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Review

Tissue engineering in endodontics

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Abstract: Tissue engineering is the science of design and manufacture of new tissues to replace impaired or damaged ones. The key ingredients for tissue engineering are stem cells, the morphogens or growth factors that regulate their differentiation, and a scaffold of extracellular matrix that constitutes the microenvironment for their growth. Recently, there has been increasing interest in applying the concept of tissue engineering to endodontics. The aim of this study was to review the body of knowledge related to dental pulp stem cells, the most common growth factors, and the scaffolds used to control their differentiation, and a clinical technique for the management of immature non-vital teeth based on this novel concept. (J Oral Sci 51, 495-507, 2009)

Keywords: tissue engineering; dental pulp stem cells; morphogens; bone morphogenetic proteins; scaffolds; regenerative endodontics; pulp revascularization.

Introduction

Although current root canal treatment modalities offer high levels of success for many conditions, an ideal form of therapy might consist of regenerative approaches in which diseased or necrotic pulp tissues are removed and replaced with healthy pulp tissues to revitalize the teeth. The creation and delivery of new tissues to replace diseased, missing, or traumatized pulp is referred to as regenerative endodontics. This approach provides an innovative and novel range of biologically-based clinical treatments for endodontic disease.

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Tissue Engineering

Tissue engineering is an emerging multidisciplinary field that applies the principles of engineering and life sciences for the development of biological substitutes that can restore, maintain, or improve tissue function. The tissues of interest in regenerative endodontics include dentin, pulp, cementum and periodontal tissues (1). The key elements of tissue engineering are stem cells, morphogens or growth factors, and an extracellular matrix scaffold (2,3).

Key Elements for Tissue Engineering

Stem cells

Stem cells are considered to be the most valuable cells for regenerative medicine. Research on stem cells is providing advanced knowledge about how an organism develops from a single cell, and how healthy cells replace damaged ones in adult organisms. Stem cells have the ability to continuously divide to either replicate themselves (self-replication), or produce specialized cells that can differentiate into various other types of cells or tissues (multilineage differentiation) (4).

Types of stem cells

Early embryonic stem cells

The first step in human development occurs when the newly fertilized egg or zygote begins to divide, producing a group of stem cells called an embryo. These early stem cells are totipotent, i.e. possess the ability to become any kind of cell in the body.

Blastocyst embryonic stem cells

Five days after fertilization, the embryo forms a hollow ball-like structure known as a blastocyst. Embryos at the blastocyst stage contain two types of cells: an outer layer

of trophoblasts that eventually form the placenta, and an inner cluster of cells known as the inner cell mass that becomes the embryo and then develops into a mature organism. The embryonic stem cells in the blastocyst are pluripotent, i.e. having the ability to become almost any kind of cell in the body.

Scientists can induce these cells to replicate themselves in an undifferentiated state for very long periods before stimulating them with appropriate signaling molecules to create specialized cells. However, the sourcing of embryonic stem cells is controversial and associated with ethical and legal issues, thus reducing their appeal for the development of new therapies (5).

Fetal stem cells

After 8 weeks of development, the embryo is referred to as a fetus. By this time it has developed a human-like form. Stem cells in the fetus are responsible for the initial development of all tissues before birth. Like embryonic stem cells, fetal stem cells are pluripotent.

Umbilical cord stem cells

The umbilical cord is the lifeline that transports nutrients and oxygen-rich blood from the placenta to the fetus. Blood from the umbilical cord contains stem cells that are genetically identical to the newborn baby. Umbilical cord stem cells are multipotent, i.e. they can differentiate into a limited range of cell types. Umbilical cord stem cells can be stored cryogenically after birth for use in future medical therapy.

Adult stem cells

This name is rather misleading, because infants and children also have stem cells. Thus the term Postnatal Stem Cells is preferable. These stem cells reside in tissues that have already developed, directing their growth and maintenance throughout life. These cells are also multipotent.

Adult stem cells typically generate the cell types of the tissue in which they reside. However, some experiments over the last few years have raised the possibility of a phenomenon known as plasticity, in which stem cells from one tissue may be able to generate cell types of a completely different tissue (6).

Postnatal stem cells have been found in almost all body tissues (7), including dental tissues (8,9). To date, four types of human dental stem cells have been isolated and characterized: i) Dental pulp stem cells (DPSCs) (10), ii) Stem cells from human exfoliated deciduous teeth (SHED) (11), iii) Stem cells from apical papillae (SCAP) (12,13), and iv) Periodontal ligament stem cells (PDLSCs) (14). Among them, all except SHED are from permanent teeth.

The identification of these dental stem cells provides better understanding of the biology of the pulp and periodontal ligament tissues, and their regenerative potential after tissue damage (1).

Progenitor cells

Stem cells generate intermediate cell types before they achieve their fully differentiated state. The intermediate cell is known as a precursor or progenitor cell. It is believed that such cells usually differentiate along a particular cellular development pathway. Generally, undifferentiated cells are considered to be progenitor cells until their multi-tissue differentiation and self-renewal properties are demonstrated and they become designated as stem cells (15).

Dental pulp stem cells (DPSCs)

DPSCs were isolated for the first time in 2000 by Gronthos et al. based on their striking ability to regenerate a dentin-pulp-like complex composed of a mineralized matrix of tubules lined with odontoblasts, and fibrous tissue containing blood vessels in an arrangement similar to the dentin-pulp complex found in normal human teeth. Then, in a later study (16), the same group demonstrated that these cells had a high proliferative capacity, a self-renewal property and a multi-lineage differentiation potential.

Laino et al. (17) isolated a selected subpopulation of DPSCs known as Stromal Bone-producing Dental Pulp Stem Cells (SBP-DPSCs). These were described as multipotential cells that were able to give rise to a variety of cell types and tissues including osteoblasts, adipocytes, myoblasts, endotheliocytes, and melanocytes, as well as neural cell progenitors (neurons and glia), being of neural crest origin (17-21).

Several studies (10,16,22-30) of DPSCs have shown that they are multipotent stromal cells that proliferate extensively, can be safely cryopreserved, are applicable with several scaffolds, have a long lifespan, posses immunosuppressive properties (31), and are capable of forming mineralized tissues similar to dentin (32,33). Paakkonen et al. (34) demonstrated that DPSCs have a general gene expression pattern similar to that of mature native odontoblasts, and are therefore a valuable human-derived cell line for *in vitro* studies of odontoblasts. However, definitive proof of their ability to produce dentin has not yet been obtained.

Recently, Takeda et al. (35) characterized hDPSCs isolated from tooth germs at the crown-completed stage and found that these cells were highly proliferative and had

the potential to generate a dentin-like matrix *in vivo*. However, these characteristics were lost in long-term culture, with a change in their gene expression profile. Meanwhile, Abe et al. (36) have described apical pulp derived cells (APDCs) present in human teeth with immature apices, and suggested that they are an effective source of cells for regeneration of hard tissue.

SHED

SHED were isolated for the first time in 2003 by Miura et al. (11), who confirmed that they were able to differentiate into a variety of cell types to a greater extent than DPSCs, including neural cells, adipocytes, osteoblast-like and odontoblast-like cells. The main task of these cells seems to be the formation of mineralized tissue (18,37,38), which can be used to enhance orofacial bone regeneration (39).

The ethical constraints associated with the use of embryonic stem cells, together with the limitations of readily accessible sources of autologous postnatal stem cells with multipotentiality, have made SHED an attractive alternative for dental tissue engineering (11). The use of SHED for tissue engineering might be more advantageous than that of stem cells from adult human teeth; they were reported to have a higher proliferation rate than stem cells from permanent teeth (11), and can also be retrieved from a tissue that is disposable and readily accessible (40). Thus, they are ideally suited for young patients at the mixed dentition stage who have suffered pulp necrosis in immature permanent teeth as a consequence of trauma (41).

SCAP

A new unique population of mesenchymal stem cells (MSCs) residing in the apical papilla of permanent immature teeth, known as stem cells from the apical papilla (SCAP), were recently discovered by Sonoyama et al. (13), who reported that these cells express various mesenchymal stem cell markers. SCAP are capable of forming odontoblast-like cells, producing dentin *in vivo*, and are likely to be the cell source of primary odontoblasts for formation of root dentin.

The discovery of stem cells in the apical papilla may also explain a clinical phenomenon described in a number of recent clinical case reports showing that apexogenesis can occur in infected immature permanent teeth with periradicular periodontitis or abscess. It is likely that the SCAP residing in the apical papilla survive such pulp necrosis because of their proximity to the vasculature of the periapical tissues. Therefore, after endodontic disinfection, and under the influence of the surviving epithelial root sheath of Hertwig, these cells can generate primary odontoblasts that complete root formation (13).

Periodontal ligament stem cells (PDLSCs)

Using a methodology similar to that utilized for isolation of MSCs from deciduous and adult pulp, Seo et al. (42) described the presence of multipotent postnatal stem cells in the human PDL (PDLSCs). Under defined culture conditions, PDLSCs differentiated into cementoblast-like cells, adipocytes, and collagen-forming cells. When transplanted into immunocompromised rodents, PDLSCs showed the capacity to generate a cementum/PDL-like structure and contributed to periodontal tissue repair.

The presence of MSCs in the periodontal ligament is also supported by the findings of Trubiani et al. (43), who isolated and characterized a population of MSCs from the periodontal ligament which expressed a variety of stromal cell markers, and Shi et al. (44), who demonstrated the generation of cementum-like structures associated with PDL-like connective tissue after transplanting PDLSCs with hydroxyapatite/tricalcium phosphate particles into immunocompromised mice.

The clinical potential for the use of PDLSCs has been further enhanced by the demonstration that these cells can be isolated from cryopreserved periodontal ligaments while maintaining their stem cell characteristics, including the expression of MSC surface markers, single-colony-strain generation, multipotential differentiation and cementum/periodontal-ligament-like tissue regeneration, thus providing a ready source of MSCs (45).

Using a minipig model, autologous SCAP and PDLSCs were loaded onto hydroxyapatite/tricalcium phosphate and gelfoam scaffolds, and implanted into sockets in the lower jaw, where they formed a bioroot encircled with periodontal ligament tissue and in a natural relationship with the surrounding bone (46).

Recently, Trubiani et al. (47) suggested that PDLSCs had regenerative potential when seeded onto a three-dimensional biocompatible scaffold, thus encouraging their use in graft biomaterials for bone tissue engineering in regenerative dentistry, whereas Li et al. (48) have reported cementum and periodontal ligament-like tissue formation when PDLSCs are seeded on bioengineered dentin.

Culturing of stem cells

Cell culture is a term that refers to the growth and maintenance of cells in a controlled environment outside an organism. A successful stem cell culture is one that keeps the cells healthy, dividing, and unspecialized.

Dental pulp stem cells can be cultured by two methods; the first is the enzyme-digestion method (10,11,13,49) in which the pulp tissue is collected under sterile conditions, digested with appropriate enzymes, and then the resulting

cell suspensions are seeded in culture dishes containing a special medium supplemented with necessary additives and incubated. Finally, the resulting colonies are subcultured before confluence and the cells are stimulated to differentiate.

The second method for isolating dental pulp stem cells is the explant outgrowth method (50-53) in which the extruded pulp tissues are cut into 2-mm^3 cubes, anchored via microcarriers onto a suitable substrate, and directly incubated in culture dishes containing the essential medium with supplements. Ample time (up to 2 weeks) is needed to allow a sufficient number of cells to migrate out of the tissues.

Haung et al. (54) compared both methods and found that cells isolated by enzyme-digestion had a higher proliferation rate than those isolated by outgrowth.

Differentiation of stem cells

Generation of specialized cells from unspecialized stem cells is a process known as differentiation, and is triggered by signals inside and outside the cells. The internal signals are controlled by the genes of one cell, which are interspersed across long strands of DNA, and carry coded instructions for all the structures and functions of a cell. The external signals for cell differentiation include chemicals secreted by other cells, physical contact with neighboring cells, and certain molecules in the microenvironment.

Cultured dental pulp stem cells can be stimulated to differentiate to more than one cell type according to the contents of the culture medium. Osteo/dentinogenic medium (10) contains dexamethasone, glycerophosphate, ascorbate phosphate and 1,25 dihydroxy vitamin D in addition to the basic elements. Adipogenic medium (55) contains dexamethasone, insulin and isobutyl methyl-xanthine, whereas for neurogenic induction (11) cells are cultured in the presence of B27 supplement, basic fibroblast growth factor, and epidermal growth factor.

Cell lines

Culturing of stem cells is the first step in establishing a stem cell line, which is a propagating collection of genetically identical cells that can be used for research and therapy development. Once a stable stem cell line has been established, stem cells can be triggered to differentiate into specialized cell types.

Odontoblasts are postmitotic terminally differentiated cells, and thus cannot be induced to undergo further differentiation. The major proteins synthesized by fully differentiated odontoblasts are type I collagen, which forms the scaffold for mineral deposition and provides strength to the mineralized dentin, and two major noncollagenous proteins (NCPs) considered to have mineralization-regulatory capacities (56), namely dentin phosphophoryn (DPP; or DMP-2) and dentin sialoprotein (DSP) (57). DPP and DSP are encoded by a single gene, DSPP or DMP-3 (58-60), which specifically defines the phenotypic characteristics of dentin (61).

Another important non-collagenous protein is dentin matrix protein-1 (DMP-1), which is found primarily in dentin and bone and has been implicated in the regulation of mineralization (62-64), being considered to act as a growth factor to induce the differentiation of DPSCs (65,66).

In order to explore the pulp wound-healing mechanism and to develop a therapeutic strategy for pulp regeneration, development of an odontoblast cell line is very important. Up to now, however, odontogenic differentiation has not been well characterized due to two major limitations: The first is the paucity of differentiation markers, which is now being overcome by the characterization of odontoblast-specific markers (DMP-1, DMP-2, and DMP-3) that can indicate the presence of a true odontoblastic cell line (61,67,68). The second is the limited life span of the primary cells (69), which is being addressed by trials of several methodologies including cell cloning and immortalization (61,70-74).

Growth factors

Growth factors are extracellularly secreted signals governing morphogenesis /organogenesis during epithelial-mesenchymal interactions. They regulate the division or specialization of stem cells to the desirable cell type, and mediate key cellular events in tissue regeneration including cell proliferation, chemotaxis, differentiation, and matrix synthesis (75). Many growth factors are quite versatile, stimulating cellular division in numerous cell types, while others are more cell-specific.

Some growth factors are used to increase stem cell numbers, as is the case for platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) (76), insulin-like growth factor (IGF), colony-stimulating factor (CSF) and epidermal growth factor (EGF). Others modulate the humoral and cellular immune responses (interleukins 1-13) while others are important regulators of angiogenesis, such as vascular endothelial growth factor (VEGF) (77,78), or are important for wound healing and tissue regeneration/ engineering, such as transforming growth factor alpha and beta (75,79,80). One distinct family of growth factors implicated in tooth development (81,82) and regeneration (3) are the bone morphogenetic proteins (BMPs) known for their ability to induce the formation of bone and

cartilage.

Bone morphogenetic proteins (BMPs)

Bone morphogenetic proteins are multi-functional growth factors belonging to the transforming growth factor β superfamily (83). The first BMPs were originally identified by their ability to induce ectopic bone formation when implanted under the skin of rodents (84). To date, about 20 BMP family members have been identified and characterized. They have different profiles of expression, different affinities for receptors and therefore unique biological activities *in vivo* (85).

During the formation of teeth, BMPs dictate when initiation, morphogenesis, cytodifferentiation, and matrix secretion will occur. Without the BMP family of growth factors, the enamel knot would not be formed, and teeth would be unlikely to develop (86).

BMPs (87-90), as well as other growth factors (91), have been successfully used for direct pulp capping. This has encouraged the addition of growth factors to stem cells to accomplish tissue engineering replacement of diseased tooth tissues.

There are two strategies for the use of BMPs for dentin regeneration. The first is *in vivo* therapy, where BMPs or BMP genes are directly applied to the exposed or amputated pulp. The second is *ex vivo* therapy, which consists of isolation of DPSCs, their differentiation into odontoblasts with recombinant BMPs or BMP genes, and finally their autogenous transplantation to regenerate dentin (86).

The role played by BMP-2 is reportedly crucial as a biological tool for dentin regeneration (92). Recombinant human BMP-2 stimulates the differentiation of adult pulp stem cells into odontoblast-like cells in culture (53,93,94), increases their alkaline phosphatase activity and accelerates expression of the dentin sialophosphoprotein (DSPP) gene in vitro (53), and enhances hard tissue formation in vivo (95). Also, autogenous transplantation of BMP-2-treated pellet culture onto amputated pulp stimulates reparative dentin formation (96).

Similar effects have been demonstrated for BMP-7, also known as osteogenic protein-1, which promotes reparative dentinogenesis and pulp mineralization in several animal models (97-103). Recently, Lin et al. (104) generated a BMP-7-expressing adenoviral vector that induced the expression of BMP-7 in primarily cultured human dental pulp cells. This expression led to a significant increase of alkaline phosphatase activity and induced the expression of DSPP, suggesting that BMP-7 can promote the differentiation of human pulp cells into odontoblast-like cells and promote mineralization *in vitro*.

However, a novel role has been suggested for BMP-4, which is secreted by mesenchymal cells, in the regulation of Hertwig's epithelial root sheath (HERS) during root development by preventing elongation and maintaining cellular proliferation. Therefore it has been utilized as an agent for regulating root formation in a variety of tissue-engineering applications (105).

Scaffolds

A scaffold can be implanted alone or in combination with stem cells and growth factors to provide a physicochemical and biological three-dimensional microenvironment or tissue construct for cell growth and differentiation (66,106-108)

Ideal requirements of a scaffold (66,109-112)

(a) Should be porous to allow placement of cells and growth factors.
(b) Should allow effective transport of nutrients, oxygen, and waste.
(c) Should be biodegradable, leaving no toxic byproducts.
(d) Should be replaced by regenerative tissue while retaining the shape and form of the final tissue structure.
(e) Should be biocompatible.
(f) Should have adequate physical and mechanical strength.

Types of scaffold

a) Biological/natural scaffolds

These consist of natural polymers such as collagen and glycosaminoglycan, which offer good biocompatibility and bioactivity. Collagen is the major component of the extracellular matrix and provides great tensile strength to tissues. As a scaffold, collagen allows easy placement of cells and growth factors and allows replacement with natural tissues after undergoing degradation (113-115). However, it has been reported that pulp cells in collagen matrices undergo marked contraction, which might affect pulp tissue regeneration (54,116).

b) Artificial scaffolds

These are synthetic polymers with controlled physicochemical features such as degradation rate, microstructure, and mechanical strength (112), for example:
•Polylactic acid (PLA), polyglycolic acid (PGA), and their copolymers, poly lactic-co-glycolic acid (PLGA).
•Synthetic hydrogels include polyethylene glycol (PEG)-based polymers.
•Scaffolds modified with cell surface adhesion peptides, such as arginine, glycine, and aspartic acid (RGD) to improve cell adhesion and matrix synthesis within the three-dimensional network (117).
•Scaffolds containing inorganic compounds such as

hydroxyapatite (HA), tricalcium phosphate (TCP) and calcium polyphosphate (CPP), which are used to enhance bone conductivity (118), and have proved to be very effective for tissue engineering of DPSCs (119,120)
•Micro-cavity-filled scaffolds to enhance cell adhesion (121,122).

Scaffolds for tissue engineering

Cumulative reports (10,22,41,66,111,123-127) have shown that pulp cells can be isolated, multiplied in culture, and seeded onto a matrix scaffold where the cultured cells form a new tissue similar to that of the native pulp.

These findings have suggested the possibility of generating pulp and dentin in pulpless canals. However, when implanting cells/scaffolds into root canals that have a blood supply only from the apical end, enhanced vascularization is needed in order to support the vitality of the implanted cells in the scaffold. This can be optimized with the addition of growth factors such as VEGF and/or platelet-derived growth factor or, further, with the addition of endothelial cells (46). Through the use of computer-aided design and three-dimensional printing technologies, scaffolds can be fabricated into precise geometries with a wide range of bioactive surfaces. Such scaffolds have the potential to provide environments conducive to the growth of specific cell types.

Clinical applications of tissue engineering concepts

A number of recent clinical case reports (128-137) have suggested that many teeth that would traditionally have undergone apexification may be treated by apexogenesis. These reports challenge the traditional approach for managing immature teeth by apexification, where there is little or even no expectation of continued root development. Instead, it is possible that alternative biologically based treatments may promote apexogenesis/maturogenesis, a term that encompasses not just the completion of root-tip formation but also the dentin of the root (138).

Although Iwaya et al. (137) and Banchs and Trope (135) applied the term 'revascularization' to describe this phenomenon, what actually occurred was physiological tissue formation and regeneration. This may be attributed to SCAP surviving the infection and contributing to this phenomenon (12,13). It is also possible that the radiographic presentation of increased dentinal wall thickness might be due to ingrowth of cementum, bone, or a dentin-like material (38,139-145). This diversity in cellular response is not surprising, given that DPSCs can develop odontogenic/osteogenic, chondrogenic, or adipogenic phenotypes, depending on their exposure to different cocktails of growth factors and morphogens (146,147).

The key procedures of the new protocol suggested for treating non-vital immature permanent teeth are (1) minimal or no instrumentation of the canal while relying on gentle but thorough irrigation of the canal system with sodium hypochlorite and chlorohexidine, (2) augmented disinfection by intra-canal medication with a triple-antibiotic paste (containing equal proportions of ciprofloxacin, metronidazol, and minocycline in a paste form at a concentration of 20 mg/ml) between appointments (148,149), and (3) sealing of the treated tooth with mineral trioxide aggregate (MTA) and glass ionomer/resin cement upon completion of the treatment. Finally, periodical follow-ups are made to observe any continued maturation of the root.

Some investigators (129-132,135-137) have induced hemorrhage in the root canal system by over-instrumentation, allowing a blood clot to form in the canal. Then MTA was placed over the blood clot. They considered that the initiation of a blood clot would provide a fibrin scaffold containing platelet-derived growth factors that would promote the regeneration of tissue within the root canal system. The induction of bleeding to facilitate healing is a common surgical procedure. It had been proposed earlier by Ostby (139) and Myers and Fountain (149) to guide tissue repair in the canal. However, there is a lack of histological evidence that a blood clot is required for the formation of repaired tissues in the canal space. Moreover, there have been no systematic clinical studies to indicate that application of this approach gives significantly better results than procedures that lack it.

There is no current evidence-based guideline to help clinicians determine the types of cases that can be treated with this conservative approach. As mentioned above, the presence of radiolucency in the periradicular region can no longer be used as a determining factor, nor can the vitality test be used. In both situations, vital pulp tissue or an apical papilla may still be present in the canal and at the apex. Clinicians are urged to consider choosing a conservative approach first, while apexification can be performed in cases of failure (1).

Concluding Remarks

•Tissue regeneration in postnatal life recapitulates events that have occurred in the normal course of embryonic development and morphogenesis.
•Both embryonic development and tissue regeneration are equally regulated through the interaction of selected and highly conserved families of proteins and gene products.
•It is now accepted that the dental pulp harbours several niches of multipotential stem cells capable of self-renewal

and differentiation.

•Techniques to isolate and characterize human pulp stem cells and manipulate their growth under defined *in vitro* conditions have to be established and optimized before cell therapy.

•Current research is exploring the perfect formula for a reliable autogenous stem cell source, appropriate signaling molecule(s) and a scaffold that will promote controlled cell growth and differentiation.

•Tissue engineering using the triad of dental pulp progenitor/stem cells, morphogens, and scaffolds may provide an innovative and novel biologically-based approach for generation of clinical materials and/or treatments for dental disease.

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