Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 5, Issue 1, 2013

Review Article

# BONE TISSUE ENGINEERING

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*Received: 22 Oct 2012, Revised and Accepted: 29 Nov 2012*

**ABSTRACT**

Bone tissue engineering is still in its infancy. Most research efforts are focused on the creation of bulk bone tissue and not on the organization of bone matrix, which is characteristic for bone tissue to carry out the weight bearing function. In long bones for instance trabecular structures are aligned along the principle stress lines. The application of MAPCs in bone regeneration is currently under investigation. The most successful materials for bone engineering are bone mimicking materials, such as demineralized bone matrix (DBM) and hydroxyapatite/tricalcium phosphat e/collagen type I ceramics. To increase the osteo induction properties of a tissue engineered construct, growth factors are incorporated into ceramics or polymer scaffolds. BMPs are the most extensively used growth factors in bioactive scaffolds, because they were discovered by their ectopic bone induction capacity. Bioreactors provide a culture environment to grow a cell-material-growth factor construct. They are designed to seed the cells homogenously in the scaffold, to control the culture medium (temperature, osmolality, levels of oxygen, nutrients, metabolites, regulatory molecules), facilitate mass transfer between the cells and the culture environment, and provide physiologically relevant physical signals (fluid flow, shear, pressure, compression/stretch, torsion). In vivo, animal models are not only used to test a bone engineering construct, but also to culture a TE-construct. Therefore, animal models can be considered as in vivo bioreactors. Interestingly, plastic surgeons have applied this principle.

**Keywords:** Scaffolds, Mesenchymal stem cells (MSC), Osteoblasts, Multipotent adult progenitor cell (MAPC), Demineralized bone matrix (DBM), Bioreactors.

## INTRODUCTION

Currently, bone tissue engineering is still in its infancy. Most research efforts are focused on the creation of bulk bone tissue and not on the organization of bone matrix, which is characteristic for bone tissue to carry out the weight bearing function. In long bones for instance trabecular structures are aligned along the principle stress lines. To create bone tissue, tissue engineers use cells, scaffolds, signaling molecules or a combination to assemble a cell-scaffold construct which can be cultured in a bioreactor prior to implantation in an in vivo model Cells[1].

The basic component of every tissue is cells. Therefore, the first step in bone tissue engineering is the choice of a reliable source of cells that allows consistent and reproducible isolation and expansion into high numbers. The ideal cell source should be easily expandable to higher passages, non-immunogeneic and have a protein expression pattern similar to bone tissue. Since bone tissue is constituted of different cell types such as osteoblasts, osteocytes, osteoclasts, osteo- and chondro-progenitors, mesenchymal stem cells and endothelial cells, it appears logical to culture and implant 'controlled' heterogeneous rather than 'pure' homogenous cell populations.

However, due to successful application of the use of purified cell populations in bone marrow transplantations in mice, baboon and human it became a general idea to select cells based on their surface markers and use homogeneous cell populations for tissue engineering
applications.

Interestingly, no relationship between membrane marker profile and the biological behavior of cells has been found. The most obvious cell type to use in bone engineering is the osteoblast. Osteoblasts produce bone matrix and when the cell is completely embedded in its own matrix it becomes an osteocyte. Unfortunately, to obtain osteoblasts one has to take a bone biopsy and thus create a bone defect to repair the other; relatively few osteoblasts are harvested from a bone biopsy; and they have a low expansion rate in vitro.

These disadvantages make primary osteoblasts less suitable for bone regeneration. A more attractive cell type for bone engineering applications is the mesenchymal stem cell (MSC). Stem cells are undifferentiated cells with high proliferation capability, being able of self renewal and multi-lineage differentiation giving rise to all mesenchymal tissues such as bone, cartilage, tendon, muscle and fat. Although the characteristic gene and protein profile of MSCs remains to be elucidated, in vitro clonal differentiation studies have demonstrated the existence of MSCs and the ability to isolate and expand MSCs from bone marrow, cartilage, synovium, periosteum, trabecular bone, fat and periodontal ligaments Identification of the MSC in vivo however, and studying its behavior in its 'niche' is still a challenge in stem cell biology.

Interestingly, a more premature stem cell, the multipotent adult progenitor cell or MAPC, was isolated from adult bone marrow (mouse, rat and human), muscle, brain and recently from adult testis (all of mouse origin). MAPCs have an extremely high expansion capacity and can differentiate to cells from the three germ layers in vitro. When injected in the early blastocyst, MAPCs contribute to most somatic tissues where they differentiate into tissue-specific cell types in response to cues provided by the different organs. Hence, MAPCs have similar expansion and differentiation characteristics as embryonic stem cells (ES), which are derived from the inner cell mass of the blastocyst[2&3].

Because MAPCs can be isolated from adult individuals their use in research as well as therapeutics is not hampered by ethical considerations as for ES cells. Nevertheless, some safety issues with respect to carcinogenicity have to be addressed, given that these multipotent stem cells are able to produce teratomas and carcinoteratomas (in case of ES cells) when injected in immune deficient mice.

The application of MAPCs in bone regeneration is currently under investigation. As for now, the use of MSCs in bone tissue engineering is a preferred cell population since these cells are already used in clinical trials for multiple applications, included bone engineering.

## SCAFFOLDS

Any tissue consists of a matrix and several cell types. In vivo, the matrix forms a 3D mesh providing cells with many anchorage molecules and signaling proteins and thus providing cells a specific physical and biochemical microenvironment. In this sense, to regenerate bone tissue one needs a template or scaffold that will act as a temporary matrix allowing cell proliferation and extra cellular matrix deposition. Moreover, the scaffold should have sufficient mechanical strength to withstand the mechanical loads during physical activity. It should also actively participate in the regeneration process. Scaffolds in bone engineering need specific

properties such as biocompatibility and biodegradability. The pore size, interconnectivity and surface to volume ratio have to be large enough to allow neo-vascularization, bone in growth and optimal diffusion of nutrients, gases and metabolic waste products; the surface properties, both chemical and topographical, should support the biological mechanisms of osteoconduction and osteoinduction; and they should ideally have the mechanical properties of bone tissue. No material meets all these criteria. The most successful materials for bone engineering are bone mimicking materials, such as demineralized bone matrix (DBM) and hydroxyapatite/tricalcium phosphat e/collagen type I ceramics. They offer either osteo induction or osteo conduction properties. However, these materials are brittle and their biodegradation depends on osteoclast activity which is difficult to control. In contrast, titanium is not biodegradable but can withstand loads during physical activity and it allows bone and vascular in growth.

Alternative materials used in bone engineering are natural and synthetic biodegradable polymers. The natural biodegradable polymers are those obtained from natural sources, either from animal or vegetal source. Synthetic biodegradable polymers are chemically synthesized and processed. The most commonly present natural biodegradable polymers are collagen, fibrinogen, chitosan, starch, hyaluronic acid and silk. Among the synthetic biodegradable polymers are poly (α-hydroxy acids), poly (ε-caprolactone), poly(propylene fumarates), poly(carbonates), poly(phosphazenes) and poly(anhydrides) the most widely used. Although these materials in combination with cells look very promising for bone engineering in vitro, limited success has been achieved when challenging the polymer cell construct in vivo. Nonetheless, major progress has been made by modifying the polymer to increase the affinity for growth factors such as BMP2 and by delivery of the scaffolds as electrospun or self assembling nanofibers.

**BIOCHEMICAL SIGNALS:**

To increase the osteo induction properties of a tissue engineered construct, growth factors are incorporated into ceramics or polymer scaffolds. BMPs are the most extensively used growth factors in bioactive scaffolds, because they were discovered by their ectopic bone induction capacity. Especially BMP2, 4, 6, 7 and 9 are considered as most potent osteo inductive agents. Two of them, BMP2 and 7 are already used in clinical applications such as spine fusion.

Interestingly, combination of BMP4 with VEGF, to improve vascularization in the scaffold, results in superior ectopic bone formation in poly(lactic-co-glycolic acid) scaffolds in vivo . Other growth factors which may be beneficial to enhance bone formation in a tissue engineering construct are Transforming Growth Factor- β (TGF- β), Insulin Growth Factor-I (IGF-I), Fibroblast Growth Factor (FGF), Platelet Derived Growth Factor (PDGF) and Placental Growth Factor (PIGF). The most rudiment procedure to bring growth factors in the scaffold is by dip coating or by mixing freeze-dried growth factor protein with the polymer during manufacturing. More advanced polymers contain glycoaminoglycans (GAGs), such as heparan sulfate or chondroitin sulfate to crosslink BMPs with the polymer. Incorporation of these GAGs will allow creating gradients of growth factors and controlled release of growth factors within the scaffold[4,5&6].

Alternatively, gene delivery of growth factors can be used instead of embedding relatively expensive growth factor protein in the scaffold. Cells are transfected with plasmid DNA encoding the growth factor of interest and seeded in the scaffold prior to implantation. Once in the scaffold the cells start to synthesize the growth factor which is entrapped in the scaffold upon secretion. Commonly, genes are delivered in a cell via adenoviral or retroviral vectors. However, viral based methods raise safety and ethical issues and it will take some time before they will be approved in clinics. To overcome these issues, non viral based methods, such as electroporation, magnetofection and lipid based techniques can be used. Another elegant method to deliver plasmid DNA is through condensation of the DNA with polyethylenimine (PEI) to form an electropositive colloidal particle.

The DNA condensate resist metabolism by endonucleases and spontaneously transfect cells by binding to their anionic surface. Once internalized, PEI permeabilizes the endocytotic vesicles which results in osmotic swelling and disruption of the endosomes, permitting DNA to escape into the cytosol. This method has been successful for the delivery of BMP4 and VEGF plasmids in polymer scaffolds.

**MECHANICAL SIGNALS**

As discussed above, musculoskeletal tissue such as bone and cartilage is highly responsive to its mechanical environment, and it is only through the interaction of mechanical and biological cues that tissue differentiation proceeds to an appropriate morphological and biochemical character that successfully functions in highly loaded situations. In practice, however, the application of mechanical stress on TE-constructs in bone engineering protocols is of limited use because

1) The mechanisms of action of mechanical stress on cells are largely unknown;

2) The effect of mechanical stress on cells is modest as compared to the influence of growth factors on cell behavior; and

3) Mechanical stress has rather a major role in remodeling processes which occurs at the organizational level of tissues which is important for the integration of the TE-construct, whereas bone tissue engineering is currently focused on production of bulk 'bone' matrix at the cellular level. Consequently, most studies investigate the influence of mechanical stress on proliferation, differentiation and matrix deposition of cells cultured on a variety of substrates. Application of stretch for instance, on monolayer human bone marrow derived cells (hBMDCs) and osteoblast cell lines (MG63, SaOs2 and MC3T3) is known to enhance proliferation and osteogenic differentiation. Similar results were obtained on cells seeded in 3D structures in vitro. Also fluid shear stress which causes higher membrane deformations of the cell than stretch, promotes in vitro osteogenic differentiation of BMDCs in 2D and 3D cultures.

Interestingly, BMDC/β-tricalciumphosphate constructs cultured under fluid flow show increased in vivo bone formation when implanted ectopically in syngeneic rats. In contrast, flow cultured BMDCs engrafted in titanium scaffolds contain equal amounts of bone tissue in vivo as the static controls in a calvarial defect rat model.

These two reports illustrate the importance of the carrier material on the cell behavior upon mechanical stimulation. At the molecular level, mechanical strains and shear stresses act through multiple signaling pathways to enhance osteoblast proliferation and differentiation. Many of these signaling pathways involve activation of one or more members of the mitogen-activated protein kinase (MAPK) family, which include extracellular signal-regulated kinases (ERK)-1 and 2 and p38 MAPK pathways ; the Nitric Oxide Synthase (NOS/NO) pathway, the Cyclo- oxygenase-2/Prostaglandine E2 (COX-2/PGE2) pathway and/or interaction with the integrin or calcium influx signaling pathway[7].

**BIOREACTORS**

Bioreactors provide a culture environment to grow a cell-material-growth factor construct. They are designed to seed the cells homogenously in the scaffold, to control the culture medium (temperature, osmolality, levels of oxygen, nutrients, metabolites, regulatory molecules), facilitate mass transfer between the cells and the culture environment, and provide physiologically relevant physical signals (fluid flow, shear, pressure, compression/stretch, torsion). Many different designs, ranging from rotating wall vessels over spinner flasks to hollow fiber reactors have been developed to create and grow tissue constructs under optimized culture conditions. In bone engineering the majority of the bioreactors are perfusion systems. A peristaltic pump pushes the medium from the reservoir through the scaffold to a waste container or back to the reservoir where the medium gets oxygenated and buffered.

Perfusion bioreactors have major advantages;

1) It is a closed loop system, which considerably reduces the risk on bacterial contamination;

2) It can be used for seeding as for culturing TE-constructs for a longer time in vitro; and

3) The fluid shear stress as the medium refresh rate can be controlled by adapting the perfusion speed.

More advanced systems also have pH, glucose and oxygen sensors integrated to track the physical parameters of the medium, which may serve as indicators for cell proliferation, cell metabolism or eventually bacterial contamination in the scaffold. In addition, some bioreactors, such as the Zetos-system, allow application of strain on the scaffolds through compression, therefore, mimicking the in vivo mechanical environment of bone tissue.

In vitro culture of bone engineered constructs in perfusion bioreactors has been successfully used to improve bone formation in vitro. Still, histological analysis of bone TE-constructs after in vitro culture or after implantation in vivo shows major differences. Successful in vivo implanted TE-constructs contain bone spicules filled with osteocytes and surrounded by clearly distinguishable osteoblast layers, blood vessels and fibrous tissue. In contrast, promising in vitro cultured constructs house engrafted osteoblast-like cells that secrete some loose connective tissue and mineralized matrix of which the origin is poorly characterized, i.e. dystrophic mineralization versus bone deposition. This observation illustrates the more advanced level of bone tissue engineering in an in vivo setting than in an ex vivo bioreactor. Consequently, animal models are not only used to test a bone engineering construct, but also to culture a TE-construct. Therefore, animal models can be considered as in vivo bioreactors. Interestingly, plastic surgeons have applied this principle[8].

An example is the reconstruction of the mandible of a 56 year old man which was resected eight years before during ablative tumor surgery. In short, a titanium mesh cage was filled with mineral blocks, collagen type I, BMP7 and a bone marrow aspirate and implanted into a pouch of the patient's right latissimus dorsi muscle. After seven weeks of 'incubation' the implant with a part of the latissimus dorsi muscle containing the thoracodorsal arteria and vein was transplanted into the defect site where the vessel pedicle was then anastomosed onto the external carotid artery and cephalic vein by microsurgical techniques. The connection of the external carotid artery and cephalic vein to the vascular network of the implant was successful since the process of active bone formation was not discontinued. The patient could enjoy his first solid meal nine weeks postoperatively. This case study illustrates the importance of careful surgery which is required to obtain regeneration and functional restoration of a critical sized bone defect.

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