Review Article

Tissue Engineering Approach for Reconstructing Bone Defects Using Mesenchymal Stem Cells

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Abstract

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Regeneration of large bone defects is considered a challenging task by facio-mandibular and orthopedic surgeons. In these circumstances, either bone grafts or metal implants are currently being used. The inherent limitations associated with these methods have directed the attention of investigators to new technologies such as bone tissue engineering, a multi-disciplinary field in which life science as well as engineering is involved to manufacture an appropriate bone construct. The objectives of scientists involved in this field are to design and manufacture scaffolds with appropriate chemical and physical features, to direct cell differentiation within the scaffold using appropriate culture conditions and finally to render the engineered construct applicable for clinical use. In this article, the main components involved in the bone tissue engineering process have been reviewed. These include cells (with an emphasis on mesenchymal stem cells), scaffolds, growth factors and bioreactors, and tissue engineering approaches to tissue regeneration.

Keywords: Tissue Engineering, Bone Diseases, Mesenchymal Stem Cells, Scaffold

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Introduction

Bone structure and development

Bone is a live, dynamic tissue with a rich blood supply and a specific capacity for regeneration (1). Bone tissue could be considered to have at least four functions. It serves as a structural scaffold to which muscles are attached and plays a major role in their contraction. Bone supports the body vital organs including brain, spinal cord and bone marrow. Bone tissue is the main source of calcium and phosphate and bone (internal spaces) provides an appropriate microenvironment for hematopoietic cell differentiation (2). To facilitate the latter function, bone is elaborated from a complex architecture composed of organic materials, inorganic materials and water. Inorganic material mainly takes the form of hydroxyapatite crystal (3). Minerals including magnesium, potassium, fluoride, phosphate and citrate constitute a minor part of the inorganic material (4). The organic matrix of bone is mainly composed of type I collagen (more than 95%-97%). Organic materials include other collagen isoforms, and non-collagenous proteins such as osteocalcin, osteonectin, osteoadherin, fibronectin, bone sialoprotein, thrombospondin, proteoglycan and glycosaminoglycan (5, 6).

There are three types of cells in bone tissue: osteoblast, osteocyte and osteoclast. Osteoblasts are bone-forming cells which have the high alkaline phosphatase activity required for bone mineralization. Osteocytes are actually ostoblastic cells that are trapped within their own deposited matrix during the osteogenesis process. Osteoclasts are bone macrophages specialized in bone resorption. (7). Bone histogenesis could be mediated by either intra-membranous or endo-chondral processes. In intra-membranous ossification, bone forms when mesenchymal precursors condense and differentiate directly into osteoblasts while in endochondral ossification, precursor cells first differentiate into cartilage cells, which are then replaced by bone cells (8).

Bone defects

Reconstruction of large defects in bone tissue is regarded as an economic and social problem, as well as an important challenge in the field of modern medicine. Most small bone defects regenerate spontaneously or with minimal intervention, due to the great capacity of bone to repair itself. In some circumstances, such as injuries following a car accident, congenital cleft palate, lesions left by removal of bone cancer and periodontal disorders, the defects are only partially repaired due either to the size of defect or the slow regeneration process inherent in the etiology of the defect. To date,

insufficient attention has been paid to the treatment of these conditions (9).

Current treatments of bone defects

Among bone repair methods, autograft transplants are considered to be the gold standard (10) since they bring osteogenic, osteoinductive and osteoconductive components to the defect sites without triggering host immune response. However, as the quantity of bone marrow that can be obtained for autograft is limited, and because it requires an extensive operation which may cause morbidity, pain and possible infection of the donor site, there is an ongoing search for alternatives (11-13). Allograft bone transplantation is a potential alternative which overcomes the problem of quantity as it could be obtained from cadaver tissue. Allograft disadvantages, however, include less osteoinductivity, possible trigger of host immune response and likely transmission of disease (11, 14). Another choice would be the use of metal implants. Although these substitutes are applicable in some cases they possess several disadvantages; including first, they do not degrade after implantation, hence, secondary surgery is necessary to remove them from the host and second, they may release toxic ions which trigger host immune response and infection (15, 16). Synthetic materials or alloplasts are being suggested as the other choice for application in bone regeneration, but they are not particularly appropriate as the host treats them as a foreign body and creates a thin fibrous membrane around them. This prevents the alloplast from being integrated into the host tissue and consequently being rejected (9). To overcome this problem, the attention of investigators has been directed to new technologies as approaches to bone tissue engineering.

Modern treatment of bone large defects

Nowadays, bone constructs elaborated according to tissue engineering principals are regarded as an ideal choice to reconstruct bone segmental defects. Tissue engineering is a multidisciplinary science, defined as the application of the principles and methods of life science and engineering in order to understand the relationships between structure and function in normal and injured mammalian tissues and to develop biological substitutes for the repair, maintenance and improvement of tissue function (17). The objective of this strategy is indeed to overcome the limitations exhibited by transplantation of tissue grafts and biomaterials.

Tissue engineering

The components that are involved in a tissue engineering process include cells, scaffolds, growth factors and bioreactors.

Cells

Osteogenic cells are essential components in all advanced bone tissue engineering strategies. These include mesenchymal stem cell (MSCs) and ordinary bone cells (18-20).

Since the proliferation capacity of specialized cells in tissue is limited, ordinary bone cells (osteoblast) would not be appropriate for tissue engineering strategies. Furthermore, long-term cultivation of these cells is usually associated with their reduced activity. For this reason, MSCs are preferred over ordinary cells in most investigations. There are many review articles of the basic biology of MSCs (21-25).

Briefly, MSCs are characterized by two properties: the ability to extensively replicate and the capacity to differentiate into multiple cell lines. These cells are resident in many tissues in adults, including bone marrow (26-28). The notion that bone marrow houses osteogenic precursor cells was first proposed by Petrakova et al. who indicated the osteogenic ability of marrow cells transplanted under the renal capsule (29). Since MSCs are usually obtained from the patient's own bone marrow aspirates, their transplantation is not associated with host versus graft reaction. Furthermore, some research has indicated that MSCs express no HLA class II receptor indicating that even non-autologous transplantation would not trigger host immune response (30).

Scaffold

Scaffolds are artificial matrices designed to mimic the mechanical and biological properties of the tissue matrix. Cells in tissues produce matrix that contains tissue specific molecules. The matrix, in turn, creates a complex network that supports the tissue and determines the shape of the tissue. In this tri-dimensional microenvironment, matrix-cell interaction initiates and promotes growth, migrations, differentiation, viability and organization of the cells as well as the remodeling of the matrix (31). In tissue engineering strategies, scaffolds provide three-dimensional spaces for the cells to undergo proliferation and differentiation. Moreover, scaffolds serve as carriers to transfer cells and bioactive materials to defect sites (32).

Characteristics of scaffolds for bone tissue engineering

In general, scaffolds must be biocompatible. Bone scaffolds should also possess mechanical proper-

ties similar to bone tissue. Scaffolds implanted in the body must be able to undergo degradation in order to be replaced by new tissue. The degradation rate must be proportional to the formation rate of the new tissue. If scaffold degrades rapidly, it would be followed by fracture, and slow degradation would prevent new tissue formation (33-36). Products resulting from scaffold degradation should not be immunogenic or cytotoxic (33, 37). Moreover, scaffold porosity should be appropriate in terms of pore numbers, size and morphology (33, 35). These parameters are crucial to the nutrition of cells and penetration of vessels (38). Different ranges of pore size including 200-400 μm (39), 100-150 μm (33) and 100-600 μm (40) have been suggested for scaffolds suitable for bone repair. In addition, scaffolds for bone tissue should possess a high surface/volume ratio (41). Interconnectivity of the pores is also a crucial factor which facilitates the diffusion of nutrients and formation of vessels (33, 35, 41). Since bone defects are irregular in shape and of different sizees, bone scaffolds should have the capacity to be manufactured in varieties of forms and sizes. The surface property of the scaffold is the other significant parameter that can influence the adherence of cells and their subsequent proliferation and differentiation. In this regard, the scaffolds' surface characteristics, chemical energy and composition are important factors (42, 43). Since a material with ideal surface properties is rare, the modification of most scaffold surfaces is inevitable (44, 45). A negative electrical charge oo the scaffold surface facilitates MSC adhesion, as well as expansion therein (46).

Seeded cells adhere to scaffolds surfaces either by receptor-independent or receptor-dependent mechanisms. In receptor-independent attachment, the adhesion is mediated by chemical bonds including hydrogen bonds and electrostatic attraction between cell surface molecules and functional chemical groups on the scaffold surfaces. Since such adhesions provide insufficient signal from the external environment into the cells, as consequence, cells may lose their viability. Receptor-dependent adhesion is mediated by extracellular matrix (ECM) molecules such as fibronectin, vitronectin, collagen and laminin (47). In order to improve cell adhesion, some researchers have focused on the incorporation of integrin domains including arginine-glycine-aspartate (RGD) oligopeptide on the scaffold's surfaces (13).

Scaffold classification

Scaffolds can be classified as ceramic scaffolds, natural scaffolds, synthetic scaffolds and Hydrogels.

Ceramic scaffolds

Hydroxy apatite and tricalcium phosphate are among the ceramics most frequently used for bone tissue engineering because they constitute the main part of the natural bone matrix (48-50). Although these materials exhibit some valuable properties for bone tissue engineering, several disadvantages limit their application. These ceramics are very fragile and possess low mechanical stability. In addition, their degradation rate is not very predictable. Degradation of these ceramics results in a considerable increase in blood levels of calcium and phosphate (51). To overcome the limitation associated with the mechanical stability of these ceramics we have recently fabricated a hybrid scaffold using tricalcium phosphate in conjunction with alginate and gelatin (52).

Natural scaffolds (polymer)

Polysaccarids such as cellulose, starch, varieties of dextran, alginates, chitin/chitosan and glycosaminoglycan (GAG); specifically hyaloronic acids or proteins such as collagen, silk fibroin would be classified as natural scaffolds. Poly hydroxyl butyrate, which is derived from bacteria, is also being considered as a natural scaffold (53, 54). The main advantages of these scaffolds are their low immunogenicity, high inherent bioactive properties, capacity for good interaction with the host tissue, high chemical diversity and, in some cases, unlimited sources (53).

Collagen, a natural polymer, can easily be obtained from tissues using enzymatic digestion and acid/salt extraction. This polymer is frequently used as bone scaffolds in tissue engineering approaches. One advantage of collagen scaffold is that it can readily be degraded by the enzymatic activity of cytoplasmic lysosomes. The degradation rate can be controlled by manipulation of the specimen density and the level of cross-linkage between collagen molecules. Arg-Gly-Asp (RGD) peptide domains in collagen serve to maintain phenotype and activity of the cells (55).

Since collagen I constitutes the major protein of bone matrix, it increases adhesion and maturation of osteogenic cells (56). The disadvantage of collagen is its poor mechanical properties. Adding hard materials as hydroxy apatite may overcome this problem. Both collagen and hydroxy apatite possess inhibitory effects on bacterial growth (57). Furthermore, collagen is being considered as ideal carrier for bone morphogenic

protein (BMP) (58). To enhance its structural characteristic and improve osteogenic capacity, collagen needs to be used in conjunction with mineral calcium (59), a non-proteinous polymer, such as chondroitin sulfate (60), poly lactic acid (61), poly lactic co-glycolic acid (62) and poly tetrafluoroethylene (63). Recently we demonstrated other properties of collagen in bone tissue engineering. According to our findings, Type I collagen gel in the seeding medium improves murine mesenchymal stem cell loading onto the scaffold, increases their subsequent proliferation, and enhances culture mineralization (64).

Among the other bone matrix molecules that are effective in osteogenesis is GAG which is composed of long carbohydrate chains (65, 66). Hyaloronan has been indicated to have both osteoconductive and osteoinductive properties (67, 68). In addition, the positive roles of sulfated GAG, their combination with collagen and perlecan in bone differentiation have been reported (69, 70).

Synthetic scaffolds (polymer)

In this group are several scaffolds including poly lactic acid (PLA), poly lactic co-glycolic acid (PLGA), poly ethylene glycol, poly caprolacton, poly vinyl alcohol and alumina (71-75).

Aliphatic polyesters such as poly glycolic acid (PGA), poly-l-lactic acid (PLLA) and poly (lactic-co-glycolic acid) (PLGA) are the most frequently used polymers in tissue engineering and have obtained Food and Drug Administration (US) approval for human use. They degrade with non-enzymatic hydrolysis (55). Degradation products are released into body fluids and excreted through natural metabolic pathways. Degradation time varies between several weeks to several months depending on their crystal structure, molecular weight and the ratio of copolymer lactic acid and glycolic acid in their structure. These scaffolds can be easily prepared in different shapes and dimensions because they are flexible at high temperatures (76). Synthetic scaffolds are preferred over natural scaffolds since comparatively they have superior mechanical properties, and an appropriate degradation rate and microstructure.

Use of synthetic scaffolds with ceramic nano particles could be a revolutionary approach in the bone tissue engineering field. Recently we have indicated that PLLA/ nano hydroxyapatite scaffolds possessed good mechanical properties and biocompatibility compared with PLLA alone. According to our findings the hybrid scaffolds supported MSC adhesion and proliferation more than PLLA alone (77).

Hydrogels

Hydrogels are polymeric systems with cross-linked structures capable of absorbing a considerable volume of water-based solutions. Cells are trapped within the hydrogel during the gelation process. The number of pores in hydrogels can be determined by adding a certain amount of cross-linker which, in turn, determines the volume of water absorbed by the hydrogels (78). The disadvantage of hydrogels is their poor mechanical properties. There are several important scaffolds that are classified as hydrogels. These include chitosan, poly vinyl alcohol, alginate and silk fibroin, the latter being considered more suitable for bone tissue engineering due to its good biocompatibility, flexibility and mechanical stability (12). Alginates, which are derived from brown seaweed and approved by the FDA as wound cover, have frequently been used in tissue engineering processes (79). Multilayer hydrogels are among the most appropriate scaffolds for cell co-culture in which one cell type secretes growth factors and matrix molecules which affect the growth and differentiation of other cell type (80).

Growth factors

Growth factors are cytokines that can be secreted by a variety of cells. They serve as signaling molecules (81). Binding growth factors on their receptors initiate intracellular signals that eventually result in either stimulation or prevention of cell adhesion, proliferation, migration and differentiation. Therefore, these molecules have crucial roles in the course of tissue formation and creation of tissue constructs (82). Growth factors that are introduced into scaffolds may have two useful effects: a) they can induce differentiation of loaded cells b) they can conduct host cell into the scaffold pore system and promote their differentiation (83). Osteogenic growth factors include BMP, transforming growth factor (TGF), fibroblast growth factor (FGF), insulin-like growth factor I/II (IGF I/II) and platelet-derived growth factor (PDGF) (84,85). BMPs are polypeptides involved in several physiologic processes, such as immune response, regulation of hormone secretion, growth and differentiation of cells, tissue morphogenesis and regeneration, and stimulation and modulation of bone tissue. BMPs, specifically BMP2, BMP4 and BMP 7, are capable of inducing endochondral ossification (86). BMP6 and BMP9 are also reported to be osteogenic inducers of MSCs (87). According to some reports repair of bone fractures can be accelerated by BMP2 and BMP7 (88-89). It seems that the main role of BMP is to recruit and

induce bone forming cells into the defect site. It has been indicated that BMP2 plays a crucial role in expression of osteogenic markers including alkaline phosphatase and osteocalcin through the mitogen-activated protein kinase (MAPK) pathway (90). BMPs have also been shown to induce the cells to express the cbaf-1/Runx2 gene (82).
In tissue engineering strategies, growth factors such as platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) are used to promote vessel formation. This is of considerable significance, especially in large constructions in which the nutrition of the cells in deep parts of the scaffold may be impaired (91). Growth factors can be immobilized on scaffold surfaces through covalent bonds. The other method would be to encapsulate growth factor within carriers which gradually destruct and release the growth factor (92, 93).

Bioreactors

Bone tissue is a tridimensional system with a complex and mechanically active structure. In this tissue, osteoblasts are in interaction with each other while experiencing mechanical forces and consistent changes in ECM architecture (94). While recent studies have indicated the usefulness of mimicking the osteoblast dynamic natural microenvironment in bone tissue engineering cultures, in most previous studies, a static cell culture was used to establish tissue engineering culture. The main disadvantage of static culture is the limitation associated with the diffusion of nutrients to deeper parts of the scaffold, leading to insufficient nutrition of the cells (95). In static culture conditions, cells cannot distribute evenly throughout the scaffolds (96, 97). In addition, static culture can not provide mechanical stimulation which is important for osteocyte activity. It has been indicated that mechanical stress could enhance Cbaf-1/Runx2 expression in bone precursor cells (98). To overcome the limitation associated with static culture, bioreactors have been developed to be used in tissue engineering processes. The objectives of bioreactors use are the followings: a) to provide a uniform distribution of cells within scaffolds, b) to provide cells deeper in the scaffold with sufficient nutrients c) to expose the cells to mechanical stimulation appropriate for bone differentiation and remodeling (96, 97). Bioreactors appropriate for bone tissue engineering include spinner flasks, rotating wall vessels and direct perfusion bioreactors (99, 101) (Fig 1).

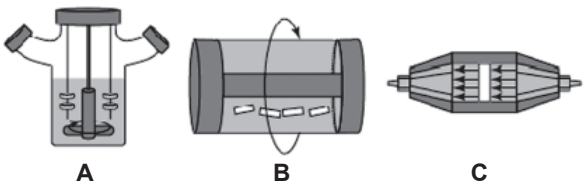

Fig 1: Some bioreactors that are appropriate for bone tissue engineering. A: spinner flask bioreactor; B: rotating wall vessels and C: direct perfusion bioreactors (99)

Tissue engineering approaches to tissue regeneration

The ultimate goal of the tissue engineering process is to reconstruct tissue defects. For this purpose, several strategies have been proposed. The strategies differ from each other in terms of the number of components (i.e. cells, scaffolds and growth factors) that each strategy utilizes to produce the construct (Fig 2).

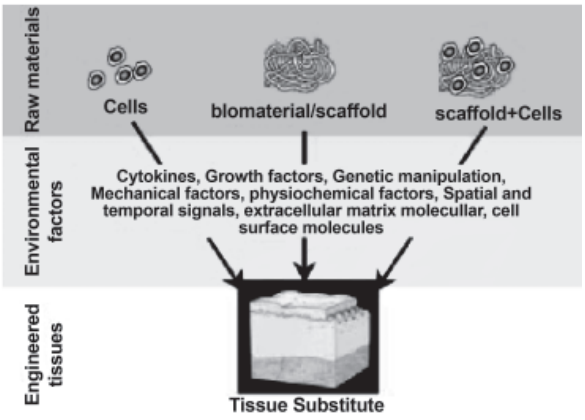

Fig 2: Tissue engineering approaches to tissue regeneration (102).

One method would be to conduct bone regeneration using scaffold alone. In this strategy, scaffold is placed in defect site and host cells migrate into the scaffold pore system (37, 39, 102). Another method would be to transplant autoulogous cells alone (without scaffold) into the defect site. It has been reported that in such a method, most cells may undergo apoptotic cell death because cell viability is dependent on surface adherence which is not provided in the absence of scaffold (37,102, 103). A third method would be the use of scaffold with growth factors such as BMPs, TGF b1 and PDGF (104, 105). This strategy is referred to as growth factor-based tissue engineering. The fourth strategy would be the use of scaffolds coated with cells. This is referred to as cell-based tissue engineering. In this approach, the scaffold serves as the carrier onto which cells can adhere, proliferate and

deposit matrix. Meanwhile, the scaffold undergoes gradual degradation and eventually it is replaced by repair tissue (106-108). In such a strategy, the scaffold serves to physically support the defect especially during the early days of transplantation when the repair tissue is not yet formed (17, 39, 102, 106).

MSC-based bone tissue engineering

Several experiments have so far been conducted using MSC-based bone tissue engineering. The presence of MSCs within scaffolds can increase the rate and amount of bone formation through two mechanisms: a) first as MSCs are osteogenic cells they can be directly involved in bone formation b) second MSCs can increase the osteoconductive properties of biomaterials by releasing osteogenic factors which, in turn, facilitate ostoeoprogenitor migration into the defect site. These effects result in enhanced bone formation in the scaffold/cell construct compared to scaffold alone.

Several animal model studies, as well as clinical trials that have provided good evidence of the efficiency of the MSC-based bone tissue engineering approach to regeneration of large bone defects are reviewed in the following paragraphs. In a study by Bruder et al constructs consisting of 65% hydroxy apatite/35% ß tricalcium phosphate and MSCs were created. These, along with scaffold alone, were transplanted into canine 21-mm femoral segmental defects. The results indicated that in femurs with cell/scaffold constructs more osteointegration of the implants with host tissue occurred compared with scaffold alone. Moreover, there were several fractures in the femur treated with scaffolds that had not been transplanted with cells (109).

In a similar study, Kon et al. transplanted hydroxy apatite ceramics coated with MSCs into large defects in ovine tibia and compared the results with those transplanted with scaffold alone. According to their results, in scaffold/MSC constructs, bone tissue appeared to form on the outer surfaces of the scaffold, as well as in the scaffold's internal spaces, while in scaffolds that had not been transplanted with cells the bone was only observed on the outer surfaces of the scaffold (110).

According to our own recent observations, both hydroxyl apatite and Bio-Oss scaffold coated with MSCs can enhance ectopic bone formation when transplanted into canine master muscle (111, 112). Furthermore, we recently reported that MSCs loaded into these ceramic scaffolds can regenerate rat calvarial bone defects more effectively than scaffold loaded with plasma-rich platelet (PRP) (113). Similarly, we found that MSC/ceramic constructs can reconstruct large defects in canine mandibolar bone better than scaffold alone (114). All these studies imply that scaffold loaded with MSCs would be more efficient than scaffold alone.

After several preliminary animal experiments, the first report on the use of bone constructs produced using the MSC-based tissue engineering approach in humans was published by Quarto et al. who prepared hydroxyl apatite ceramics the shape of the defects and loaded them with the patient own MSCs. Good integration has been observed when such constructs have been transplanted into patient long bone with 4-8 cm segmental defects (115).

In a clinical trial to reconstruct a large defect in the mandible of a 56 year old man, Gronthos designed a titanium mesh in the form and size of the patient's mandible defect and filled it with hydroxyl apatite porous scaffolds loaded with MSCs and rhBMP-7. The construct was then implanted into the patient's latissimus dorsi muscle for 7 weeks at the end of which the implants with acquired vascular bundles were transplanted into the patient's defect site. A few months later, the patient was able to initiate chewing. Unfortunately, after 15 months, the patient died of a myocardial infarction. However, this procedure was a breakthrough in regenerative medicine using tissue engineering approaches (116).

Cell loading into scaffold pore systems

In cell-based tissue engineering approaches, an essential step in establishing a 3D culture is to seed the cells into the scaffold's internal pore system. Successful and efficient loading of the cells is one of the key steps and challenges in the tissue engineering field. In this regard, several strategies have been proposed including: placing the cells suspended in medium as a drop on the top surface of the scaffold; immersing the scaffold in a medium containing the cells under study (12); injection of the cells into the pores of scaffold using a pipette tip (12,117), agitation of the cell and scaffold complex using an orbital shaker (118); loading the cells by perfusion using an appropriate bioreactor(119); and using the centrifuge to force the cells into scaffold pores (120). The use of nano particles and magnetic forces would be an alterative way to load the cells onto scaffolds. In this method, cells are first labeled with magnetite cationic liposomes and then placed on the scaffold surfaces. A magnet placed underneath the scaffold draws the cells into the scaffold pore

system (121). Recently we have reported that the presence of collagen gel in the seeding medium can significantly enhance the efficiency of cell loading into the scaffold pores (64).

Future Prospects

The objective of bone tissue engineering is to cure large bone fractures and defects using osteoconductive scaffolds, osteogenic cells and inducing growth factors. Despite all the progress that has been made in this field, clinical use remains limited due to some outstanding problems. These include the shortage of biomaterials with appropriate mechanical, biological and chemical characteristics, problems associated with angiogenesis in large constructs and finally the ability to produce scaffolds that correspond to the complex shape of bone defects. To overcome these limitations close collaboration between the biological sciences and engineering sciences is needed.

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