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REVIEW

Recent advances in bone regeneration using adult stem cells

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Abstract

Bone is a highly vascularized tissue reliant on the close spatial and temporal association between blood vessels and bone cells. Therefore, cells that participate in vasculogenesis and osteogenesis play a pivotal role in bone formation during prenatal and postnatal periods. Nevertheless, spontaneous healing of bone fracture is occasionally impaired due to insufficient blood and cellular supply to the site of injury. In these cases, bone regeneration process is interrupted, which might result in delayed union or even nonunion of the fracture. Nonunion fracture is difficult to treat and have a high financial impact. In the last decade, numerous technological advancements in bone tissue engineering and cell-therapy opened new horizon in the field of bone regeneration. This review starts with presentation of the biological processes involved in bone development, bone remodeling, fracture healing process and the microenvironment at bone healing sites. Then, we discuss the rationale for using adult stem cells and listed the characteristics of the available cells for bone regeneration. The mechanism of action and epigenetic regulations for osteogenic differentiation are also described. Finally, we review the literature for translational and clinical trials that investigated the use of adult stem cells (mesenchymal stem cells, endothelial progenitor cells and $CD34^+$ blood progenitors) for bone regeneration.

Key words: Bone tissue; Mesenchymal stem cells; Tissue engineering; Osteogenesis; Angiogenesis; Endothelial progenitor cells

Core tip: Mesenchymal stem cells and blood derived progenitor cells have the potential to open new horizon for the treatment of bone defects. Numerous preclinical and clinical studies demonstrated angiogenic and osteogenic potential of these cells. Additional research is required in order to improve isolation and expansion techniques, as well as long term studies to follow the safety of this cellular approach.

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INTRODUCTION

Incidence of bone fractures that require hospitalization is estimated to be 1 million in the United States alone. About 5%-10% of these fractures present delayed healing or nonunion and represent a major treatment challenge in orthopedic reconstructive surgery[1]. Current gold standard surgical technique for reconstruction of large bony defects involves autologous bone blocks harvested from the fibula, iliac crest, scapula, and radius[2-5]. These procedures require extended hospitalization and a secondary donor site with associated morbidity and complications. In addition to their surgical complications, these techniques are unpredictable and associated with significant graft resorption[6,7]. As an alternative to autologous bone block, recent developments in bone tissue engineering, gene and stem cell therapy are currently examined. A tissue engineering approach offers several potential benefits, including the lack of donor site morbidity, decrease in technical sensitivity of the repair, and most importantly, the ability to closely mimic the *in vivo* microenvironment in an attempt to recapitulate normal bone development[8].

BONE COMPOSITION

The bone comprises of inorganic and organic components. Two thirds of the bone volume consists of inorganic mineral salts: calcium, phosphate, carbonate, citrate, hydroxyl and ions (magnesium, sodium and fluoride) in the form of hydroxyapatite crystals[9]. The organic component is composed of 99% collagen type-1[10] as well as several growth factors osteocalcin, osteonectin, phosphoproteins, proteoglycans and bone morphogenic proteins (BMPs)[11].

In addition, the bone consists of cellular components: pre-osteoblasts, osteoblasts, osteocytes and osteoclasts. Osteoblasts originate from pluripotent mesenchymal cells, characterized as mononucleated, cuboidal cells located at bone margins. Their life span is between 3 d (in young rabbits) and 8 wk (in humans). Osteoblasts produce the inorganic bone matrix (un-mineralized osteoid) and supervise the mineralization process by secretion of alkaline phosphatase. At the end of the process several osteoblasts remain entrapped in the matrix that they have secreted, and develop into osteocytes[12]. These cells are considered mature osteoblasts. Only 10%-20% of the osteoblasts develop into osteocytes. Osteocytes are smaller than osteoblasts, have a larger nucleus to cytoplasm ratio and have a large number of processes which provide intercellular communication. Osteocytes are thought to be the cells responsible for bone regeneration, thus their presence is crucial[13,14]. The process of bone formation is followed by bone resorption. This process is carried out by osteoclasts that secrete enzymes and reduce the pH at the resorption site. Osteoclasts are large, multinucleated, polarized cells with a life span of 15-20 d. These cells originate from hematopoietic precursors of monocytes in the bone marrow, thus comprise phagocytic properties[15].

EMBRYONIC BONE DEVELOPMENT

The development of bone tissue is controlled by tightly regulated programs of cell proliferation, differentiation, survival, and organization. The bone tissue derives from mesenchymal condensations in which osteochondral progenitors give rise to either chondrocytes to form the cartilage or osteoblasts to form the bone[16]. Within the mesenchymal condensations, cells differentiate into chondrocytes or osteoblasts, and form cartilage and bone under the control of various transcription factors. Differentiation of mesenchymal cells into chondrocytes leads to the production of cartilage structures of the future bones of all the skeleton, except for the craniofacial skeleton and the clavicle. Through the process of endochondral ossification, the cartilage structures are subsequently replaced by bone and bone marrow. In the craniofacial skeleton and the clavicle, differentiation of mesenchymal cells into osteoblasts produces intramembranous bones directly from the mesenchymal condensations through the process of intramembraneous ossification[17]. It is well known that bone mineralization during endochondral and intramembranous ossification is tightly coupled with angiogenesis[18].

BONE REMODELING

Bone remodeling occurs throughout life. During this process small amounts of bone are resorbed by the activity of osteoclasts, followed by the recruitment of osteoblast precursors that differentiate and replace the amount of the removed bone[19]. The remodeling process takes place at anatomically distinct sites, termed basic multicellular units, that are dispersed throughout the skeleton[20]. An exact coupling between bone resorption and bone replacement is essential for successful bone remodeling. Thus, bone resorption by osteoclasts has to be matched with the generation of osteoblasts from precursor cells in order to replace the resorbed bone[21]. In the coupling between bone resorption and bone formation several factors are involved, such as growth factors released from the matrix, secreted and membrane-bound factors of osteoclasts, signals from osteocytes, signals from immune cells and signaling taking place within the osteoblast lineage[22].

FRACTURE HEALING AND TREATMENT OF NONUNION

The biologic process of fracture healing is complex and impacted by various factors including the recruitment of stem/progenitor cells from the bone marrow to the fracture site. The healing occurs *via* rapid bone regeneration followed by osteoclast-mediated remodeling, resulting in new bone with structural integrity and a geometrical configuration similar to that prior to the injury[2,23]. In most cases, healing is uneventful, but in some cases delayed or impaired healing occurs. The absence of proper blood supply, improper stability, soft tissue damage and different host features such as age, osteoporosis and smoking have been found to contribute to failure in achieving complete restoration. Nonunion fracture is a dramatic example of poor healing and is defined as non-consolidation at the fracture site within 6 mo[4,5,24]. Autologous bone graft is the gold standard treatment for nonunion fractures. Bone block may be harvested from the iliac crest, fibula or calvaria and transplanted at the recipient site. Bone block stimulates bone regeneration by serving as a scaffold for new bone growth (osteoconduction); moreover it contains cells [osteogenesis, *e.g.,* mesenchymal stem cells (MSCs) and osteoblasts] and growth factors (osteoinduction). However, this technique has several disadvantage such as donor-site morbidity, pain and prolonged hospitalization[2-5].

MICROENVIRONMENT AT OSSEOUS TISSUE HEALING SITE

Healing of small bone defects and bone fractures involves complex and well coordinated interactions between cells, cytokines, and a mechanically stable environment with adequate blood supply[25] to generate new bone instead of a fibrous scar[26]. This complex dynamic process begins with secretion of pro-inflammatory cytokines from aggregated platelets, such as interleukin-1 (IL-1), IL-6 or tumor necrosis factor-α, which have chemotactic activity towards endothelial cells, fibroblasts, lymphocytes and monocytes-macrophages[27]. Transforming growth factor β1 (TGFβ1) plays a key role in this process due to its potent chemotactic and proliferative effect on mesenchymal stem cells, therefore modulate the recruitment of bone cells to the healing site[28]. In addition, TGFβ1 induces the production of extracellular bone matrix proteins such as collagen, osteopontin, and alkaline phosphatase from MSCs and osteoblasts[29]. BMPs and vascular endothelial growth factor (VEGF) are additional growth factors that induce osteogenesis and angiogenesis[30,31]. BMPs belong to the TGF-β superfamily and induce the formation of both bone and cartilage by stimulating the cellular events of mesenchymal progenitor cells. VEGF induces angiogenesis that is crucial for delivering oxygen and progenitor cells to the site[26].

RATIONALE FOR USING ADULT STEM/PROGENITOR CELLS FOR BONE REGENERATION

In the process of bone development and bone healing MSCs and adequate vasculature play a central role. Therefore, cell-based therapy strategies to enhance bone regeneration utilize MSCs and endothelial progenitor cells (EPCs).

MSCs contribute to the maintenance of various tissues, especially bone, in adults[32]. In 2006, the International Society for Cellular Therapy proposed that cells with the following characteristics should be considered as MSCs: (1) cells adherent to plastic in culture; (2) presence of CD105, CD73 and CD90 but absence of CD34, CD45, CD14 or CD11b, CD79α or CD19, and HLA-DR molecules; and (3) cells with the capacity to differentiate into osteoblasts, chondrocytes and adipocytes[33]. MSCs can be isolated from the bone marrow, placenta, umbilical cord blood, adipose tissue, muscle[34], brain[35], kidney, heart and many others[36]. While these cells share common characteristics, they are distinctive, and reflect aspects of their tissue of origin[37]. In animal models, transplantation of MSCs formed ectopic bone and improved healing of bone defects[38,39]. In clinical trials, MSCs regenerate bone in non-union long bone defects[40] in children with osteogenesis imperfecta[41] and in maxillofacial surgeries[42,43]. However, several difficulties concerning the use of MSCs still exist: (1) their aspiration involves an invasive procedure and morbidity[44]; (2) age-dependent decline exists in their proliferation and osteogenic differentiation potentials[45]; and (3) inadequate vascularization to MSC composite graft that causes malfunction and death of the transplanted cells[46].

In 2009, a novel study presented a successful use of blood derived endothelial progenitor cells to heal critical size bone defects[47]. Circulating EPCs were discovered in 1997 by Asahara *et al*[48] and Murohara *et al*[49]. EPCs participate in neovascularization[50], angiogenesis, vascular repair, blood-flow recovery after tissue ischemia, distraction osteogenesis[51,52], fracture healing[53], bone regeneration[54], and have osteogenic potential[55]. EPCs are mainly located in bone marrow and mobilize into the circulation under the guidance of signals such as granulocyte colony-stimulating factor (G-CSF), VEGF, basic fibroblast growth factor, placental growth factor, erythropoietin, or stromal-derived factor-1 (SDF-1) in order to home to ischemic sites[56,57]. EPCs can be isolated from peripheral blood, cord blood or bone marrow[58] and classically described as cells expressing a combination of an endothelial marker (VEGF receptor-2) and hematopoietic progenitor markers (CD34/CD133). However, this definition is under debate, since none of these markers are fully specific[59,60].

In vitro culture of mononuclear cell fraction in specific

conditions consistently produces two distinct EPC subtypes: early EPCs and outgrowth endothelial cells[61] (that are also known as endothelial colony-forming cells[62] or late EPCs because of their late appearance in culture). Early EPCs live < 7 d in culture and have a spindle shape morphology and limited proliferation potential. These cells are CD133, CD31, CD45, VEGFR-2 and CD14 positive[63]. Late outgrowth EPCs emerge 2-3 wk after seeding from the nonmyeloid cell population (CD14 and CD45 negative), present a cobblestone morphology, have a high proliferation potential, express CD34, CD144, and VEGF-R2, but are negative for CD133, CD45 and CD14[63].

Both populations are capable of inducing neovascularization, but with a different mode of action. Whereas early EPCs have limited capacity for population doubling and induce only transient angiogenesis, late outgrowth EPCs can expand to more than 100 population doublings[64]. Early EPCs exert an angiogenic effect mainly by paracrine effect (secretion of proangiogenic growth factors), whereas late outgrowth cells were thought to produce the effect by direct engraftment and may differentiate to osteoblasts in a proper environment[55].

G-CSF-mobilized CD34 positive cells

The use of G-CSF was suggested as a favorable method to harvest abundant $CD34^+$ cells from adult peripheral blood[65]. This alternative approach to isolate EPC was introduced in order to avoid EPC culture and to overcome the scarcity of these cells in the circulation (0.0001% of total mononuclear cells in peripheral blood of human adults)[66]. These cells are also known as endothelial and hematopoietic progenitor cell-rich population and present phenotypic characterization that includes endothelial and hematopoetic markers: positive for CD133, CD31, c-Kit, and CD45 but negative for kinase insert domain protein receptor and CD14. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of these cell revealed weak expression of CD31 and osteocalcin[65]. *In vitro* and *in vivo* experiments using these enriched $CD34^+$ cells demonstrated angiogenic and osteogenic properties similar to expanded EPCs[67]. Peripheral blood and cord blood $CD34^+$ cells were used to enhance tissue neovascularization in several preclinical models: stroke induction in immuno-compromised mice, wound healing in diabetic mice, peripheral nerve injuries and spinal cord injuries[68-71]. Based on the belief that improved neovascularization may be an attractive strategy for bone healing, several studies were conducted to investigate the efficacy of circulating $CD34^+$ cells for bone fracture healing and found promising results[67,72,73].

MECHANISMS OF ACTION IN BONE REGENERATION

The idea of stem cell therapy relies on two possible mechanisms of action: (1) Direct pathway - this process starts with homing of the transplanted cells to ischemic or damaged site. This is followed by integration of the cells into the site and differentiation into cells specific to the tissue[74]; and (2) Indirect, paracrine pathway-transplanted cells secrete peptides (growth factors, interleukins, *etc*.) that modify the environment and recruit resident cells (MSCs and EPCs) to the regenerating site[75].

Homing of transplanted cells into ischemic or injured site allows systemic application of the cells. This includes the recruitment of the cells along a gradient of chemokines and their receptors. The CXCR4-CXCL12 and CX3CR1-CX3CL1 systems are reported to be involved in MSC migration, and among them, SDF-1 alpha and CXCR4 (the SDF-1 receptor) are strong mediators of MSCs and EPCs[76-80].

Following integration into the damaged site, the fate of the cells will be determined by environmental cues and allow osteogenic differentiation of the cells. The osteoblastic differentiation of MSCs was demonstrated *in vitro* by culturing the cells in the presence of ascorbic acid, inorganic phosphate (beta glycerol phosphate), and dexamethasone. *In vivo* studies have suggested that transplantation of adult stem cells derived from one tissue are able to engraft into developmentally unrelated tissues and, in certain cases, contribute to their regeneration[81]. Nevertheless, since *in vivo* transdifferentiation of the cells is more difficult to prove, it was suggested that the supposed differentiation was actually a result of fusion between donor and host cells[82,83]. The second mechanism by which MSCs contribute to bone formation is through indirect paracrine effect. MSCs secrete trophic agents and pro-angiogenic factors [angiopoietin (Ang)-1, -2, Ang-like-1, -2, -3, -4, VEGF, and fibroblast growth factor-2] that recruit resident MSCs to the site of injury and promote angiogenesis that is indispensable for tissue reconstruction[84].

There is only little data regarding the role of EPCs in bone healing. Early outgrowth EPCs exert an angiogenic effect mainly by product secretion, whereas late outgrowth cells were thought to produce the effect by direct engraftment. Direct incorporation of transplanted EPCs into intima layer of blood vessels was demonstrated in several *in vivo* models[48,54,85]. The indirect role of late EPCs to sustain MSC survival and function was demonstrated in an ectopic subcutaneous model[46]. According to this study, EPC-derived paracrine factors *via* platelet-derived growth factor BB/platelet-derived growth factor receptor-β signaling regulate MSC engraftment[46]. The mechanism of action of $CD34^+$ cells was suggested by Kuroda *et al*[86] and includes differentiation of the cells into osteoblasts or endothelial cells and engraftment in the fracture healing site. To confirm this hypothesis, transplanted human cells were labeled in the fracture site using human specific antibodies (immunohistochemestry). Furthermore, human specific endothelial and osteoblastic markers

were identified in the fracture site using RT-PCR. Additional mechanism of $CD34^+$ cells in bone healing can be attributed to their paracrine effect, especially VEGF secretion. Recruitment of resident rat endothelial cells and osteoblasts to the fracture site was demonstrated following human $CD34^+$ cell transplantation. Moreover, injection of VEGF antagonist reduced the vasculogenic and osteogenic effect that was observed following transplantation of $CD34^+$ cells into fracture healing sites[86].

EPIGENETIC REGULATION IN BONE REGENERATION

Epigenetic mechanisms play essential roles in stem cell maintenance and differentiation during bone regeneration processes[87,88]. During the differentiation of stem cells into osteogenic cells specific genes are up-regulated, while others are repressed. Epigenetic regulation is a crucial mechanism involved in the control of the complex, dynamic gene expression pattern during stem cell differentiation. Epigenetic modifications lead to functional relevant alterations in the genome without any changes in the nucleotide sequence[89]. Well known examples for epigenetic modifications are DNA methylation and histone modifications[90]. During DNA methylation, through the enzymatic activity of DNA methyl-transferases (Dnmts), a methyl group is covalently bound to the carbon 5 of the cytosine in the CpG sites of the genome[91]. Methylation of cytosine results in the down-regulation of gene expression[90]. In contrast, the absence of DNA methylation is crucial for gene expression[92]. During bone regeneration DNA methylation is essential. As such it has been documented that CpG methylation of the osteocalcin promoter significantly decreases during *in vitro* osteoblast differentiation of MSCs and thus leads to the up-regulation of osteocalcin expression[87]. In addition, hyper-methylation of the LIN28 promoter (a gene responsible for the maintenance of stemness) reduces LIN28 expression during osteoblast differentiation and hence facilitates osteogenesis[93].

Gene transcription is also regulated by histone modifications[90]. Histones are positively charged proteins that tightly bind to the negatively charged phosphate-sugar backbone of the double stranded DNA. Histone binding to the DNA modulates the accessibility of the DNA for transcription factors[94]. The most investigated histone modifications are acetylation and methylation. Acetylation, mediated by histone acetylases, of the histon's lysin residue reduces the positive charge of the histone and thus decreases histone binding to the DNA. This in turn increases DNA accessibility for transcription factors and hence leads to increased gene expression. On the other hand, histone deacetylases remove acetyl from histones and therefore enhance histone binding to the DNA. This leads to a closed chromatin structure, reduced DNA accessibility for transcription factors and thus decreased gene expression[95]. Histone acetylation plays an important role during bone regeneration. It has been shown that during osteoblast differentiation both the promoter and the coding region of osteocalcin contain high levels of acetylated H4 and H3 histones, pointing to increased transcriptional accessibility to these sites. Furthermore, it has been demonstrated that down-regulation of histone deacetylase 1 is an important process for osteogenesis[96].

Histone methylation often occurs on lysine and arginine residues. Histone methylation is tightly controlled by the balanced activity between methyl transferases and demethylases[95]. Histone methylation is involved in osteogenic differentiation of MSCs. It has been demonstrated that the activation of CDK1 promotes osteogenic differentiation of MSCs through phosphorylation of EZH2, the catalytic subunit of PRC2, that in turn catalyzes the methylation of histone H3 on lysine 27 (H3K27)[97].

The above mentioned examples are only a few describing the complex involvement of epigenetic regulation in bone regeneration. The importance for the understanding of epigenetics in osteogenic differentiation of stem cells gains growing attendance among scientists coping with the development of new technologies for bone regeneration.

TRANSLATIONAL STUDIES

Cell-based pioneer studies in the field of bone regeneration used non-expanded bone marrow derived MSCs. Autologous bone marrow MSCs were used to treat patients with genetic bone metabolism diseases[41] as well as nonunion fractures[40]. However, due to the limited number of MSCs in the bone marrow (0.01% of bone marrow cell population) the results following non-expanded MSC transplantation are unpredictable[98]. Few years later, technological developments in MSC culture allowed expansion and characterization of these cells. The osteogenic potential of expanded, purified and identified MSCs was extensively studied, with conflicting results[42,99]. These inconsistent results can be related to several factors that influence MSC function: donor related differences (species, age, systemic disorders), insufficient vasculature to the grafted area that caused malfunction and apoptosis of the cells[46] and inadequate osteoblastic differentiation of the transplanted cells *in situ*. To overcome these disadvantages, attempts were made to improve osteogenic differentiation of MSCs by genetic modification[100] and to enhance vascularization of the graft[101,102]. MSCs that were engineered to express an osteogenic growth factor such as BMP2 and implanted *in vivo* were shown to differentiate and contribute to the process of bone formation[103-106]. According to their results MSC-BMP2 induced new bone formation in orthotopic (bone defect) and ectopic (intramuscular) models. Histological analysis revealed that MSCs-BMP2 differentiated into osteoblasts and became integrated

in newly formed bone alongside recipient cells that also had differentiated into osteoblasts. In contrast, MSCs that were not genetically transformed survived in the damaged tissue as undifferentiated or fibroblastic cells[98]. Since early vascularization of the graft is crucial for successful bone regeneration, Kumar *et al*[101] examined the vasculogenic and osteogenic potential of MSC expressing BMP2 and VEGF (alone or in combination). In the dual therapy group, improved vasculogenesis preceded increased bone healing, suggesting that increased vascularity during the bone-healing process allows continuous flow of all the necessary nutrients and cells needed for bone regeneration. Another approach to improve vascularization of MSC-composite graft was presented by Koob *et al*[102] who used co-culture of human umbilical vein endothelial cells (HUVECs) and MSCs. HUVECs and MSCs were seeded alone or in combination into xenograft scaffolds and implanted into calvarial critical-sized defects. Neo-vessel formation was considerably higher in the co-implantation group, suggesting that implanted MSCs supported HUVEC-triggered neovascularization. In addition, implanted MSCs effectively supported bone formation in calvarial defects. However, the human HUVEC-derived neo-vasculature did not improve MSC-triggered bone regeneration in this orthotopic critical-sized defect model[102].

With the novel discovery of circulating EPCs[48], and the detection of cells expressing endothelial markers in newly regenerating bone tissue[107], a new idea to treat bone defects using EPCs was considered. Transplantation of autologous late outgrowth EPCs into critical size gap in sheep tibiae revealed full bridging at 3 mo in 6 out of 7 EPC-transplanted defects while non or minimal new bone formation was observed radiographically in 8 sham-operated defects[47]. These authors also suggested that the effect of EPC is not limited to vasculogenesis. When sheep EPCs were sub-cultured under osteogenic conditions, they changed their morphology and formed nodular aggregates (1-2 mm diameter) following 1-2 wk incubation and stained positively for markers of osteoblastic differentiation[55]. These results were confirmed by other research groups that showed promising results with late EPCs[108,109]. However, none of them utilized expanded human late EPCs. This could be related to difficulties in human late EPC culturing that demands further improvement. The negligible concentration of circulating EPCs in humans is probably the main obstacle in this process; however, their concentration increases following trauma, bone fractures, malignancy, tissue ischemia, physical activity and GMCSF administration[110]. Contrary to the difficulties arising with human late EPC culture, early human EPCs (that have only limited proliferation capacity) are more accessible, therefore can alternatively be used. Seebach *et al*[54] compared healing of critical size defect in athymic rats following transplantation of human early EPCs/bone marrow MSCs/and coculture of EPCs + MSCs. Early vascularization was significantly improved in the EPC + MSC group or EPC group. Bone formation increased significantly when treated with co-culture of MSCs + EPCs and to a lesser extent in the MSC group. Surprisingly, transplantation of early EPCs that increased early vascularization failed to enhance bone formation. Therefore, it is suggested that there is a synergistic effect and that the initial stage of neovascularization by EPCs is considered to be crucial for complete bone regeneration by MSCs in the late phase[54].

EARLY CLINICAL FINDINGS - BONE REGENERATION USING AUTOLOGOUS STEM/PROGENITOR CELLS

One of the leading research groups in the field of bone regeneration recently published a randomized controlled clinical trial in which human autologous cells were transplanted into bone defects with promising results. Bone repair cells were produced from bone marrow aspirates, isolated, cultured and characterized. Flow cytometry demonstrated that these cells were enriched for mesenchymal and vascular phenotypes. Cell suspension was absorbed onto a gelatin sponge that was transplanted into the extraction site and covered by a bio-absorbable collagen barrier membrane. Six weeks later, a biopsy of the regenerated site revealed highly vascularized, mineralized bone tissue formation[111]. Promising results were also reported following transplantation of human autologous bone marrow derived MSCs to augment cleft palate and maxillary sinus[112,113]. Orthopedic use of bone marrow MSCs to treat long bone fractures was also recently reported[114]. Autologous osteoblasts, isolated from bone marrow and cultured in osteogenic conditioned medium, were transplanted into the fracture two weeks post internal fixation. They showed statistically significant acceleration of fracture healing in the experimental group that was treated with cells compared with the nontransplanted group. Importantly, none of these studies reported on significant inflammatory reaction or serious adverse effects.

From a clinical point of view peripheral blood progenitor cells are more accessible compared with bone marrow derived MSCs. Therefore attempts are made to isolate, culture and transplant blood derived progenitor cells into bone defects. The first clinical case of tibial nonunion treated with peripheral blood progenitors (G-CSF-mobilized $CD34^+$ cells) was published by Kuroda *et al*[115]. In this case report, delayed healing and nonunion were observed following primary operation that included transplantation of autologous iliac bone block. Nine months later, mobilized $CD34^+$ cells were transplanted into the site and resulted in bone union without any symptoms including pain and gait disturbance[115]. Encouraged by these results, the same research group began a phase Ⅰ/Ⅱa clinical trial: Autologous local transplantation of G-CSF-mobilized

peripheral blood $CD34^+$ cells for patients with tibial or femoral nonunion[110]. Five days following G-CSF injection, each patient's cells were magnet sorted to separate the $CD34^+$ cells. Treatment included autologous transplantation of G-CSF-mobilized peripheral blood $CD34^+$ cells suspended in atherocollagen gel. Radiological fracture healing at 12 wk was achieved in five of seven (71.4%) patients, which was greater than the threshold (18.1%) predefined by the historical outcome of standard care.

SAFTEY OF ADULT STEM CELL TRANSPLANTATION

Clinical trials that assessed the safety of MSC transplantation for the treatment of graft-*vs*-host disease, ischemic heart disease, spinal cord injury, and systemic lupus erythematosus, did not find any significant adverse effect[116-120]. Nevertheless, studies that investigated the role of MSCs in tumor-genesis found that MSCs have the ability to interact with tumor cells, support tumor angiogenesis and tumor development by providing the matrix required for new vessels and tumor cell scaffolding[121,122]. Therefore, the potential of MSCs to facilitate growth of preexisting occult tumors raised concerns[123,124]. *In vitro* transdiffrentiation of MSCs was also reported but may be related to contamination with cancer cells[125,126].

EPCs initiate vasculogenesis, as such, these cells play a critical role in pathological vasculature in tumor growth[121] and metastasis[127]. Recent clinical studies demonstrated an increase in the amount of circulating EPCs in patients suffering from malignant tumors and in cases of invasive or recurrence of the disease[128,129]. These observations underline the need for careful phenotypic, functional, and genetic characterization of EPCs/MSCs before cell administration or therapy. Also, the question of whether patients should be treated with autologous, or donor-derived progenitor-cells remains to be addressed.

PROSPECTIVE

There is a significant clinical need to develop innovative approaches for the treatment of large bone defects, nonunion fractures and alveolar bone atrophy. In recent years, many efforts are being made to regenerate bone using stem cells and progenitor cells. While animal studies and limited human studies have shown efficacy of bone marrow derived MSCs and circulating EPCs, the mechanisms by which these cells improve bone regeneration are still unclear. Numerous factors restrict the clinical use of stem cells: immune rejection of cells that are not of autologous origin; limited amount of available cells for transplantation that requires *in vitro* cell expansion with xenograft materials; donor related differences (*e.g.*, age and systemic conditions) that affect cell function and the absence of potency test that can predict the *in vivo* function of cells before transplantation. Moreover, safety and regulation of these procedures have to be performed before clinical trials. To overcome these drawbacks, new technologies that will allow increasing the amount of cells available for transplantation and standardization of growth conditions need to be developed. In addition, understanding the mechanisms of action will enable a breakthrough in bone regeneration induced by cells.

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