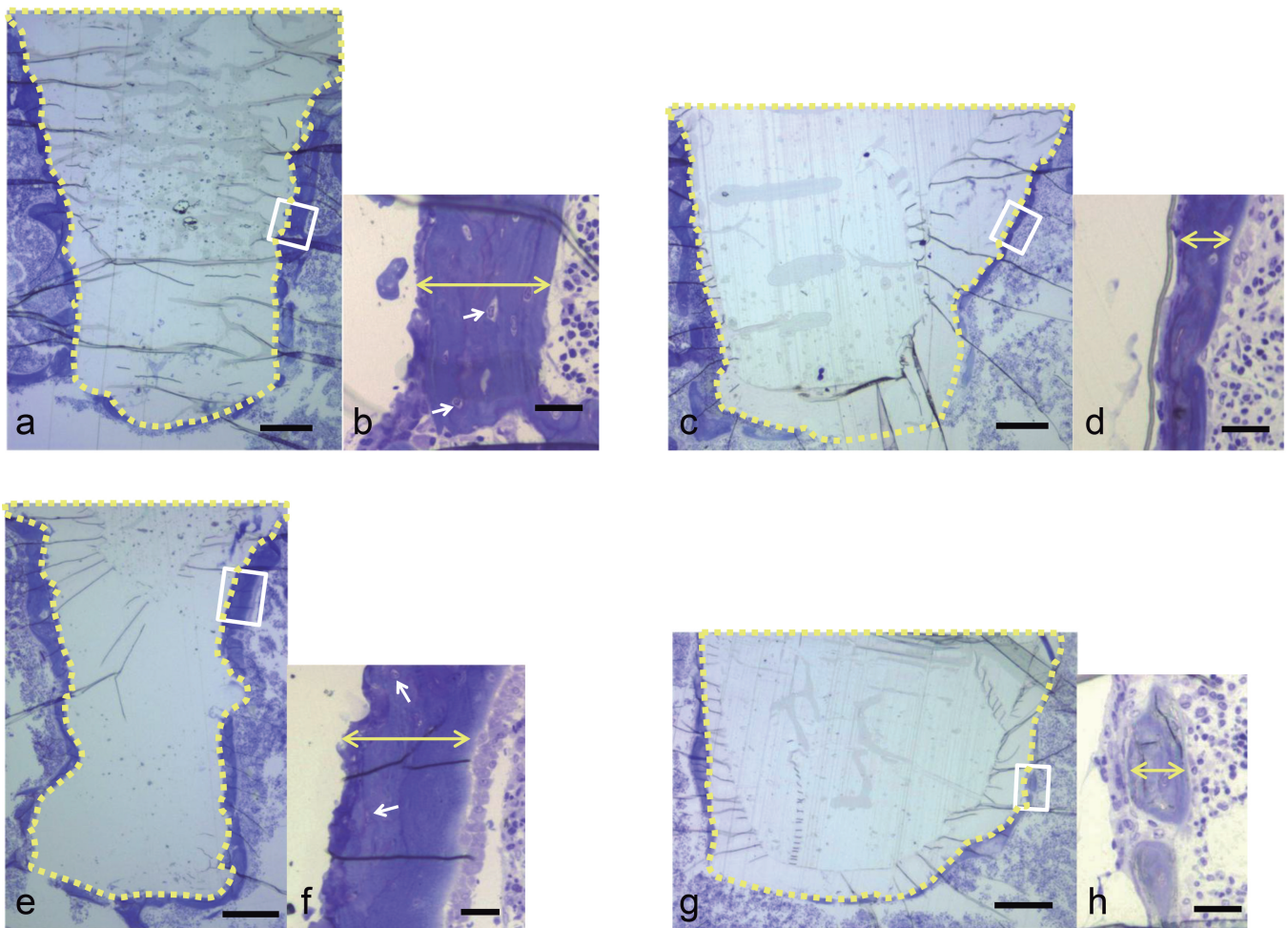


Fig. 5. Osteoconductivity of Ca/4MMT and HA/4MMT transplanted in a rat tibia. Both materials were transplanted into a bone cavity (1.5 mm in diameter) prepared in six male 6-week-old Sprague-Dawley rats. Each experimental material (1.3 mm in diameter and 1 mm in length) was transplanted into the left proximal epiphysis of the tibia, while 4MMT was placed into the right side in the same rat as a control. Three rats were used for each material. Four weeks later, the animals were sacrificed and each specimen was decalcified and embedded into Epon 812. One-micrometer sections were prepared, stained with toluidine blue, and evaluated under a light microscope. Specimens of Ca/4MMT (**a, b**), HA/4MMT (**e, f**), and 4MMT (**c, d, g, h**) were prepared from the same rats and sectioned horizontally. The area surrounded by the yellow dotted line indicates the implanted material. Higher magnification of the areas (**b, d, f, h**) within the white rectangles in **a, c, e, and g** is shown. The two-headed arrow indicates the width of newly formed hard tissue around the material. Representative data are shown. Arrows indicate osteocyte-like cells. Bars: a, c, e, g, 250 μ m; b, d, f, h, 30 μ m.

implantation of β -TCP/gelatin incorporating BMP-2 or bFGF into bone defects promoted significantly higher bone regeneration at the defect site than did growth factor-free composite scaffolds (Tsuzuki et al., 2012).

Angiogenesis

Angiogenesis plays crucial roles in physiological processes that range from fetal development and growth to tissue repair and regeneration. Therapeutic angiogenesis represents a broad range of interventions that generate new blood vessel growth to promote neovascularization and tissue regeneration (Risau, 1997). Vascular endothelial growth factors (VEGFs) are a family of secreted polypeptides and act as key regulators of physiological angiogenesis, skeletal growth, and reproduction (Leung et al., 1989). VEGF-A, the founding member of the family, regulates the progression phase of angiogenesis and induces proliferation, sprouting, migration, and tubular formation of endothelial cells (ECs) (Ferrara and Davis-Smyth, 1997). VEGF-A is released from ECs or neighboring cells in an autocrine and paracrine manner, and released VEGF-A induces proliferation of ECs and blood vessel formation. In PDL tissue, VEGF-A was identified in ECs, osteoblasts, fibroblasts (Miyagawa et al., 2009), and PDL stem cells (Yeasmin et al., 2014). Interestingly, *in vitro* co-culture of PDL cells with ECs promoted VEGF-A production by PDL cells and tubular formation of ECs (Yanagita et al., 2013). Moreover, *in vivo* co-transplantation of PDLSCs with ECs significantly increased the number of newly formed blood vessels that were larger and functionally more mature than those produced by EC transplantation alone (Yeasmin et al., 2014). Taken together, these findings suggest that PDL cell-derived VEGF-A directly regulates angiogenesis in PDL tissue, thereby indirectly contributing to regeneration of the periodontium.

Neurogenesis

The nervous system plays a central role in regulating pain, inflammation, and tissue repair processes through both efferent neuronal pathways and sensory functions. Innervation and reinnervation are important in the regeneration, maintenance, or repair of PDL tissue (Fujiyama et al., 2004), wounded ear tissue (Buckley et al., 2012), muscle (Kaariainen and Kauhanen, 2012), tendons, and bone (Ackermann, 2013). A recent study revealed the functional contribution of nerves and neuropeptides to PDL regeneration (Lv et al., 2014).

Bioactive features of 4-META/MMA-TBB resin

Tooth fracture is associated with PDL rupture. In such cases, infection develops and the microorganisms spread, resulting in the loss of both PDL tissue and bone. In general, such teeth are extracted and implant replacement is performed. However, recent studies have assessed the potential for preservation of teeth affected

by vertical root fracture (Sugaya et al., 2001; Unver et al., 2011). 4MMT resin, which has shown resistance to hydrolysis regardless of extensive thermocycling in a wet environment (Gendusa, 1992), was used as an adhesive agent to bond fractured parts of tooth and exhibited a good clinical prognosis. However, the generation of PDL tissue has not been demonstrated on the surface of this adhesive because cementum is not fabricated on its surface. Although PDL cells exhibit proliferation on 4MMT resin *in vitro*, it seems difficult to create a scaffold using this resin to induce cementum fabrication *in vivo*. Therefore, an adhesive with properties that overcome these problems is required.

We previously demonstrated new bone formation around 4MMT resin that was implanted into a surgically prepared rat alveolar bone cavity (Maeda et al., 1999). We also reported the biocompatibility of this resin against human PDL cells (Maeda et al., 2011). Moreover, elevated extracellular calcium enhanced the osteogenic differentiation of human PDL cells via calcification and upregulated BMP-2 expression (Maeda et al., 2010). These findings led us to examine the biological features of calcium-including 4MMT resin. 4MMT containing calcium chloride (Ca/4MMT) induced HA deposition on its surface after immersion in culture medium (Fig. 3). In addition, it promoted the expression of BMP-2 when cultured with human PDL cells and exhibited biocompatibility for the cells (Fig. 4). Finally, we implanted Ca/4MMT and 4MMT containing HA (HA/4MMT) into surgically prepared bone cavities of rat tibias. Both materials clearly induced newly formed hard tissue within 4 weeks in contrast to 4MMT alone (Fig. 5). We also found that these newly formed bone-like tissues in Ca/4MMT and HA/4MMT were thicker than those in 4MMT, in which osteocyte-like cells were embedded. These findings indicate that Ca/4MMT and HA/4MMT display both biocompatibility and osteoconductivity. The potential of these materials to induce PDL generation followed by cementum formation requires further clarification.

Conclusion

We must continually and aggressively explore the best therapeutic methods of regeneration and preservation of PDL tissue for patients. To address this complicated issue, we must analyze the tooth and periodontal tissues in more detail, identify the optimal combinations of the aforementioned elements, and develop suitable new biomaterials and experimental models. These efforts will certainly open the door to new and ideal treatments.

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