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Mesenchymal Stem Cells for Bone Tissue Regeneration

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Abstract

There are different approaches for treatment of bone fractures; one of them is bone tissue engineering. There are great varieties of clinical cases when the bone grafts are needed. All of them implies to fill a relevant big gap of bone with bone tissue or to supply the bone with a good bioconductive material. One of the most successful approaches for treatment such defects is - bone tissue engineering, which implies cells and scaffold interactions. The most promising cells - are mesenchymal stem cells. They fill a porous scaffold and are cultivated *in vitro*, after that this construct is transplanted into the defect. There are different approaches in creation of bone tissue transplant. All of them means to use different materials for scaffolds (bioceramics, bioglass, demineralized bone matrix etc.), with different properties, different cells (differentiated or stem cells), and different methods of stem cell placement and retention in those materials — it implies different number of cells, different methods of loading those cells, usage of some factors that helps bone formation and blood vessels invasion. Here we would like to make a review of different approaches in bone tissue engineering and to tell what was done in our laboratory in this area.

Introduction

There are numerous clinical cases when the treatment of large bone defects is needed - tumor excision, bone substitution after revision prosthesis surgery, treating bone fractures after different traumas, nonunion, alveoli filling after extraction of teeth, correction of nose defects, legs lengthening in aesthetic surgery. All these cases require a relatively large bone graft to fill such defect.

There are several approaches to treat large bone defects - Ilizarov method (bone transport) and bone graft transplant. Ilizarov technique implies osteotomy and the following bone distraction, and is based on the bone's potential to regeneration. This method is successful in treating of large bone defects up to ten centimeters [1]. But this method has several considerable disadvantages, it is very inconvenient for the patient, requires a long recovery period, and numerous complications are encountered; the most common are wire-site sepsis and fixation instability [2, 3]. Another method is the usage of bone graft, it can be autologous, allogenic or xenogenic origin, or different biological implants of natural or synthetic origin. All of them have certain advantages and disadvantages. But the main purpose of these grafts is to fill the defect and to provide bone's mechanical integrity. Their osteoconductive (they support cell and nutrition infiltration through the three-dimensional porous structure) properties provide bone ingrowths, but some of them are also osteoinductive (contain special proteins, facilitating bone ingrowths and differentiation of osteogenic precursors). And only filled with mesenchymal stem cells these materials provide osteogenic (supply the graft with bone- forming cells) properties.

MESENCHYMAL STEM CELLS

Mesenchymal stem cells (MSCs) are nonhematopoietic cells, plastic adherent, with fibroblast-like morphology (Figure 1). They were first obtained and characterized by Fridenstein in 60s years of 20th century [4]. The number of these cells in bone marrow - the main source of MSCs - is rather low - 0.01-3% [4 - 8]. But they can be cultivated in vitro to increase their number, after 2-3 passages there is about 50-300 mln cells.

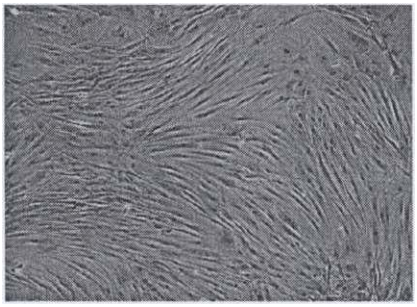

Figure 1. Monolayer of MSC-like cells (x 200) (from bone marrow).

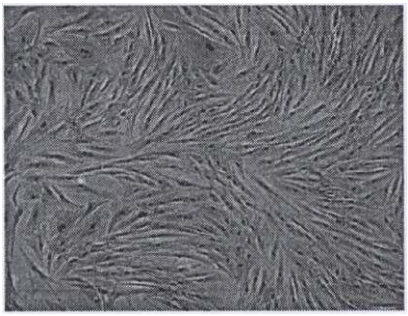

Figure 2. Monolayer of MSC-like cells (x 200) (from adipose tissue).

Apart from bone marrow, MSCs are also located in other tissues of human body. The number of reports of new tissue sources increases intensively. These cells can be obtained from adipose tissue (Figure 2) [9], skin (Figure 3) [10], muscles, heart, liver [11], umbilical cord blood [12, 13], placenta (Figure 4) [14, 15], and peripheral blood [16].

The amount of obtained MSCs varies due to method of their isolation and cultivation. There are also several data that the number of MSCs decreases with age [17].

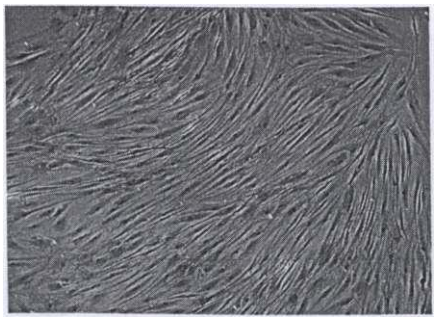

Figure 3. Monolayer of MSC-like cells (x 200) (from derma).

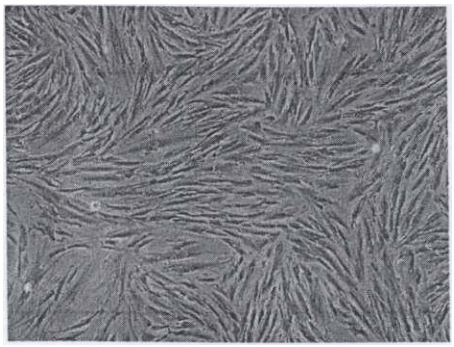

Figure 4. Monolayer of MSC-like cells (x 200) (from placenta).

MSC is a heterogeneous population - they differ in morphology, physiology and expression of surface antigens. Still, there is no single specific marker to identify MCSs. They express a complex of markers, characteristic for mesenchymal, epithelial and muscle cells, and lack hemopoietic and endothelial surface antigens [18]. MSCs isolated from bone marrow express: CD44, CD105 (SH2; endoglin), CD106 (vascular cell adhesion molecule; VCAM-1), CD166, CD29, CD73 (SH3 and SH4), CD90 (Thy-1), CD

117, and STRO-1 [19, 20], At the same time, MSCs do not possess markers typical for hematopoietic and endothelial cell lineages: CD11b, CD14, CD31, CD33, CD34, CD133 and CD45 [20].

MSCs are shown to possess a potential to multilineage differentiation. Under appropriate conditions they can be differentiated into osteoblasts (Figure 5) [21], **adipocytes and chondrocytes (Figure 6, 7) [22, 23, 18]. But it is still not clear if there is one MSC that gives rise to each cell of mesenchymal origin, or a mixture of progenitor cells committed to different cell lineages. There are also controversial data about the ability of MSCs to differentiate into cells of three germ layers [24 - 26].**

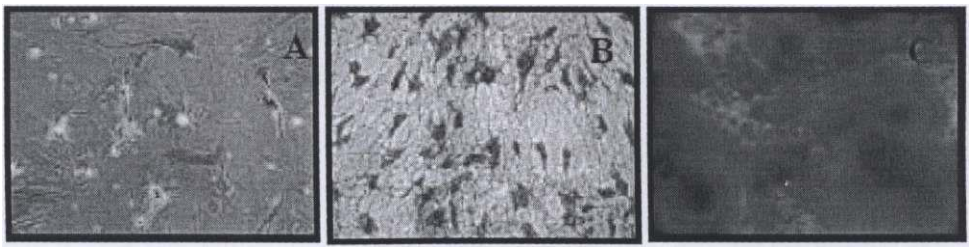

Figure 5. A - Alkaline Phosphatase 14 days after transfer to osteogenic medium (x 200) (MSCs from bone marrow). B - Von Kossa 21 days after transfer to osteogenic medium (x 200) (MSCs from bone marrow). C - Alizarin Red 21 days after transfer to osteogenic medium (x 200) (MSCs from bone marrow).

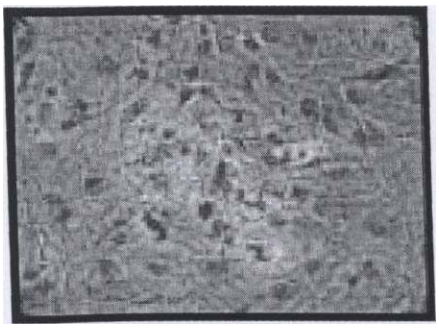

Figure 6. Oil Red 21 days after transfer to adipogenic medium (x 400) (MSCs from bone marrow).

One of the main MSCs advantages is their immunological properties. Immunosuppressive effects of MSCs were shown [27]. Their injections could cure severe graft versus host disease (GVHD) [28]. Furthermore, MSCs injection in immunocompetent baboons prolonged skin allograft survival [29].

MSCs lacks MHC II expressions, so they could not be recognized with immune system, these antigens are absent on the surface, but are detected in the cell. [30], So these cells can be applied as allogenic transplant [31].

Taking into account all above mentioned characteristics of mesenchymal stem cells it becomes clear, that they will be a convenient and promising tool for bone tissue engineering.

These cells have already been shown to be effective in bone tissue formation. MSC showed good osteoinductive properties while they where systematically transplanted into patients with ontogenesis imperfecta [32, 33]. Goshima was the first to show new bone formation in porous bioceramic scaffold, filled with mesenchymal stem cells; new bone deposition was evaluated in mouse subcutaneous model [34]. Different studies were obtained ever since. And numerous studies were undertaken for developing a transplant with seeded mesenchymal stem cells. Though mesenchymal stem cells can be obtained from different tissues, bone marrow MSCs are more commonly used for bone tissue engineering. Still, it is provided, that MSCs from different resources have almost common characteristics [35]. In our research we've compared bone marrow derived mesenchymal stem cells with MSCs from adipose tissue and showed, that although the multilineage potential of bone marrow and adipose tissue derived MSCs was similar according to cell morphology and histology, some minor differences in marker gene expression occurred before and after induction of diverse differentiation pathways. So, BM MSCs are more preferable for bone tissue engineering [36, 37].

So, these cells, with an effective potential to bone formation, are widely used in development of bone tissue transplant.

To deliver cells into the defect, there should be a suitable vehicle. There are a great variety of scaffolds to take; the appropriate one should be chosen for the study. In general, all these materials, which are used for scaffolds fabrication, can be divided into two big groups - natural and synthetic.

NATURAL MATERIALS

Demineralized bone matrix (DBM) is a product of banked allograft, which is prepared by a standardized process. The bone is treated with acid, but first, it is chopped, crushed into particles, then the residual acid is eliminated by rinsing in sterile water, ethanol, and ethyl ether. These are the basic steps, but variables may be in time, acid application, or temperature [38, 39]. DBM has osteoinductive potential, because it still contains growth factors such as bone morphogenic proteins and BMP-7 is most abundant, but, it should be noted that its osteoinductive capacity differs from donor to donor. [39]. Form of demineralized bone matrix vary a lot, it is available as a freeze-dried powder, crushed granules, chips, gel or paste [40], These different forms allow perfect matrix application depending on the injury site shape.

Another natural source of material for graft is natural coral exoskeleton. The structure of the commonly used coral, Porites, is similar to that of cancellous bone, and is used as a bone graft substitute [41 - 43]. Natural coral exhibited progressive resorption, it's biocompatible and osteoinductive properties were evaluated in different animals and in human studies. Coral exoskeleton is just cut into the blocks of necessary size and shape and sterilized [44]. Also it is commonly used for hydroxyapatite (HA) obtainment. But the heating makes the coral HA very brittle, which becomes powder even under the slightest pressure. There is another source of HA of natural origin - bovine-derived hydroxyapatite is also widely used in treatment of bone defects [45, 46].

The other natural material, used for bone tissue engineering - collagen I, fibrin, hyaluronic acid, chitosan - are often xenogenic origin and are used for drugs delivery were there is now need in mechanical support.

SYNTHETIC MATERIALS

Almost all natural materials have significant disadvantages, and the main inconvenience is based on the inability to change their parameters, for example, it is hard to control the rate of their resorption, which is sufficient for new bone growth and growth factors delivery.

So, the alternatives to natural materials are synthetic ones. Based on their chemical composition, synthetic bone grafts can be divided into several groups: metallic implants, ceramics, glass ceramics, and polymers.

Metallic materials are always used in orthopedic or dental surgery for implants fabrication (plates, screws, nails to fix up the entire bone particles). Besides, there are several indications of porous metallic scaffolds with interconnected pores for easy bone ingrowths and vascularize-

986tion. The main materials are titan and its alloys, and stainless steel. The study of these scaffold showed good osteointegration [47 - 49]. Metallic porous scaffold also can be used as growth factors delivery device. These scaffolds where good enough to release TGF-**B**1 (tissue growth factor) [50]. The main disadvantages of metal scaffolds is their inability to biodegrade, the lack of biological recognition on the material surface, release of toxic metallic ions, and so called - stress shielding. But many of these difficulties can be overcome by coating with biocompatible materials. [51 - 55, 56-60].

The most promising materials in bone tissue regeneration are biodegradable synthetic materials.

Synthetic polymer materials are commonly used for implant manufacture and represent an effective alternative to standard metallic fixation. The use of resorbable plates and screw for fixation of pediatric scaffolds is very effective and numerously documented [61, 62]. But there are several complications, concerning late degradation tissue response to polymer particles. It is thought that poly-L-lactic acid slowly degrades into particles with a high crystallinity, and the degradation rate is very low. These particles are still not fully resorbed even after 5, 7 years of implantation [63, 64].

The most commonly used biodegradable polymers for bone tissue engineering are - polyesters: poly(glycolic acid) (PGA), poly-L-lactic acid (PLLA) and their copolymers (PLGA), poly(caprolactone) (PCL) and poly(propylene fumarat) (PPF); and others: polyurethanes (PU), polyethylene glycol (PEG).

Besides implants manufacturing, biodegradable polymers are also used in bone tissue engineering. Biodegradable, with porous structure that can be modified for appropriate bone ingrowths, these polymers represent a promising material for scaffold fabrication [65 - 69].

But the most widely used material is bioceramics. They are well-studied materials for hard tissue, such as bone, teeth, joints regeneration. According to a type of reaction with bone tissue, bioceramics can be divided into bioinert, bioactive and resorbable.

Nearly inert bioceramic, such as AI_2O_3, $Zr0_2$, is nontoxic, non-allergenic, but is less reactive compared with other synthetic materials. These implants bind to the bone due to bone's attachment to the roughness of the material [70, 71].

Bioactive materials consist of two groups - slowly and rapidly resorbing ceramics. They allow forming a chemical bond with living tissue. It is considered, that on the boundary of bioglass and bone, as a result of ion change, Si-OH groups are formed on the surface of the glass, than amorphous calcium phosphate formation occurs which results in hydroxyapadte crystallization and formation of apatite layer. Hydroxyapatite is also bioactive, but there is no silicon in HA implants. It is considered that during incubation biological silicon could be concentrated at the alkaline pH HA interface, this fact produce a surface similar to that of bioactive glasses [72]. Bioactive materials are used as coating on metal implants, providing appropriate biocompatible properties [73].

Rapidly resorbable ceramics - tricalcium phosphate constructs - are used for bone replacement, which especially effective if the velocity of composites degradation is similar to bone growth. Slowly resorbable ceramic - hydroxyapatite - has a rather low rate of degradability. It was evaluated that 100% synthetic HA ceramics have not been resorbed after more than five years from the implantation [74]. The rate of degradation of resorbable bioactive ceramics can be controlled by the creation of composite material with different proportion of HA/TCP content. The higher concentration of HA - less resorbing the composite is, on the contrary, more TCP - more resorbable scaffold is [75].

Bioceramics, of course, have sufficient advantages - it is resorbable and this property can be manipulated, this scaffold can be highly porous, and their size, degree of porosity can be modified. They can be performed in different state and shape. But bioceramics has several disadvantages - it is very brittle, and it does not provide any osteogenic properties.

There are enormous kinds of materials, which are used for scaffolds fabrication and bone defects treatment. They all have advantages and disadvantages, so there was made an approach

to try to avoid some disadvantages of one material by making a composite scaffold with another. Such composite materials are - composites of ceramics with polymer materials, ceramics with metals, natural polymers with bioceramic [76 - 79].

As we can see, all these materials provide osteoconductive and/or osteoinductive properties, and seeding with MSCs supply these composites with osteogenic properties also.

BONE TISSUE ENGINEERING

To make a tissue transplant one should think about the scaffolds characteristic, the type of cells and the way of cells "seeding" the scaffold.

The scaffold is porous and its pores should be interconnected for cell contact, good nutrients delivery and vascularization. Concerning on literature data, the mean pore size should be about 400pm, and the poristosy of the scaffold should be about 60% - these parameters are the most appropriate for cell loading and bone formation [31, 74, 80, 81].

These cells should be appropriately seeded into the matrix. Their number should be sufficient for uniform filling of the whole scaffold's volume. The cell number varies a lot, not only because of the size of scaffold; it varies from $4xl0^6$ cells/cm^3 to $185x10^6/cm^3$ [31, 80, 82, 83]. Our laboratory results show that seeding cell concentration influences not only on settling scaffolds with cells, but also on a differentiation of BM SSCs (bone marrow derived stromal stem cells) in osteogenic pathway. We compared 2.5 x 10^5, $1x10^6$, $2x10^6$ concentrations to assess the efficiency of seeding concentration of cells on bone formation with BM SSCs (bone marrow stromal stem cells) in calcium phosphate scaffolds (Table 1) [84], The seeding cell concentration 2 x $10^6/cm^3$ was optimal for engineering of bone tissue in vitro (Figure 8). For uniform cell loading we used orbital shaker for 2 hours. And this approach showed good results in cells distribution throughout the scaffold. This method was found to be effective not only *in vitro,* but also in *in vivo* studies in sheep's critical size defect model. This study revealed that MSC affect active process of bone reparation, influence protein metabolism state, and determine the way of biochemical reaction during osteogenesis [85].

Table 1.

Number of cells	**2. $5x10^5$**	**lxl O^6**	**2 $xl0^6$**
Effectiveness of load, %	2,5	5,0	7,0
Number of cells out of matrix after 24 hour	3750	50000	38000

But it is easy to load cells onto a small scaffold, about 3x3x3 or 4x4x4 or 7x7x7 mm, which are commonly used for subcutaneous implantation to study ectopic bone formation. They are just poured onto the scaffold and are allowed to attach for several hours, this method allows to spread cells uniformly only when the scaffold is relatively small [67]. And it is more difficult to achieve uniform loading when the defect is big. In this case, the scaffold should be big enough to fill the defect in critical size-defect animal model, and pouring the cell suspension is noneffective. Such big scaffolds are filled with cells by the method of vacuum cell loading. The cell suspension is added to a syringe containing the implant. All loaded carriers are subjected to a vacuum to allow penetration of the mesenchymal stem cell suspension. They also can be seeded with the help of orbital shaker, as we did, and this method turned out to be the most effective one [31, 86]. MSC cells are sometimes suspended in Tissucol (Baxter) or fibrin and after loading, they are treated with thrombin to make a fibrin clot around and within ceramic, to entrap the cells [82, 87]. For the best adhesion of cells, the scaffold can be treated with patient's serum [83], fibronectin, laminin and other extracellular proteins, which help cells to attach.

The effectiveness of regeneration can be increased when activators of osteogenesis are supplemented. It can be bone morphogenic proteins (BMP: BMP-2, BMP-3, BMP-4, BMP-6 or 7) which in their turn are members of TGF-β superfamily proteins. These inductive molecules trigger endochondrial cascade in non differentiated mesenchymal cells to bone formation. It was shown, that BMP-2 directly stimulates differentiation into osteoblasts, and this process can be stimulated with dexamethasone [88]. Therefore, protein containing implants, which stimulate bone regeneration, are more effective in compare with mineral implant [89 -91].

It is still not decided what will be preferable, to use non-differentiated mesenchymal stem cells, or to trigger their differentiation pathway. Mostly, MSCs are used without differentiation [74, 87]. But there are enough literature data, evidencing the differentiation of MSCs, loaded into the scaffold before implantation [92], An interesting approach was undertaken by Kim et.al., who used porous synthetic polymer scaffolds that released biologically active dexamethasone and ascorbate-2-phosphate. These scaffolds were seeded with human MSC and implanted subcutaneously into athymic mice. The successful formation of mineralized bone tissue in vivo was achieved [67].

Bone is a well vascularized organ, so it is important if a bone transplant will be also vascularized, to supply the cells in the inner part of scaffold with nutrients, otherwise, they will be eliminated.

Itself scaffold should be porous, and the size of these pores should be enough to allow vessels to grow. But it is not sufficient when the scaffold is rather big. One of the most difficult problems of bone tissue engineered scaffolds is vascularization of bone implants. One of the approaches is - arteriovenous loop fabrication. But for large defects this method could be applied only with the use of bioreactor [93]. The most common is VEGF (vascular endothelial growth factor) treatment. Biodegradable polymer scaffold are loaded with VEGF, which is released in vivo, attracting endothelial cells [94]. There alternative method is to use endothelial differentiated cells. These cells are mesenchymal stem cells, obtained from different tissues, for example Wharton's jelly, bone marrow, or umbilical cord blood [95, 96]. Fabrication of such tissue engineered constructs is one of the most promising in tissue engineering. It would be more effective to transplant such full-grown bio-construct, to provide the defect with an excellent bioactive, osteoinductive and osteogenic implant, to achieve the most rapid and accurate bone tissue regeneration.

CONCLUSION

In this article we reviewed one of the bone fracture treatments - bone tissue engineering. This technique requires a scaffold to be filled with MSCs in different methods. Scaffold itself can be made from a great variety of materials, natural, synthetic or it can be a composite matrix. All of them have their advantages and disadvantages for bone healing, when they are used alone. But these materials do not have any osteogenic properties, on themselves. MSCs with their proliferative abilities provide these materials with osteogenic properties. The immunologic status allows allogenic application of these cells. So, these cells, with an effective potential to bone formation, are widely used in development of bone tissue transplant. And now it is the most effective and promising method for large bone defects, nonunion and fractures healing.

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