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RESEARCH ARTICLE

TISSUE ENGINEERING IN PERIODONTICS

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ABSTRACT

Mankind is advancing beyond the ability to create inanimate objects, towards the capability of replacing and regenerating our own body tissues. The amalgamation of bioengineering and dentistry will result in an explosion of knowledge. Current approaches to tissue engineering can be divided broadly into two main types:ex vivo and in vivo .For a successful attempt for tissue engineering the following three components are of utmost importance ie Matrix or Scaffold, Appropriate cells, and Signaling molecules or soluble mediators.

INTRODUCTION

Tissue engineering in periodontics

Mankind is advancing beyond the ability to create inanimate objects, towards the capability of replacing and regenerating our own body tissues. The amalgamation of bioengineering and dentistry will result in an explosion of knowledge that will enhance our understanding craniofacial development and cullminate in a new era in dentistry, enabling us to restore lost tissue function. Tissue engineering is a contemporary area of applied biochemical research aimed at developing procedures and biomaterial for the fabrication of the new tissues to replace the damaged tissues and is based on principle of cell biology, developmental biology and biochemical science. Tissue engineering is also reffered to as “Regenerative Dentistry” because the goal of tissue engineering is to restore the tissue function through the delivery of stem cells, bioactive molecules or the synthetic tissue constructs engineered in laboratory. The application of regenerative dentistry in dental clinics can produce wonderful treatments to dramatically improve patient‟s quality of life.

The earliest clinical application of human cells in tissue engineering may be for the skin tissue using fibroblasts, keratinocytes, or a scaffold. It started around 1980. A little later, periodontal and alveolar bone tissues were attempted to regenerate with use of membranes that ensure the maintenance of the site for tissue regeneration by preventing fibroblasts from invasion there eg: guided tissue regeneration (GTR) and guided bone regeneration (GBR)).[1] In dental implantology guided bone regeneration membranes are used for bone augmentation of proposed implant-placement sites. The term "Tissue engineering" was coined at a meeting sponsored by the National Science Foundation (NSF) in 1987. Langer and Vacanti in 1993 defined tissue engineering as "an interdisciplinary field that applies the principles of engineering and the life sciences towards the development of biological substitutes that restore, maintain or improve tissue function."[2] Current approaches to tissue engineering can be devided broadly into two main types:*ex vivo* and *in vivo*. [3] In former, the target tissue is created in laboratory by culturing cells on biodegradable scaffolds in presence of specific trophic factors before their transplantation in to the body. In the latter approach the elements mentioned above are placed into a tissue defect “ in situ “ and the tissue is restored by maximizing the natural healing capacity of the body by creating a local environment that is favorable for regeneration.

The disadvantages of in vitro engineering the tissues are

i) Absence of a physiological, load bearing, natural environment during the formation of the tissues. It is well known that mechanical forces serve as a critical regulator of cell function and can profoundly alter tissue architechture,[4] and

ii) The incorporation of engineered tissue after being implanted into the host organ requires mechanical coupling with the surrounding structures. It requires remodeling, degradation and new tissue-formation at the interface of the host tissue and the implant surface. This incorporation is a crucial event in the de novo

regeneration of tissues *in vivo* following implantation.[5]

These disadvantages of *in vivo* strategy for tissue engineering lead to introduction of a suitable scaffold carrying appropriate cells and growth factors to facilitate tissue regeneration *in vivo*. However, one disadvantage of this approach is that the regenerating tissue may be dislodged or degraded by the mechanical forces normally acting at that site, before the regenerating tissue is fully formed and incorporated. The advantage of *in vivo* strategy is:i) The ability to examine the material as it is formed, ii) The ability to perform specific measurements prior to implantation

Five main categories of therapeutics in the development of Tissue engineering are

***Cell-based therapeutics*:-** Delivery of undifferentiated or partially differentiated progenitor cells. The regenerative potential of the stem-cell based therapy is the highest, but limited by the scarce availability of sources for cells. Bone auto grafts may be considered as cell-based therapeutics for periodontal regeneration.

***Conductive therapeutics*:-** Developing biocompatible scaffold. Regenerative potential is limited by the lack of biologically active factors and sufficient progenitor cells within the defect. Examples - dental implants, guided tissue regeneration, hydroxyapatite, tricalcium phosphate, and calcium sulfate fillers.

***Inductive approaches*:-** Biocompatible scaffold that guides the regeneration of the tissue by carrying one or more biologically active factors that recruit vascular events and progenitor stem cells from the immediate vicinity to the tissue defect. Regenerative potential is higher than the conductive biomaterial. Examples -allogenic bone grafts or biomaterials carrying recombinant proteins /EMD

***Gene-based therapeutic*:-** Biocompatible scaffold carrying single or multiple genes that transform the non-progenitor cells already present within the tissue defect into both progenitor and mature tissue-specific cells. It is able to signal the cells present in the defect to differentiate into a phenotype more favorable to the regenerative process.

***RNA-based therapeutics*:-** RNA-based therapeutics may be considered a fifth category of regeneration therapy even though this approach remains in the conceptual stage of development. RNA interference- Fire and Mellow in 2006.[6] For a successful attempt for tissue engineering the following three components are of utmost importance –Matrix or Scaffold, Appropriate cells, and Signaling molecules or soluble mediators.

Matrix or scaffold

Tissue engineering inherently involves recreation of a three dimensional structure from an endogenous source in the patient or from a donor. Matrices or biomaterials are used to guide the organization, growth and differentiation of the cells and the soluble mediators in the process of forming functional tissues and provide both physical and chemical uses. A *biomaterial* is a substance which is compatible with the physiology of the body; typically designed for use in tissue therapy and/or tissue engineering. Few terms which are used synonymously and interchangeably with biomaterial are - *bioabsorbable material*, which is a material whose breakdown products are incorporated into the normal physiologic and biochemical process; a *biocompatible material* can function in a biologic environment without known or significant detrimental effects on either the material or the living system; a *biodegradable material* breaks down when placed in a biologic environment; and a *bioabsorbable material* is a material that is broken down *in vivo* and removed from the implant site.[6]

Basic requirements of a matrix: The basic requirements to be fulfilled by a material to act as a scaffold for tissue engineering can be classified as Biomechanical requirements 1) space maintenance within the defect 2) barrier or exclusionary function Biological requirements 1) biocompatibility 2) incorporation of cells 3) incorporation of instructive messages

Biomechanical Requirements*:-*i) Space maintenance within a defect

The engineered material should be of sufficient form to allow the placement into a defect and prevent subsequent collapse of the repositioned tissue into the defect site. Thus, the material should act in a manner consistent with the principle of guide tissue regeneration and have similar design features.[7] .As a result a sufficient wound space and a suitable environment for regeneration will act synergistically to permit the cascade of molecular events needed to drive the regenerative process.

Design features to obtain satisfactory space maintenance would include –an ability to be easily cut or moulded into a desired shape and be of a consistency compatible with easy handling.[8]The scaffold should be of sufficient rigidity to withstand soft tissue collapse into the defect.[7]the scaffold should structurally reinforce the site so as to maintain the shape of the defect and prevent distortion of surrounding tissues.[6]

Barrier or exclusionary function

The engineered tissue should act as a barrier to the in growth of unwanted tissues, for example, gingival epithelium and connective tissue, y*et al*so permit selective in growth of tissues (cementum, periodontal ligament and bone). To achieve this, design features have to be incorporated whereby the external surface may be exclusionary yet the internal scaffold remains conducive to new tissue in growth. Perhaps, of interest here is the important sealing role the epithelium plays. Thus, a principle aim of tissue engineering for the periodontium should not be total exclusion of epithelium from the site but rather encouraging it to form a rapid and successful biologic seal to protect the fragile underlying regenerative events.[9]

Biological requirement

Biocompatibility

The scaffold material should be either biocompatible with the tissues to be regenerated or biodegradable, allowing for gradual replacement with regenerated tissues.[10] Also, the design features needs to include consideration of pore size for rapid colonization of cells *in vitro* as well as subsequent tissue maturation during *in situ* regeneration. These material should be free from transmissible diseases, immunologically inert and should not induce an over-exuberant inflammatory response.[11]

Incorporation of cells

The cells with 'periodontal regeneration phenotype' should be possible to culture and subsequently incorporate into a suitable biodegradable scaffold for immediate introduction into a periodontal defect.

Incorporation of instructive messages

The synthetic material should be biodegradable but constructed from a material with suitable affinity for the adsorption of appropriate growth/differentiation factors as well as integrins, cell receptors and other instructive molecules normally found in regenerating tissues. As more data becomes available, the precise growth, differentiation and subsequent gene expression for particular extracellular matrix protein in an orderly manner may be incorporated into engineered matrices for regenerative purposes.

***Materials used for Scaffold*:** A variety of materials have been used in tissue engineering as scaffolds. They can be classified as:Absorbable-Natural polymers - Collagen, Chitosan, Synthetic polymers -Polylactic acid (PLA), Polyglycolic acid (PGA), Co-Polymers of PLA-PGA, etc. Natural mineral – Absorbable-Inorganic bone. Non-absorbable - Calcium phosphates Tricalcium phosphate (TCP), Hydroxyapatite (HA).

Natural Polymers: Type I collagen constitutes 90% of total body collagen type. Hence, type I collagen, the main structural protein of extracellular matrix and mixtures of type I collagen with other matrix components have been successfully used in several tissue engineering applications such as artificial skin engineering. Collagen used for this purpose has been derived from bovine skin, cartilage, tendon, intestine and also from sheep's intestine. Type I collagen combined with ceramics have also been fabricated for bone regeneration in orthopaedics. A combined collagen and mineral based product for bone regeneration is marketed by Orquest Co. Also, demineralised bone matrix (DMBM) consisting of type I collagen is being marketed by the name of 'OsteograforR' for bone regeneration. It is available in granules, sponge and gel form. Chitosan is a biodegradable natural biopolymer extracted from chitin.[12] It has also been used as scaffold for hepatocytes and fibroblasts.[13] Chitosan-tricalcium phosphates sponges has also been found to be feasible scaffolding material to grow osteoblasts in a three dimensional structure for transplantation into a site for bone regeneration.[14]

Synthetic Polymers: Synthetic degradable polyesters were adopted in surgery 30 years ago as materials for sutures and bone fixation devices.[15] These polymers are developed in microorganisms and produced post-purification. The acidic degradation products of classical polyesters and their co-polymers have been implicated in adverse tissue reaction, particularly in bony sites. As a result, synthesis of polymers that yield less acidic degradation products and still have suitable strength and degradation properties are being developed. James and Kohn have developed such a material from tyrosine carbonates.[16] These multiple co-polymers have crystallizable hard segments of PHB and non-crystallizing oligoesters (adipic acid, ethylene glycol, 1,4- butanediol, and diol terminated PCL) as soft segments. Materials with trimethylene carbonate and caprolactone and composites of degradable polyesters with hydroxyapatite are also being utilized for scaffolds. The apparent advantage of poly (α-hydroxy) acids is their degradation by hydrolysis resulting in decomposition products that are mostly metabolized to CO2 and H2O through Kreb's cycle. Polyglycolic acids degrade faster while polylactic acid is most stable *in vitro*. Thus, modification of poly(l-lactide) by cross linking or addition of D-lactide or glycolide results in material that have more rapid degradation, thus diminishing the disadvantage of slow resorption. The use of synthetic gels is emerging primarily as a way to deliver cells or scaffolds *in situ*. A predominant approach, pioneered by Hubbell, is the formation of photopolymerizable gel using PEO-based substrate.[17] Kushida and co-workers**.**[18] are using thermal reversible gels to create cell-sheets that can detach and be used for tissue engineering. A degradable polymer of PEO-PBT that forms a hydrogel with properties that can be modulated by the relative ratios of the two contributing monomers and termed 'Polyactive' is being investigated at a number of sites for application ranging from skin to bone. But it has been recently reported to be poor bone-bonding material in human study by Rosseler*et al*.**[19]** A major form of research is also on developing materials that control cell behavior via specific receptor-ligand interactions. The prototypical adhesion sequence, RGD, was derived from fibronectin. Since then, hundreds of new adhesion sequences from extracellular matrix have been identified. The use of adhesion peptides is also emerging with controlled presentation of growth factors as either bound or unbound to the substrate.

Natural Mineral: Anorganic bone or decalcified bone is the hydroxyapatite skeleton that retains the microporous and macroporous structure of cortical and cancellous bone, which remains after chemical or low heat extraction of the organic component of bone. Usually bovine bone-mineral particles implanted in defects show a greater degree of incorporation into host osseous tissue and have a composite modulus of elasticity closer to that of natural bone.[20]

Historically, bovine anorganic bone grafts have failed due to rejection, probably because earlier methods used chemical detergent extraction which left residual protein and therefore, produced adverse reactions and clinically unacceptable results.[21]However, currently available bovine derived anorganic bone is deproteinized, which supports cell mediated resorption.

Calcium Phosphates: They were implemented as matrix materials for facilitating bone regeneration *in vivo*.[22] The two most widely used forms of these bioceramics are: Tricalcium phosphates (TCP), Hydroxyapatite (HA).

***Tricalcium phosphate*:** This is a porous form of calcium phosphate, the most commonly used form being β - TCP. Potential problem with the use of this material was, it often underwent physiochemical dissolution too soon after implantation. The suggested mechanism of dissolution was that TCP particles might provoke the activation of phagocytes that in-turn stimulates other cells and an inflammatory response. Agents produced by this process could accelerate the degradation process.

Hydroxyapatite: The problem associated with TCP led to the development of this second type of bioceramic. The rationale for developing this material relied in part on the fact that because the naturally occurring mineral in the bone was HA, synthetic implant with the same material would be compatible with osseous tissue. While, it is generally being considered a non-resorbable material, synthetic HA has been shown to undergo physiochemical dissolution, albeit at a very slow rate. Hence, this substance can functionally be considered as long lasting scaffold, especially when they are incorporated into the bone. The problem with HA is that it has a very high modulus of elasticity, which makes it a very stiff structure. This generally alters the distribution of mechanical forces in surrounding tissues and thereby affects the stress-induced

remodeling of neighboring bone .[22] Carbonate can substitute into the HA lattice by replacing either the hydroxide or the phosphate ions, producing either type A or type B carbonate-apatite, respectively. Bone mineral is of the type B form - also referred to as 'dahllite'. However, the carbonate content was comparable to that of bone (approximately 4.3%) the dahllite cement had a higher Crystallinity than bone mineral. While there has been some success in producing carbonate apatite in the laboratory, it is still not possible to produce a substance that has same composition and structure as bone mineral. This formed the rationale for developing deorganified bone to produce natural bone mineral as implant substance. Even today, there has been no single scaffold material which fulfills all the criteria for tissue engineering.

Appropriate Cells:- Cells are basic constituents of any tissue. A tissue cannot be found without the contribution of cells. It is a fact that periodontal regeneration would be impossible, if cells are about in the vicinity of regenerating area. For a periodontal tissue to regenerate following cell types are required: Epithelial cells: for formation of junctional epithelium, Fibroblasts: for formation of fibrous component tissue and periodontal ligament. Cementoblasts: for formation of cementum. Osteoblast: for osteogenesis. Endothelial cell: for angiogenesis. In many tissues, the number and mitotic activity of precursor cells is so high, that there is an ample source of endogenous cells. However, during periodontal regeneration, it is of utmost importance that which cells repopulate the site of regeneration so as to form appropriate tissues. Hence, tissue engineering so as to form appropriate tissues. Hence, tissue engineering provides an extraneous source of required cells, seeded in a suitable matrix, which in the presence of appropriate growth factors will produce periodontal tissues. Therefore, successful tissue engineering hinges on the ability to: Accurately predict cell response, acquire proper cells, cultivate the cells for proliferation and cell differentiation to an appropriate phenotype function.[23]

***Ability to predict cell response*:** Accurate prediction of cell response relies on the understanding of cell biology, Extracellular matrix biology, developmental biology and physiology, as well as immunology and inflammation. To be competitive, tissue engineering must incorporate principles of biology. These are relatively new analytical tools that will play important roles to predict cell responses. Polymerase chain reaction (PCR) analysis and gene technology will allow in depth study of gene expression. This will be used to characterize cellular phenotype and understand cause and effect relationship at genetic level.[24] protein chip technology will enable rapid identification and screening of functional parameters, novel cellular markers (phenomics), and autocrine and paracrine factors influencing cell population. It has the potential to reduce to the time period of few hours, what usually would take days to weeks to accomplish using standard laboratory techniques.

***Ability to acquire appropriate cells*:** Cell sourcing is a key element enabling or prohibiting potential application in tissue engineering. There are a variety of choices depending on application Autologous cells, Allogenic cells, Xenogenic cells ,Immortalized cell line, either allogenic or xenogenic Stem cells, either autologous (adult derived) or allogenic (fetal derived). The choice of cell source influences many design parameters such as culture requirements and delivery strategies.[25] It will also influence time to clinical implementation, government regulation and commercial strategy.[26]

Stem Cells

A Stem cell is a cell that is capable of developing into other types of body cells. They function to replace dysfunctional cells, naturally maintaining optimal growth. There are two types of stem cells; Adult stem cells And Embryonic stem cells. Embryonic stem cells are of three types out of which two are capable of developing into any type of cell in the body. They are called Totipotent cells and Pleuripotent respectively. The third type of stem cell is called multipotent stem cells. Stem cells that are Pluripotent have the capability of forming virtually all of the possible tissue types found in human beings. These stem cells can only be found in a particular stage a (blastocyst) in human embryos. Multipotent stem cells are partially differentiated, so that they can form a restricted number of tissue types. Multipotent stem cells can be found in fetus, in numerous adult tissues and in umbilical cord blood. The third type of stem cells have less regenerative potential and can only develop into a limited number of other types of cells.

A stem cell is capable of undergoing differentiation and multiplication to form a fully differentiated cell. First it undergoes a symmetric cell division to form a pair of stem cells, which further undergoes asymmetric cell division to form one stem cell and one progenitor cell. The progenitor cell formed undergoes progenitor cell division to form two progenitor cells which further undergoes terminal cell division to form differentiated cells.[27]

***Cell Sources*:** Over the last decade, the regenerative capacity of postnatal progenitor cells has increasingly emerged making these cells an attractive candidate for use in tissue-engineering applications. In particular, cell-based periodontal regeneration has been performed using various approaches and principles, and several excellent reviews have been published recently.

Periodontal ligament-derived cells

`Because periodontal ligament-derived cells have multipotential characteristics, the cells are regarded as useful sources for the regeneration of periodontal tissues containing bone, cementum and periodontal ligament. *Nakaharaet al.* implanted autologous dog periodontal ligament cells that were seeded onto a collagen sponge scaffold into a periodontal fenestration defect model in dogs, and showed regeneration of alveolar bone and cementum in uniform layers on the root surface.

Periodontal ligament-derived mesenchymal stromal cells

Seo et al. isolated a population of multipotent stem cells in human periodontal ligament and indicated that periodontal ligament-derived mesenchymal stromal cells exhibited some characteristics similar to those of mesenchymal stromal cells, such as multipotency, clonogenic ability, high proliferation and the expression of the putative stem cell marker STRO-1, as well as the perivascular cell marker CD146.

Periosteal cells

The cultured periosteum has the capacity to differentiate into an osteoblastic lineage and expresses periodontal tissue related genes. *Yamamiya et al*. showed cultured periosteum combined with platelet-rich plasma and hydroxyapatite induced clinical improvements in human infrabony defects.

Gingival epithelium and fibroblast

Gingival epithelial sheets derived from human gingival tissues were developed and applied clinically as a treatment for chronic desquamative gingivitis. Transplantation of gingival epithelial sheets induced a reduction in inflammation and the gain of a healthy epithelial junction and connective tissue. *Mohammadi et al*. applied autologous gingival fibroblasts for patients with insufficient attached gingiva and showed the increase in width of keratinized tissue.

Bone marrow-derived mesenchymal stem cells

Using bone marrow aspirates from over 350 human donors, *Pittenge*r and colleagues (1999) showed lineage specific differentiation of MSCs into fat, cartilage, and bone under appropriate *in vitro* culture conditions. Not only did the human bone marrow derived MSCs demonstrate ability to extensively proliferate, but these cells also were capable of guided differentiation into multiple cell types, establishing a provocative cell source for potential tissue engineering. *Kawaguchi et al*. demonstrated that auto transplantation of bone marrow derived mesenchymal stem cells induced periodontal regeneration in experimental class III furcation defects in dogs.

The use of allogenic and xenogenic sources present unique immunologic and safety considerations. Once past the immunological issues, the use of allogenic cells should be biologically identical to the use of autologous cells. However another important aspect is the purity of cell population following culture.. In vitro, studies confirm the inability of the keratinocytes or fibroblasts to elicit a cell- mediated immune response, even in the presence of cytokines known to stimulate T cell response.[28] These data presents the possibility of using many allogenic parenchymal cell types for tissue engineering. The use of xenogenic cells has been viewed as an important alternative in the problem of cell sourcing. Xenogenic hepatocytes are incorporated in extracorporeal liver assist devices, designed with membrane separation between patient plasma and porcine cells.[28] There are various methods of immune isolation involving: :Gel encapsulation of cell aggregates, Microencapsulation of cells, Conformational coatings of cell clusters.

A molecular approach to blocking rejection of xenogenic cells has been made through genetic manipulation of donor animals to reduce aspects of acute and chronic rejection.[29] A novel approach to using xenogenic cells is to co-culture the cell of interest with testes- derived sertoli cells to confer immune privilege.[30] However, use of xenogenic cells must be balanced with the risk associated with the possible transmission of animal viruses. The use of immortalized cells is limited to extracorporeal liver assist devices and in genetic manipulation of beta cells and other insulin producing cells in treatment of diabetes. Stem cells have the potential to revolutionize cell therapy and tissue engineering. There has been a great deal of both interest and concern over the use of human embryonic stem cells (ES). The ability to cultivate ES cells combined with their potential to give rise to virtually all cell types, has opened the door to the possible generation of almost limitless cell sources for a variety of tissues. However, it is limited by ethical concerns and the limited ability to control or direct cell response. And alternative is the identification of potential multipotent progenitor cells in adult organs. In contrast to ES cells, the challenges are in the identification and isolation of progenitor among the complex array of cells in the tissues, in the targeted stimulation of their proliferation and then in the differentiation of the cell toward a functional phenotype.

Control of cell proliferation and differentiation

The ability to control cell proliferation and differentiation is one of the most limiting but important aspects of cellular tissue engineering.[31]The effects of growth factors on cells need to be characterized. These effects are divided into three categories: Growth factors that favoured the differentiation of mesodermal cells (Activin-A & Transforming growth factor-β). Factors that activated ectodermal and mesodermal markers (retinoic acid, epidermal growth factor, bone morphogenic protein-4, and basic fibroblast growth factor). Factors that allowed differentiation of all three embryonic germ layers; ectoderm. mesoderm and endoderm (nerve growth factor and hepatocyte growth factor). This demonstrated that specific factors favour certain lineages, primarily through an inhibition of certain lineages rather than promotion of a specific one. It is also important to develop suitably defined culture systems and permissive environments to not only promote proliferation but as importantly, promote true differentiation and organotypic properties. The ability to control cell proliferation and differentiation is one of the most limiting but important aspect. Technical knowledge and skill must develop in this area if tissue engineering is to become a successful reality.

***Signaling Molecules*:** Signaling molecules are materials or protein and factors that have potential to alter the host tissue so as to stimulate or regulate the wound healing process. These signaling molecules can be broadly classified as: Growth factors. Biologic modifier & Extracellular matrix proteins.

***Growth Factors*:** These are the class of naturally occurring polypeptide that function in the body to promote itogenesis (proliferation) directed migration and metabolic activity of cells.The principle growth factors used to promote wound healing are given in table 1

Few other growth have been recognized namely VEGF (Vascular endothelial growth factors), CSF (Colony stimulating factor), SGF (Skeletal growth factor) & PTH-P (Parathyroid hormone related protein).

Biologic modifiers & Extracellular matrix protein

This class of molecules includes various bone morphogenic proteins, enamel matrix derivative and various attachment proteins.Properties, role and location of bone morphogenic protein. Table 2. Enamel matrix derivatives (EMD) is composed primarily of acidically extracted amelogenesis. These amelogenins are thought to regulate the initiation and growth of hydroxyapatite crystals during mineralization of enamel. In vitro, EMD affects cellular attachment, mitogenesis, biosynthesis, and differentiation. EMD coated tooth surfaces improved attachment of PDL fibroblasts20 and has no effect on gingival fibroblasts, thus, indicating a selective behavior. Also studies have demonstrated that mesenchymal cells of the dental follicle develops a hard tissue

Table 1 Principle growth factors used to promote wound healing

Sr.no.	Factor	Source	Action
1.	PDGF (AA, AB, BB) (Platelet derived growth factor)	Platelets and macrophages, epithelial cells, endothelial cells, smooth muscle cells, bone matrix	• competence factor • increases protein synthesis • increases mitogenic activity
2.	TGF-α (Transforming growth factor- α)	Platelets, Macrophages, Epithelial cells, Eosinophils.	• stimulates epithelialization
3.	TGF-β1 (Transforming growth factor- β)	Platelets, macrophages, activated T-lymphocytes, osteoblasts, immature chondrocytes, bone matrix	• effects depend on stage of cell differentiation. • Inhibits growth of epithelium.stimulate growth of mesenchymal cells. immunosuppressive
4.	EGF (Epidermal growth factor)	Submandibular glands	• increases keratinocytes proliferation.
5.	IGF-I (Insulin like growth factor - 1)	Blood, liver, bone	• progression factor • increases fibroblast growth • increases DNA synthesis
6.	IGF-II	Bone	• proliferation, differentiation • increases DNA synthesis
7.	FGF-a (Fibroblast growth factor-a)	Brain, pituitary gland	• competence factor • competence factor, mitogenic for endothelial cells
8.	FGF-b	Brain, pituitary gland	• competence factor • mitogenic for endothelial cells • less potent cartilage repairer

Table 2 Properties, role and location of bone morphogenic protein.

Sr.no	BMP	Properties, role & location
1.	BMP-1	Protease (member of astacin family); may function as a procollagen C- proteinase I,II & III; activates BMPs; non osteoconductive; may be involved with Langer-Giedon syndrome (rare inheritable disorder involving skull and long bones)
2.	BMP-2	Osteoconductive; embryogenesis; apical ectodermal ridge; pattern formation; differentiation; osteoblasts, adipocytes, chondrocytes; may influence osteoclast activity; neuronal differentiation; may inhibit bone healing; repair of long bone, spleen, liver, brain, kidney, heart, placenta.
3.	BMP-3 (osteogenin)	Osteoinductive; promotes chondrogenic phenotype; located in lung, kidney, brain, intestine.
4.	BMP-4	Osteoinductive; embryogenesis; gastrulation and mesoderm formation (mouse); produced by dorsal aorta (direct sympathetic neuron differentiation);fracture repair; over expression associated with ectopic ossification of fibrodysplasiaossificans progressive; located in apical ectodermal ridge, meninges, lung, kidney, liver.
5.	BMP-5	Osteoinductive, embryogenesis, located in lung, kidney, liver.
6.	BMP-6	Non-osteoinductive, embryogenesis, neuron maturation; regulates chondrocytic differentiation, found in lung, brain, kidney, uterus, muscle, skin.
7.	BMP-7 (osteogenic-1)	Osteoinductive, embryogenesis; repair of long bone, alveolar bone, spine, fusion, differentiation of osteoblasts, chondroblasts, adipocytes; located in adrenal glands, bladder, brain, eye, heart, kidney, lung, placenta, spleen, skeletal muscle.
8.	BMP-8 (osteogenic-2)	Osteoinductive; embryogenesis; spermatogenesis (mouse)
9.	BMP-8B (osteogenic protein-3)	Initiation and maintenance of spermatogenesis (mouse)
10.	BMP-9	Osteoinductive; stimulates hepatocytes proliferation, hepatocyte growth and function
11.	BMP-12 & BMP-13	Inhibition of terminal differentiation of myoblasts

material believed to be acellularcementum when exposed to enamel matrix proteins.[32] Fibronectin, and attachment protein, is a large glycoprotein present in serum, whose major function is to aid in the attachment of cells to the intracellular matrix. It has been applied on root surfaces to enhance the cellular attachment. However, the results were not promising, as serum already contains high levels of fibronectin.

Platelet Rich Plasma

***Rationale*:** PDGF and TGF-β are well established wound healing "hormones". One of the highest concentrations of PDGF & TGF-β in the body is found within granules of blood platelets (concentration being about 50 ng/ml of whole blood sequestered within the platelets). Thus, concentrating the platelets would also result in concentration of the growth factors, thereby enhancing wound healing on application.

***Processing of PRP*:** Autologous platelet rich plasma (PRP) was developed in the 1970's as a by-product of multi-component pheresis. Techniques and equipment have dramatically improved since then.

***Platelet concentration required in PRP*:** According to Lynch, the platelet concentration in PRP should be increased to at least 3 folds compared to whole blood. According to Obarrio *et al*, count between 5,00,000-10,00,000/mm is acceptable.

Principle: Platelets + Plasma + Thrombin / CaCl2 → Platelet Rich Plasma (PRP) gel. When thrombin and Ca are added to PRP, the platelets are activated and release the contents of their α-granules. The thrombin and calcium also initiate clotting, thus converting the fibrinogen to fibrin, resulting in a clinically useful PRP gel that can improve the handling and efficacy of particulate autographs and bone substitutes. Earlier, the thrombin used was lyophilized bovine thrombin.[33] The use

of bovine thrombin may be associated with the development of antibodies to factors V & XI resulting in risk of life threatening coagulopathies.[34] In order to eliminate this antigenic response, autologous human thrombin, prepared from the same blood used for PRP procurement, is used nowadays.

Advantages of the use of autologous PRP: It is safe as it is an autologous preparation, it promotes adhesiveness and tensile strength for clot stabilization. It is biologically acceptable to the root surface. It contains growth factors: PDGF & TGF-β released by the platelets. It promotes angiogenesis. It has haemostatic properties. It contains a dense fibrin net that is highly osteoconductive. It contains high concentration of leukocyte which acts as an 'autologous antibiotic' reducing the risk of infection. It enhances wound healing.

The use of PRP has been shown to increase the rate of bone formation in a graft and enhance the density of formed bone.[35] Also, the amount of bone regenerated in a PRP enhanced graft is considerably greater. Radiographically, investigators have assessed PRP grafts to be 2.16 times more mature at 2 months, 1.88times more mature at 4 months and 1.62 times more mature at 6 months. PRP also modulates and upregulates one growth factors function in the presence of second or third factor. It is this specific feature that separates PRP growth factors from recombinant growth factors, which are single growth factors that focus only on a single regeneration pathway.

This technique fulfills all the 3 criteria of tissue engineering

The localized degranulation of concentrated platelets provides the necessary signaling molecules required for regeneration.2) The use of autologous cancellous bone or anorganic bovine bone provides a rigid framework, thus serving as a scaffold. Also, the formation of a fibrin clot serves as a natural scaffold in the defect.3) The use of autologous cancellous bone and marrow provides an increased population of osteo-progenitor cells.

Thus, this technique allows clinicians to use principles of tissue engineering even before reconstructing growth factors and BMPs become available in private clinical practice. In the future, platelet gels may be enriched with recombinant 'genetically engineered' tissue growth factors to further enhance their potency.

Yet, it is safe to say that currently PRP is neither fully understood nor fully utilized. It is a technology in its infancy. The technology must be improved to allow the clinician to sequester platelets more quickly and to greater densities, using minimal quantities of blood. Only then can this modality be established as a routine chair-side procedure as part of contemporary periodontal regenerative therapy.

***Platelet-Rich Fibrin*:** Platelet-rich fibrin (PRF) was first described by *Choukroun et al*. in France. It is referred as a second-generation plateletconcentrate, which has been shown to have several advantages over traditionally prepared Platelet rich plasma (PRP). PRF is a fibrin matrix in which platelets, cytokines, growth factors are trapped and may be released after a certain time and aids in periodontal regeneration.[36]

Advantages of PRF over PRP preparation: No biochemical handling of blood . Simplified and cost-effective process. Use of bovine thrombin and anticoagulants not required. Favorable healing due to slow polymerization. More efficient cell migration and proliferation. PRF has supportive effect on immune system. PRF helps in hemostasis.[37]

***Disadvantages*:** Increased time of procedure, increased cost of equipment, Knowledge of phlebotomy required.[36]

***Indication for using PRF*:** Ridge augmentation. Palatal wound coverage following soft tissue harvest. Combined periodontic endodontic lesion, furcation defect. Socket preservation. Alveolar cleft palate repair.Implant surgeries, oral nasal fistula repair. Intrabony, furcation defects .Jaw reconstruction surgeries.Soft tissue procedures.

***Contraindications*:** Platelet dysfunction syndrome, Critical thrombocytopenia, Hemodynamically unstable patients, Pregnancy.

***Various forms of PRF*:** PRF gel, PRF membrane, PRF plug, PRF fragments

***Preparation of PRF*:** Required quantity of blood is drawn in 10 ml test tubes without an anticoagulant and centrifuged immediately using a tabletop centrifuge for 12 minutes at 2,700 rpm. The resultant product consist of the following three layers namely, Top most layer consisting of platelet poor plasma, PRF clot in the middle, Red Blood Corpuscles at the bottom.[36] .The upper straw-colored layer is then removed and middle fraction is collected, 2 mm below to the lower dividing line, which is the PRF.

Growth factor delivery by gene transfer

A novel approach for tissue engineering. The ultimate success of tissue inducing substances and growth factors depends on the large scale purification and production of these molecules, as well as methods to deliver these factors to their targets. A problem with current delivery of these growth factors to periodontal wounds includes the extremely short half-lives of the factors. These factors remain for a very short duration, presumably because of proteolytic breakdown, receptor mediated endocytosis, and the solubility of the delivery vehicle. Therefore, the use of DNA delivery systems may arise as another method of targeting proteins to a wound site. Furthermore, assessing the longer term exposure of wounds to growth factors may provide better understanding of the mechanism of periodontal wound healing.

The transduction of appropriate target cells represents the critical first step in gene therapy; consequently, the development of the methods of gene transfer suitable for different forms of therapy has been a major focus of research. The single common feature of the method is the effective delivery of genes into cells. In the case of retroviral vectors and adeno-associated viral vectors, the transferred DNA sequences are stably integrated into the chromosomal DNA of the target cell.

Several groups have delivered GF genes to healing skin and bone wounds using plasmid DNA. *Eriksson et al*[38]and *Winkler et al* [39]have developed unique methods of transducing wounds by the in vivo microseeding techniques *Giannobile et al* studied in collaboration with *Eriksson* and have shown that gene transfer of human epidermal growth factor (4EGF) to procrine periodontal wounds shows greater levels of EGF protein than do mock-transduced wounds, 24 and 96 hours after surgery.

Bone morphogenic proteins gene delivery

An experimental study in rodents by *Lieberman* and Colleagues demonstrated gene therapy for bone regeneration, with results revealing that the transduction of bone marrow stromal cells with rh BMP-2 lead to bone formation within an experimental defect comparable to skeletal bone. Another group was similarly able to regenerate skeletal bone by directly administering Ad5/BMP-2 providing further evidence for the ability of *in vivo* and *ex vivo* bone engineering. *Francheshi* and colleagues investigated *in vitro* and *in vivo* Ad gene transfer of BMP-7 for bone formation. Ad transduced non osteogenic cells also were found to differentiate into bone-forming cells and produce BMP-7 or BMP-2 *in vitro* and *in vivo*. When genes that encoded the BMP antagonist were delivered, inhibition of periodontal tissue formation resulted .A recent study by Dunn and colleagues showed that direct *in vivo* gene delivery of Ad/BMP-7 in a collagen gel carrier promoted successful regeneration of alveolar bone defects around dental implants.

Gene therapy presents certain advantages when compared with other therapies. Because both cell transplantation and laboratory cell culturing are not needed, gene therapy may be safer and more cost-effective than cell-based therapies. Moreover, when compared with the existing recombinant single-protein-based therapies gene therapy may mimic the complex natural process of periodontal tissue formation, because multiple genes, and multiple factors, can be delivered within the bone defect.

Ribonucleic acid mediated silencing

The ribonucleic acid (RNA)-mediated silencing process is defined as RNAi, a discovery for which *Fire and Mellow* received the 2006 Nobel Prize. It is based on the principle of RNA interference (RNAi), a novel mechanism of action whereby the expression of certain genes detrimental to the tissue regeneration process is silenced by RNAs. RNAi works through small RNAs of approximately 20 to 30 nucleotides that guide the degradation of complementary or semi complementary molecules of messenger RNAs (posttranscriptional gene silencing) or interfere with the expression of certain genes at the promoter level (transcriptional gene silencing). Artificially, transcribed short hairpin RNAs (shRNAs) can be introduced into the cell by plasmid transfection or viral transduction, or small linear RNAs (siRNA) can be directly transfected into the cells. In the cytoplasm, the shRNAs or siRNA participate in endogenous posttranscriptional gene silencing. The synthetic RNAs are recognized and processed by an endoribonuclease named Dicer and incorporated into the RNA-induced silencing complex. Then, silencing occurs through the AGO2-mediated cleavage of target messenger RNAs. Most RNA-based therapeutics currently under investigation uses siRNAs because they are safe and cost-effective. They can be introduced into the cells without the aid of viruses and can be chemically synthesized. The first siRNA-based therapeutic tested in human clinical trials was the VEGF-targeted RNA for the treatment of macular degeneration of the retina. Tumor necrosis factor-α-targeted siRNA can suppress osteolysis induced by metal particles in a murine calvaria model, opening the way to the application of RNAi in orthopaedic and dental implant therapy. In terms of bone regeneration, *Gazzerro* and colleagues have demonstrated that downregulation of *Gremlin* by RNAi in ST-2 stromal and MC3T3 osteoblastic cells increases the BMP-2 stimulatory effect on alkaline phosphatase activity and on Smad 1/5/8 phosphorylation, enhances osteocalcin and Runx-2 expression, and increases Wnt signaling, with the potential to increase bone formation *in vivo*. Taken together, these studies prove that RNAi, when adequately used, can foster tissue regeneration. The use of RNA-based therapeutics for tissue regeneration is still in its early stages. Nevertheless, RNAi promises to be an effective therapeutic tool and may be successful in periodontal regeneration.

Bilayered cell therapy: A tissue engineered skin substitute as an alternative to tissue from palate

Bilayered cell therapy is a living bilayered tissue engineered skin substitute constructed of type 1 bovine collagen and viable allogenic human fibroblasts and keratinocytes isolated from human foreskin. BCT is morphologically, biochemically and metabolically similar to human skin. Its cell proliferation rate is similar to that of human skin. Mitotic activity occurs in the basal keratinocytes of the epidermis and in the fibroblasts within the matrix. The keratinocytes produce growth factors and cytokines that act as signals between cells and help to regulate normal wound healing. Bilayered cell therapy exhibits a synergistic interaction between epidermal and dermal layers. It enhances cell and tissue differentiation through cell: Matrix, cell: Cell and cell: Environment interactions. BCT is safe and capable of generating keratinized tissue without the morbidity and potential difficulties associated with donor site surgery (*Mc Guire and Todd Scheyer*).

Cell Sheet Engineering

This is a recently discovered technology for the regeneration of tissues. [40] This technique is superior to the conventional technique as it involves detachment of cultured cells without using enzymatic approach. Cell sheet engineering by the use of temperature-responsive dishes provides a novel strategy to produce tissues without a specific scaffold. The resulting cell sheets retain their original extracellular matrix and cell-cell contact. *Okano et al.* [40] utilized change in cell culture temperature and a surface-grafted temperature-responsive polymer named poly (Nisopropylacylamide) (PNIPAAm) to control cell surface adhesion. Cell sheet engineering is commercially available under the name of UpCellTM (Cell Seed Inc., Tokyo, Japan).

The ultimate goal of periodontal therapy remains the predictable three-dimensional repair of an intact and functional periodontal attachment that replicates its pre-disease structure. While periodontal treatment, aimed at removing the bacterial cause of the disease is generally very successful. However, the ability predictably to regenerate the damaged tissues still remains a major unmet objective for conventional treatment strategies.

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