Review article

Cell therapy for bone repair

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\textbf{A B S T R A C T}

When natural bone repair mechanisms fail, autologous bone grafting is the current standard of care. The osteogenic cells and bone matrix in the graft provide the osteo-inductive and osteo-conductive properties required for successful bone repair. Bone marrow (BM) mesenchymal stem cells (MSCs) can differentiate into osteogenic cells. MSC-based cell therapy holds promise for promoting bone repair. The amount of MSCs available from iliac-crest aspirates is too small to be clinically useful, and either concentration or culture must therefore be used to expand the MSC population. MSCs can be administered alone via percutaneous injection or implanted during open surgery with a biomaterial, usually biphasic hydroxyapatite/calcium-triphosphate granules. Encouraging preliminary results have been obtained in patients with delayed healing of long bone fractures or avascular necrosis of the femoral head. Bone tissue engineering involves in vitro MSC culturing on biomaterials to obtain colonisation of the biomaterial and differentiation of the cells. The biomaterial-cell construct is then implanted into the zone to be treated. Few published data are available on bone tissue engineering. Much work remains to be done before determining whether this method is suitable for the routine filling of bone tissue defects. Increasing cell survival and promoting implant vascularisation are major challenges. Improved expertise with culturing techniques, together with the incorporation of regulatory requirements, will open the way to high-quality clinical trials investigating the usefulness of cell therapy as a method for achieving bone repair. Cell therapy avoids the drawbacks of autologous bone grafting, preserving the bone stock and diminishing treatment invasiveness.

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Physiological bone repair results in the production of normal bone. Unfavourable local conditions (e.g., inadequate blood supply, soft tissue injury, or mechanical instability) and/or extensive bone tissue loss may result in failure of physiological bone repair with delayed healing, nonunion, or a persistent bone defect. In these situations, autologous bone grafting is the current standard of care. The osteogenic cells and bone matrix in the graft provide the osteo-inductive and osteo-conductive properties required for new bone formation. However, drawbacks of autologous bone grafting include donor-site morbidity [1], limited availability of autologous bone, and loss of bone stock. Attention has therefore turned to other options, such as allogeneic bone grafts and bone substitutes, which supply an osteo-conductive matrix. Cytokines, most notably bone morphogenetic proteins (BMPs) can be added to produce osteo-inductive effects. Physical methods (e.g., electromagnetic fields and ultrasounds) remain to be evaluated.

Cell therapy holds promise as an alternative to autologous bone grafting for promoting bone repair. Bone progenitor cells are supplied to the injury site, either alone or in combination with a mineral or protein matrix and/or cytokines. In bone tissue engineering, the cells are cultured, alone or on a biomaterial, before implantation. Cell therapy spares the bone stock and diminishes treatment invasiveness.

This conference reviews the current use of cell therapy for bone repair in humans, chiefly at long bone sites, to achieve either fracture healing or bone defect filling. Cell therapy for disorders of bone metabolism (osteoporosis), osteogenesis imperfecta, and gene therapy will not be discussed.

1. Physiology of bone repair

Bone tissue is capable of self-repair, which results in the production of new bone exhibiting all the characteristics of normal bone. Fracture healing or bone defect filling by an autologous cancellous bone graft results from interactions among osteogenic cells,
cytokines, an osteo-conductive matrix, and a mechanically stable environment with a good blood supply, according to the ‘diamond concept’ [2].

In rare cases, the cortices undergo primary healing after perfect fracture reduction and stabilisation. Usually, however, fracture healing involves intra-membranous and enchondral ossification. This complex dynamic process requires the precise orchestration of various events during four overlapping stages [3] having distinctive histological characteristics: an inflammatory response, formation of a cartilaginous soft callus, formation of a bone hard callus, and bone union with remodelling. This process involves a sequence of anabolic and catabolic events, some of which are non-specific (production then remodelling of the cartilaginous callus) and others specific (formation of the bone callus, which is then remodelled into normal bone). Thus, bone resorption plays a crucial role, and the resorption and formation processes are not separate or independent in time and space. The final result of the bone repair process is the production by the cells of a collagen matrix, whose ossification restores the normal mechanical properties of the bone. These histologically defined stages of bone repair require a number of cellular events (migration, proliferation, and differentiation), whose coordination is ensured by cytokines and growth factors.

Inflammation plays a role of paramount importance at the beginning of the bone repair process. The injury triggers the release of pro-inflammatory cytokines (interleukins IL-1β and IL-6, TNFα), whose chemotactic effects attract inflammatory cells and stimulate angiogenesis at the fracture site. Cell types that are more specific to the bone repair process are involved subsequently. Although the molecular mechanisms that regulate cell proliferation and differentiation have been partly elucidated, no biological markers of use for the clinical monitoring of bone healing have been identified to date.

2. Cell types involved in bone repair

The bone repair process mobilises many cell types. Despite having no direct role in bone formation, the cell types involved in the inflammatory and angiogenic responses are indispensable to the development of the bone formation mechanisms. They release cytokines and growth factors (PDGF, BMPs, VEGF, and interleukins) that attract and activate the mesenchymal stem cells (MSCs) directly involved in bone repair.

MSCs are precursors of osteoprogenitor cells. They play a key role in cell therapy for bone repair, as they are the best characterised multipotent cells and can now be produced reliably for clinical purposes. The osteoblast lineage makes a major contribution to bone remodelling but is not currently used for clinical applications.

Friedenstein et al. [4] were the first to demonstrate new bone formation from cultured bone marrow (BM) cells. The BM cells proliferated in vitro, generating colonies of fibroblast-like cells, or ‘colony-forming unit fibroblasts’ (CFU-Fs). MSCs are defined as multipotent non-haematopoietic cells capable of differentiating into functional cell types found in various mesenchymatous tissues (bone, cartilage, muscle, tendon, adipose tissue, and haematopoietic stroma) [5]. The self-renewal capacity of MSCs ensures that they maintain their multipotency throughout their life span.

MSCs can be identified in vitro based on their ability to adhere to plastic culture dishes and to generate CFU-Fs after several days of culture in standard medium containing foetal calf serum. Then, depending on the available induction influences, MSCs can differentiate into bone tissue cells (osteoblasts), cartilage cells (chondrocytes), and adipose cells (adipocytes) [5]. In the in vitro MSC phenotype is characterised by absence of expression of membrane molecules specific of haematopoietic cells (CD45, CD14, and CD34), contrasting with the presence of other molecules (CD73, CD44, CD105, CD90, and CD146). No marker strictly specific of MSCs is available [6], a fact that complicates the reliable identification of MSCs and their extraction from the pool of nucleate BM cells.

MSCs were first identified in BM [5] then in adipose tissue [7], cord blood, the placenta [8], the periosteum [9], and other tissues. MSCs from these different sources share similar phenotypic characteristics but differ regarding their differentiation and proliferation properties. It should be noted that these MSCs are identified only after culturing, Native MSCs (naturally found in tissues), in contrast, are poorly characterised and difficult to identify. Native MSCs have been identified in blood vessel walls [10].

The source of the MSCs present at sites of bone repair, particularly after a fracture, is difficult to determine. In animal models, these cells come from the periosteum, BM, and neighbouring soft tissues. The most obvious source of MSCs is the BM, in which MSCs contribute 0.001% to 0.01% of all mononuclear cells in healthy adults, with a decrease over the life span [11,12]. One millilitre of BM contains only 18 ± 7 × 10⁶ mononuclear cells including 612 ± 134 MSCs [13]. BM concentration and culturing techniques are therefore valuable to expand the MSC population available for clinical use.

Methods are now available for expanding MSCs in compliance with current regulatory requirements for use in clinical applications [14]. Current culture media contain no animal products and are based on human platelet lysates designed for optimal safety. Within 2–3 weeks, a 30-ml sample of iliac-c crest BM generates several million MSCs, depending on the available culture surface area. During culturing, MSC differentiation to cartilage, adipose, or bone cells can be induced. Acquiring a high level of expertise with MSC production and differentiation to osteoprogenitor cells is crucial to successful bone repair. Differentiation of cultured MSCs to the osteoblastic lineage (osteoid-induction) can be obtained by adding BMP (BMP2 or BMP4) or dexamethasone.

Differentiating MSCs release growth factors and cytokines that contribute to regulate the bone repair process. The fluctuations over time in the production of growth factors and cytokines are poorly known, a fact that limits our ability to obtain precise therapeutic effects by using these molecules.

An interesting characteristic of MSCs pertains to the immune system: MSCs are not immunogenic, because they express little or no major histocompatibility complex Class II molecules and induce no T-cell proliferation. On the contrary, MSCs have immunosuppressive properties related to their ability to inhibit T-cell proliferation and NK-cell lysis under allogeneic conditions [15,16]. These properties may enable allogeneic MSC transplantation without immunosuppressive therapy of the recipient and suggest a role for universal MSC banks for regenerative medicine.

Another major advantage of MSCs is a high level of resilience with preservation of bone repair capabilities even after several hours of transport.

3. Cell therapy approaches to bone repair

According to the diamond concept [2], MSCs play a crucial role in bone repair. Cell therapy can serve as an alternative to autologous bone grafting. A large number of osteoprogenitor cells are implanted at the injury site, either alone or combined with a matrix. BM MSCs are currently the most appropriate cells for inducing bone repair, as they have a strong osteogenic potential and are easily obtained by culturing iliac-crest aspirates.

Several MSC-based cell therapy modalities have been developed, i.e., with and without cell culturing and with or without a matrix.

The mononuclear cell fraction of the BM, which contains the MSCs, can be used directly by percutaneous injection of
aspirated BM into the injury site. BM is aspirated into heparin syringes, in small fractions of 2–4 mL, as blood aspirated in larger fractions dilutes the BM and therefore decreases the MSC concentration [13]. To increase the number of injected mononuclear cells and consequently of MSCs, the aspirated BM can be concentrated by centrifugation. After concentration, Hernigou et al. [13] obtained $2579 \pm 1121$ MSCs per mL, i.e., 3-fold to 6-fold the number obtained after aspiration. Concentrated or unconcentrated mononuclear cells can be combined intraoperatively with a synthetic or natural osteo-conducting matrix (e.g., allogeneic bone graft or coral) (Fig. 1) before implantation.

Mononuclear cells can be cultured in vitro to enable the selection and expansion of MSCs. This method generates millions of MSCs, whereas only a few thousand are present initially in the aspirate. Cultured MSCs can be injected percutaneously or combined with biomaterials to produce a construct appropriate for bone grafting (Fig. 1).

MSCs can be cultured in vitro on biomaterials for a few days or weeks. Biomaterial colonisation and cell differentiation is obtained, and the construct is then implanted into the injury site. This procedure follows the bioreactor concept used in bone tissue engineering (Fig. 2).

One option consists in implanting the construct into a muscle for a few weeks to promote angiogenesis. The muscle containing the construct is then harvested and transplanted into the injury site. An anastomosis with the muscle vascular pedicle is created to ensure an adequate blood supply. Thus, the patient becomes his or her own bioreactor [17].

Several biomaterials can be chosen for combination with the cells, depending on the goal (mechanical strength or filling) and approach (percutaneous or surgical). The most widely used biomaterials are calcium-phosphate ceramics, which usually combine hydroxyapatite and β-tricalcium phosphate as granules or, more rarely, sticks, and exhibit interconnected pores each measuring 100–400 µm. These biomaterials promote the adhesion,
proliferation, and osteoblastic differentiation of MSCs, as well as the production of the collagen matrix that subsequently undergoes mineralisation. Collagen sponges and biodegradable polymers can be used also. The biomaterials must be absorbable, at a variable rate depending on their anticipated biomechanical role, and must allow the ingrowth of newly formed blood vessels from the neighbouring tissues. Good-quality vascularisation of the tissue in contact with the implant is crucial.

4. Cell therapy for bone repair in humans

Factors that influence bone union or bone defect filling include the site, defect size, quality of the overlying skin and muscle, and quality of the blood supply. Translation research from animal bone repair models to humans remains limited, as detailed in a previous article [18]. Only the most significant case-series are discussed here.

4.1. Delayed union or nonunion with no bone defect

Delayed union and nonunion are common complications for which the current standard of care is autologous cancellous bone grafting, combined with internal fixation. A muscle flap is used if the bone is exposed. Autologous bone grafting is a form of cell therapy, since the graft supplies BM cells including MSCs, as well as bone matrix.

4.1.1. Initial attempts at cell therapy relied on BM alone

One method consisted in aspirating the BM and re-injecting it percutaneously. In 1991, Connolly et al. reported a positive correlation between bone union and the cell concentration in the BM aspirates [19]. The treatment was successful in 18 of their 20 patients with tibial nonunion. In a study by Garg et al. [20] in patients with long bone nonunion treated with two BM injections 3 weeks apart, bone union was achieved within 5 months in 17 of the 20 patients.

Another option consists in concentrating the BM aspirates to increase the number of injected MSCs. In patients with nonunion, Hernigou and Beaujean [21] observed decreased MSC densities not only at the nonunion site, but also in the iliac-crest BM aspirates, a finding replicated recently by Mathieu et al. [22], who also reported decreased cytokine and growth factor levels. Hernigou et al. [23] used BM concentrates prepared from a mean aspirate volume of 300 mL and obtained healing of 53/60 (88%) tibial nonunions. The healing rate increased with the injected MSC concentration. Patients whose fractures did not heal received fewer than 1000 MSCs per mL and fewer than 30,000 MSCs in all, whereas those who healed received significantly higher MSC concentrations and counts, with a mean of 1500 MSCs per mL and 54,000 MSCs in all, in a volume of 20 mL.

BM concentration can be performed from BM aspirates of 300–500 mL, by accredited cell therapy laboratories. A fraction of the recovered mononuclear cells is kept at the laboratory for determination of the CFU-F count, which reflects the MSC concentration within the re-injected mononuclear cells. Concentration of smaller BM samples, performed in the operating room, produces fewer mononuclear cells, mirroring the smaller initial population, and does not allow CFU-F quantification to evaluate the number of re-injected MSCs. This procedure can be used, however, in situations that do not require a large number of MSCs.

Few published studies assessed the combined use of concentrated or unconcentrated BM with a biomaterial. This method is a valid option for everyday practice, provided CE-marked biomaterials are used and concentration (if used) is achieved via an approved procedure.

Ateşchâr et al. [24] studied 15 patients with infected tibial nonunion. After eradication of the infection, cancellous bone allografts vitalised with an injection of autologous BM were implanted. They obtained infection control in 14 patients and bone union in 11 patients. Dallari et al. [25] compared bone chips alone, bone chips with platelet gel, and bone chips with platelet gel and BM cell concentrations in patients treated with tibial osteotomy for genu varum. The outcomes were better in the groups that received platelet gel with or without BM cells.

4.1.2. The MSC population can be expanded in vitro

During lengthening of 51 femurs or tibias, the use of culture-expanded MSCs and platelet-rich plasma was associated with a significant improvement in the healing index compared to 60 control bones [26]. The healing index was better for the femur than for the tibia, suggesting a role for the blood supply and soft tissues.

In a prospective randomised trial of 64 closed long bone fractures with delayed healing, callus formation after 2 months was improved in the group given cultured MSCs compared to the control group [27].

4.2. Treatment of bone defects

Bone defects are more challenging to treat. The success rate depends on the size and quality of the adjacent soft tissues. For bone defects no larger than 2–3 cm, autologous bone grafting alone may be sufficient. A very useful technique for larger defects is the Masquelet procedure, in which cement is implanted to induce the formation of a membrane, whose osteo-inductive properties then promote the union of the autologous cancellous bone graft that is implanted subsequently. The use of vascularised bone grafts such as the fibula averts the risk of graft necrosis. The Ilizarov technique often requires an additional graft at the end of the procedure. Drawbacks of allografts implanted into large bone defects include partial re-inhabitation, a risk of absorption, and a risk of infectious agent transmission.

Percutaneous injection of unconcentrated BM into unicameral cysts was inferior to glucocorticoid injections in one study [28] and successful in another [29]. Park et al. [30] compared surgery with bone chip and autologous BM implantation to percutaneous injection of allogeneic demineralized bone matrix and autologous BM in 23 patients with calcaneal unicameral cysts. The outcomes were similar, with a lower morbidity rate in the percutaneous injection group.

In a study of 79 acetabular defects with 87% of type III (American Academy of Orthopaedic Surgeons classification) during revision hip replacement surgery, Ochs et al. [31] obtained similar outcomes with frozen non-irradiated allografts and with allografts vitalised by autologous bone marrow after irradiation to eliminate the risk of pathogen transmission.

Quarto et al. [32] were the first to report the use of cultured BM MSCs combined intra-operatively with hydroxyapatite blocks to fill large bone defects (4–7 cm). They successfully treated 3 patients, with defects in the tibia, humerus, and ulna, respectively. A subsequent study confirmed healing of the defects after 6–7 years [33].

The next level of complexity involves culturing the MSCs for several days or weeks on a biomaterial, which is then implanted into the bone defect.

Morishita et al. [34] used this technique successfully in 3 patients with benign bone tumours. BM MSCs were allowed to proliferate for 2 weeks then cultured on hydroxyapatite granules for 2 weeks in a medium designed to promote osteoblastic differentiation. The construct was then implanted into the defects. Osteo-integration was satisfactory 29 months later.

Meijer et al. [35] reported outcomes in 6 patients with jaw defects treated using this technique. In biopsies obtained after 4 months, bone formation was detected in 3 patients but was induced by the construct in a single patient. The authors pointed out...
the importance of differentiating bone formation induced by cells from the border of the defect and bone production by the implanted cells. Nevertheless, disappearance of the implanted MSCs does not rule out a key role for these cells in the initial osteo-induction process.

A radically different approach consists in using the patient as his or her own bioreactor. Warnke et al. [17] successfully reconstructed a 7-cm mandibular defect using a bone and muscle construct that was previously prepared in vivo. Titanium mesh designed to fit the bone defect was loaded with hydroxyapatite granules coated with recombinant human BMP-7 and un-concentrated BM. This construct was then implanted into the latissimus dorsi muscle to allow for ingrowth of vessels from the muscle. After 7 weeks, the construct was transferred to the bone defect, with an anastomosis of the muscle vascular pedicle to the external carotid artery. Bone density increased over time, and new bone was detected throughout the replacement. Unfortunately, a fracture and an infection developed, and the patient died of cardiac arrest 15 months after the implantation.

4.3. Avascular necrosis of the femoral head

Cell therapy undoubtedly has a place in the treatment of avascular femoral head necrosis, as re-inhabitable matrix persists within the necrotic bone. Hernigou et al. [36] reported a decrease in the MSC population within the proximal femurs of patients with glucocorticoid-induced femoral head necrosis. Avascular necrosis can be viewed as a stromal disease, suggesting that injection or implantation of BM or MSCs may hold therapeutic potential. This hypothesis was confirmed [37] in a study of 534 hips with early stage avascular necrosis of the femoral head, without loss of sphericity. Core decompression was combined with grafting of concentrated autologous BM aspirated from the iliac crest. After 8–18 years, only 94 hips had required replacement surgery [37]. Also in early avascular femoral head necrosis, a randomised study of 18 hips showed better 24-month outcomes with core decompression and autologous BM mononuclear cell grafting than with core decompression alone [38].

Another study compared 30 hips treated by implantation within the decompression tunnel of a hydroxyapatite cylinder loaded with concentrated BM mononuclear cells to 8 control hips treated with the cylinder alone [39]. After a mean follow-up of 29 months, the necrotic lesion was smaller in the BM group, in which only 3/30 hips showed progression to extensive collapse, compared to 6/9 controls. Another randomised trial in early stage avascular femoral head necrosis showed significantly better outcomes after the injection of 2 × 10⁶ ex vivo-expanded autologous BM MSCs than after core decompression [40].

These studies establish that cell therapy can help to achieve bone repair. Additional studies with better designs are needed. Technical challenges and regulatory requirements contribute to explain the paucity of published data. Cell therapy indications are recapitulated on Fig. 3.

5. Safety and regulatory requirements

At present, only autologous MSCs are used for bone repair cell therapy. Intra-operative BM concentration in the operating room using small centrifuges and CE-marked kits does not require authorisation and is performed under the responsibility of the surgeon. If the sample is taken out of the operating room for concentration at another facility, this facility must hold accreditation from the French Drug and Healthcare Product Safety Agency (ANSM), which is the case of cell therapy departments and most French Blood Product Units. In a study conducted in France by Hernigou et al. [37] in over 1000 hips treated with concentrated BM, no procedure-related complications were recorded.

MSC culturing for use in humans is classified in Europe (European Commission 1394/2007) as an ‘advanced therapy medicinal product’ and can be performed only in facilities that comply with Good Manufacturing Practices [14]. In France, accreditation by the Drug and Healthcare Product Safety Agency is required also. Specific rules designed to ensure safety and quality must be followed during harvesting and culturing, and tests must be performed on the cells produced (sterility, viability, and characteristic membrane markers) before their implantation. Cases of aneuploidy related to cell senescence have been reported. Extensively cultured cells are at risk for senescence, which can impair their self-renewing potential. However, senescence has been reported only with far longer culturing times than those used in clinical practice. In addition, no cases of tumour development have been reported with human MSCs. Tarte et al. [41] found no evidence of deleterious changes or malignant transformation of cultured MSCs used in two national multicentre immune-haematology trials.

However, the immunomodulating effects of MSCs and their stromal properties (ability to maintain the survival and growth of associated cells) warrant caution in patients treated for neoplastic diseases, most notably bone malignancies.

6. The future

Delayed union, non-union, and osteonecrosis will no doubt be the leading indications for cell therapy bone repair in everyday practice. As part of the European REBORNE programme (www.reborne.org), a multicentre trial was started in March 2013 to assess the use of cultured autologous BM cells loaded intra-operatively onto biphasic calcium-phosphate granules as an alternative to autologous cancellous bone grafting in patients with delayed union. In addition, a multicentre trial of cultured autologous MSC injection in patients with avascular femoral head necrosis is scheduled to start in 2014.

The development of hydrogel-based solutions for supplying cells and biomaterials percutaneously can be expected in the future. The immunosuppressive effects of MSCs may allow the transplantation of allogeneic MSCs in various orthopaedic conditions, with the establishment of cell banks for regenerative medicine. Trials evaluating allogeneic MSCs (Mesoblast) in delayed union are under way in Australia.

The next step will consist in medico-economic evaluations of the benefits of bone repair cell therapy.

The development of tissue engineering techniques for the treatment of large bone defects [42] raises far greater challenges. A multidisciplinary approach will be required to improve implanted cell survival and to ensure prompt vessel ingrowth into the biomaterial via careful selection of structure and shape, together with addition of cytokines and growth factors.
The regulatory requirements derived from the field of medications will have to evolve in accordance with the specific characteristics of cell therapy trials in surgery.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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