**REVIEW ARTICLE**

Exploring the application of mesenchymal stem cells in bone repair and regeneration

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Failure of bone repair is a challenging problem in the management of fractures. There is a limited supply of autologous bone grafts for treating nonunions, with associated morbidity after harvesting. There is need for a better source of cells for repair. Mesenchymal stem cells (MSCs) hold promise for healing of bone because of their capacity to differentiate into osteoblasts and their availability from a wide variety of sources. Our review aims to evaluate the available clinical evidence and recent progress in strategies which attempt to use autologous and heterologous MSCs in clinical practice, including genetically-modified MSCs and those grown on scaffolds. We have compared various procedures for isolating and expanding a sufficient number of MSCs for use in a clinical setting.

There are now a number of clinical studies which have shown that implantation of MSCs is an effective, safe and durable method for aiding the repair and regeneration of bone.

MSCs and bone repair

After a fracture, a haematoma forms which prevents excessive extravasation. The platelets, inflammatory cells and macrophages arriving at the site of injury secrete cytokines and growth factors, including platelet-derived-growth factor, bone-morphogenetic proteins (BMPs), vascular-endothelial-growth-factor and interleukin-1 to -6. This cellular response triggers the invasion of the MSCs which differentiate into osteoblasts and chondrocytes in order to complete the repair. MSCs from the periosteum, bone marrow, circulating blood and the surrounding soft tissues have been shown to contribute to bone repair in rodent models. The management of a defect in the...
femur of athymic rats showed that a ceramic scaffold loaded with expanded MSCs gave significantly more new bone formation at 12 weeks and stronger bone than a scaffold without MSCs. This study confirmed that the MSCs formed bone by differentiating into osteoblasts.\(^8\)

MSCs are non-immunogenic. They do not express major histocompatibility class II and co-stimulatory molecules, including CD40, CD80, and CD86.\(^12\) Hence, allogeneic transplantation of MSCs should not require immunosuppression of the host. Most importantly, MSCs do not induce the proliferation of lymphocytes.\(^13\) They have immunosuppressive properties and suppress the proliferation of T-cells and cytokine production in response to alloantigens or insignificant mitogens, as well as inhibiting the function of B cells,\(^14\) dendritic cells\(^15\) and the natural killer cells.\(^16\)

MSCs show homing potential which is affected by numerous cytokines and growth factors, including stromal cell derived growth factor-1 (SDF-1) and its receptor CXCR4, which have been shown to act as a potential homing signal for MSCs in bone healing. In a mouse model of a femoral defect, Kitaori et al\(^17\) showed that bone formation was mediated by expression of SDF-1 in the periostum, causing recruitment of MSCs to the bone lesion. However, some studies have reported low levels of CXCR4 expressed by MSCs and have found that blocking CXCR4 had no effect on the migration of MSCs, suggesting that other molecules may be involved. There is no consensus on the mode of migration of MSCs since some studies have shown them in the circulation,\(^18\) whereas others have found no evidence of this.\(^19\)

**Isolation and expansion of MSCs**

MSCs are frequently isolated from the marrow of the superior iliac crest, but MSCs from the femur and the tibia during hip and knee replacements are a suitable alternative. However, since only 0.001% to 0.01% of mononuclear cells from bone marrow are MSCs,\(^20\) an efficient method of isolation is required. This is usually achieved by density gradient centrifugation using Ficoll or Percoll.\(^21\) When cells are layered over Ficoll or Percoll and centrifuged, layers of red blood cells, fat cells and mononuclear cells (MNCs) are formed according to their densities. The MNCs containing potential MSCs which form the middle layer can be aspirated, purified (Fig. 1a) and expanded (Fig. 1b) in a short period of time. With optimal conditions they can be cultured up to passage 30, although the proliferation and differentiation potentials of MSCs are reduced because of senescence, the Hayflick effect and telomere shortening.\(^22\) Early passage cells (< 10) are considered to be most useful and their senescence can be circumvented by the addition of growth factors or by expanding them in a three-dimensional bioreactor which mimics the environment in vivo.\(^24\) An important barrier to the use of ex vivo expanded MSCs is the risk of introducing pathogens and xenoinmunisation because of the use of fetal bovine serum for their culture. Increasingly, serum-free media are used for culture thus making it possible to use them for clinical applications.\(^23\) The main therapeutic usefulness of MSCs is their ability to differentiate into osteocytes, chondrocytes and adipocytes in the ex vivo environment (Fig. 2). They can be differentiated in vitro into osteoblasts by the addition of dexamethasone and ascorbic acid, although no study has compared whether differentiated or undifferentiated MSCs differ in their ability to aid bone regeneration.

**Application of stem cells in bone regeneration**

After MSCs are expanded ex vivo they are either introduced by systemic infusion, or growth on a scaffold and
applied directly to the site of the lesion, or genetically modified before being used in a scaffold.

**Expanded MSCs introduced by systemic infusion.** Animal models have shown that MSCs can migrate to the bone marrow after peripheral injection and remain there for an extended duration. Studies have shown the successful infusion of *ex vivo* expanded MSCs into human volunteers indicating that this is feasible and well tolerated. Systemic infusion of MSCs for bone regeneration has been successfully used by Horwitz et al. in treating osteogenesis imperfecta. Six children with severe osteogenesis imperfecta received two infusions of allogeneic MSCs. Five children showed homing of MSCs into one or more sites including bone, skin and marrow stroma with an acceleration of bone growth of between 60% and 94% compared with matched unaffected children. However, this technique has not been used in the repair of fractures. Direct application of MSCs to the fracture is deemed to be more practical, with research into systemic infusion of MSCs being more academically driven.

**Application of MSCs grown on scaffolds.** Scaffolds serve as carriers for cultured MSCs before implantation. Scaffolds need to mimic the natural environment of the bone matrix and should be safe to be used in clinical practice. The synergistic effect of using composites of scaffolds with growth factors has been shown to increase the formation and vascularisation of bone. Numerous scaffolds have been investigated in pre-clinical studies, although hydroxyapatite (HA) and calcium phosphate seem to be favoured because of their excellent osteoconductive properties. HA provides good strength but is not resorbed, while beta-tricalcium phosphate (β-TCP) is fragile but has a greater capacity for resorption. Hence, a combination of HA and β-TCP, biphasic calcium phosphate, is typically used. HA also has poor mechanical properties and bone formed using an HA composite cannot maintain the mechanical loading needed for remodelling. In order to overcome this, HA can be combined with biodegradable polymer/bioceramic composites, including polyactic-co-glycolic acid, which allows for better control over shaping micro- and macrostructure composites for bone regeneration.
The fibres of the extracellular matrix (ECM), their interconnecting pores and HA crystals making up the bone tissue all have nano-scale dimensions (<100 nm in one dimension). Therefore manufacturing nano-composite materials is of interest since it provides three-dimensional (3D) constructs which fit the size of the surrounding matrix, promoting cell adhesion and matrix interactions. More recently, mechanical stability has been identified as an important factor in the repair of fractures. Three-dimensional polymer scaffolds with dimensions of 150 μm to 500 μm have been shown to have excellent stability. Ex vivo mechanical loading has an additive effect on BMP2-induced osteogenesis in genetically-engineered MSC-like cells, and mechanical stimulation of MSCs has shown them to differentiate into adipocytes, chondrocytes and osteocytes.

Quarto et al expanded bone-marrow-derived stem cells for three weeks and seeded them on to macroporous HA scaffolds to treat nonunion. At seven months the three treated patients showed good integration of the implant. Angiographic evaluation after seven years showed vascularisation of the grafted zone, which is vital for the survival and future stability of the graft. Before such scaffolds can be used in clinical practice, they need to be tested for long-term stability and biocompatibility. The ex vivo differentiation of MSCs into osteoblasts is another technique which has emerged. Morishita et al used an HA scaffold to differentiate MSCs ex vivo into osteoblasts to heal the defect in a patient after curettage of a tumour, which illustrated that tissue-engineered osteogenic ceramics may be an alternative to autologous bone grafting. A tissue-engineered prosthesis has been used successfully in three patients with osteoarthritis of the ankle with no adverse reactions and high clinical scores. Kitoh et al injected differentiated bone-marrow-derived stem cells with platelet-rich plasma without a scaffold into three femora and two tibiae in two patients undergoing distraction osteogenesis to obtain the target lengths without major complications.

MSCs have been successfully used in the treatment of osteonecrosis of the femoral head. Kawate et al cultured MSCs and applied MSCs/beta-TCP composite granules for steroid-induced osteonecrosis of the femoral head and found that this treatment prevented progression and showed early bone regeneration at 34 months, but it was not useful in the presence of pre-operative collapse.
Allograft bone chips containing bone-marrow-derived cells have been used for spinal fusion. Expanded MSCs have also been used in spinal fusion using porous β-TCP scaffolds seeded with MSCs. Of 41 patients, fusion was demonstrated in 95%. With limited evidence supporting scaffolds seeded with MSCs. Of 41 patients, fusion was demonstrated in 95%.54 With limited evidence supporting the role of MSCs in spinal fusion, further investigation using randomised control trials is required.

Genetically modified MSCs. Although the combination of growth factors and scaffolds remains a promising approach, there are limitations in the long-term release of growth factors to promote the proliferation and maintenance of MSCs. Therefore genetic modification of MSCs to express growth factors, which involves either transfection of MSCs through viral vectors or by the use of non-viral vectors, is a suitable alternative. Viral vectors have been shown to elicit immune reactions and have variable ability to transfect dividing and non-dividing cells efficiently.55 However, compared with non-viral vectors, they show better expression of the desired protein and their efficiency in transfection is better. Viral vectors are often the optimal choice for gene delivery in MSCs.

Numerous osteoinductive growth factors have been used to modify MSCs and have been shown to give successful bone induction in vivo. BMP-2-transfected MSCs showed bone formation in mouse hindlimbs and in bony union of critical-sized mouse radial defects. In another study, Lin et al compared adipose- and bone-marrow-derived stem cells (ADSC/BMSCs) which had been genetically modified with BMP-4 to repair defects in the calvarial bone in rabbits and found no significant difference in bone regeneration. Furthermore, in vitro studies showed that deposition of ECM was significantly higher in differentiated ADSCs than in BMSCs. Fat was detected in ADSC-seeded ECM, thus requiring further investigation of their use in bone defects especially in those bearing load. To date, no clinical studies have applied ex vivo-expanded genetically-modified MSCs because of the need to identify the optimal growth factor and the vector to ensure effective, safe and consistent treatment.

Application of non-expanded MSCs for bone regeneration
MSCs can be applied for bone regeneration without expansion ex vivo in order to avoid cost and time. After the preliminary work by Herzog in 1951 which demonstrated the procedure of percutaneous bone grafting, many clinical studies have successfully applied MSCs for bone regeneration. In 1995 Connolly demonstrated a series of 100 skeletal healing problems, including delayed unions and nonunions of fractures, arthrodeses, and bone defects that MSCs were effective in bone repair when applied in this way. However, this and earlier studies did not report the number of MSCs needed to give optimum healing.

Hernigou et al showed that bone healing depended on the number and concentration of transplanted MSCs. They found that seven of 60 patients with defects of the shaft of the tibia did not achieve union. In these patients the mean number of MSCs in the graft was < 1000 cells per cm³ and < 30 000 cells in total. Both the mean concentration and the mean number in patients who had not achieved union were significantly lower (p < 0.01) than in those in whom union was successful. Therefore they considered that a graft needed to contain at least > 1000 MSCs per cm³ to achieve union. This has implications for the technique used to isolate MSCs since the aspirate is not guaranteed to contain the required total cell count. However, recent studies by Wongchueonsoontorn et al have demonstrated two techniques for increasing the volume of bone marrow applied to a bone defect. First, small volumes preferably within the range of 2 ml to 4 ml should be aspirated from the site since larger volumes dilute the bone marrow with blood, and secondly, the concentration of MSCs should be increased by centrifuging the aspirate before injection. In contrast to the classic laboratory procedure, separation by density gradient does not require training and allows the processing of stem cells at the bedside. Although Hernigou et al demonstrated an approximate estimate for the number of MSCs required for bone regeneration, the number of viable cells after implantation into man has not yet been analysed.

The future of MSCs for clinical practice
The process of harvesting MSCs is a simple, stepwise process as illustrated in Figure 4. It is clear from the various
studies discussed above that the application of MSCs to bone defects enhances bone formation without any adverse reactions to the patients. However, the success is limited so far because of the small numbers in the trials, the lack of controls and the short follow-up. Furthermore, most studies have investigated the application of MSCs in ‘worst-case’ clinical situations. Future studies need to explore the use of MSCs as a first-line treatment for bone defects, after the acquisition of adequate data to verify their effectiveness in bone repair and regeneration.

The transition of MSCs to clinical practice is developing fast as evidenced by major advances in studies performed on animals by introducing scaffolds and gene therapy. However, there are aspects of the application of MSCs which need further investigation (Fig. 5). Despite the advances in using MSCs on scaffolds, few studies have applied this technique in clinical trials. Studies in man can evolve by comparative studies to discover the optimal scaffold and by expansion into nano-scaffolds, particularly under strain models to determine mechanical stability. Genetic modification of MSCs has not been addressed in human bone healing because further studies are required to find the optimal and best combination of growth factors along with finding optimal non-viral vectors.

The future of stem cells looks promising as advances in tissue engineering, biomaterials and cell biology converge to enable stem cells to play a major role in the repair and regeneration of bone.
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References


