Mesenchymal stem cells: from biology to clinical use

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Introduction

Stem cells are immature progenitor cells capable of self-renewal and multilineage differentiation through a process of asymmetric mitosis that leads to two daughter cells, one identical to the stem cell and one capable of differentiation into more mature cells.

Stem cells may be: 1) *totipotent*, i.e. early embryonic cells (1-3 days from oocyte fertilization), which can give rise to all the embryonic tissues and placenta; 2) *pluripotent*, i.e. embryonic cells from blastocystis (days 4-14 after oocyte fertilization), which can differentiate only into embryonic tissues belonging to the inner cell mass (ectoderm, mesoderm, and endoderm); or 3) *multipotent*, i.e. embryonic cells from the 14th day onwards, fetal stem cells, cord blood stem cells, and adult stem cells, which can give rise only to tissues belonging to one embryonic germ layer (ectoderm or mesoderm or endoderm).

Mesenchymal stem cells (MSC) are non-haematopoietic cell precursors initially found in the bone marrow, but actually present in many other tissues. MSC in culture are adherent, proliferating, and capable of multilineage differentiation into several tissues of mesenchymal origin, such as bone marrow stroma, adipose tissue, bone, cartilage, tendon, skeletal muscle, visceral mesoderm, and endothelial cells[1-5]. Well known and used for bone regeneration for many years, MSC came in the limelight at the end of the 1990s thanks to the evidence that, despite their adult stem cell nature, these cells are capable of pluripotent differentiation, which may be useful for regenerative medicine. In addition, since the beginning of 2000 it has become clear that MSC possess immune regulatory properties that may make them useful in autoimmune diseases.

Mesenchymal stem cells

The presence of MSC of bone marrow origin was formally demonstrated in the second half of the 1970s[1], by seeding whole bone marrow samples in culture plastic disks and removing non-adherent cells after some hours. The few adherent "fibroblastic-like" cells formed small cell clusters, defined fibroblast-colony forming units (CFU-F)[1,6]. After several culture passages, surviving cells became homogeneous and retained their ability to replicate and form cartilage and bone cells[1].

Several studies later confirmed the multipotency of these cells. In the presence of adequate stimuli they differentiate into adipocytes (with formation of cytoplasmic vacuoles containing lipids), osteoblasts (with deposits of hydroxyapatite crystals), chondrocytes (with synthesis of cartilage matrix) and muscle cells (rich in myotubes). This differentiation is detectable through the use of appropriate cell staining and immunochemistry reactions[2-5,7,8]. MSC are also capable of expressing genes of embryonic origin, cell-cell contact molecules, extracellular matrix, such as interstitial type I collagen, fibronectin, type IV collagen and basal membrane laminin. MSC may also secrete cytokines such as interleukin (IL)-7, IL-8, IL-11, stem cell factor (SCF), and stromal-derived-factor-1 (SDF-1) that regulates the homing of haematopoietic stem cells into the bone marrow[2-5,7-11]. MSC normally renew the stromal microenvironment necessary for haematopoiesis. Indeed, MSC are capable of supporting *in vitro* long-term haematopoietic cultures very efficiently[12]. Patients undergoing allogeneic bone marrow transplantation show a defect oin the stromal cells' capacity to support the growth of haematopoietic progenitors[13]; a reduced support to granulocyte-monocyte-colony-forming unit (CFU-GM) formation by bone marrow stroma is well documentable even in patients undergoing autologous and/or chemotherapeutic treatments[14]. Moreover, co-infusion of MSC and haematopoietic stem cells leads to more rapid haematological recovery after high-dose chemotherapy as compared to haematopoietic stem cell transplant alone[15].

Blood Transfus 2007; 5: 120-129 DOI 10.2450/2007.0029-07
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MSC are relatively rare in the bone marrow ($1/10^5$ mononuclear cells), but they can proliferate very efficiently preserving their stem cell properties *in vivo*[16,17]. The progressive loss of differentiation potential because of senescence generally occurs after about 40 doublings[16,17]. MSC may also differentiate *in vitro* into cells of non-mesodermal origin, such as neurons, skin and gut epithelial cells, hepatocytes and pneumocytes[1-5,18-22], although there is a lack of precision regarding terminology in some papers. MSC are considered different from: (i) *multipotent adult progenitor cells* (MAPC), which may differentiate *in vitro* into endothelial, epithelial, and neural cells, as well as cells of mesenchymal origin[5], and are probably the common progenitors of haematopoietic and mesenchymal stem cells; (ii) *marrow stromal cells* or *multipotent mesenchymal stromal cells*, which possess multilineage differentiation potential restricted only to tissues deriving from mesoderm (fat, bone, cartilage, muscle)[23]. The discrepancy between terminology and biological features is probably due to variability in methodologies used by different researchers, rather than to the real co-existence of different stem cells of mesenchymal origin, even though a gradient of MSC differentiation potential probably exists, similarly to that for haematopoietic stem cell precursors. Some tissue factors, such as basic fibroblast growth factor (bFGF) or heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF), besides enhancing proliferation, may interfere with the differentiation potential of MSC, thus influencing their multipotency[24].

Some Authors have shown that very small populations of MSC circulate in the peripheral blood[25,26]. More recently, MSC have also been detected in tissues other than bone marrow, such as subcutaneous fat (adipose tissue-derived adult stem cells, ADAS)[27-29], scalp subcutaneous tissues[30], periodontal ligament[31], umbilical cord blood[32], foetal tissues[33-35], as well as lymphoid tissues such as lymph nodes[36], and adult human and mouse spleen and thymus[37,38], thus suggesting that a *"mesenchymal system"* is virtually present in all adult tissues[39]. In practice, however, only adipose tissue- and cord blood-derived MSC seem to be alternatives to bone marrow-derived MSC for clinical use, although with some differences in terms of CFU-F frequency (higher for adipose tissue-derived MSC, very low for cord blood-derived MSC), immunophenotype (lower expression of CD106 in adipose tissue-derived MSC and of CD90 and CD105 in cord blood-derived MSC), differentiation potential (reduced in cord blood-derived MSC), and gene expression[27-29,40-44].

MSC can be obtained *ex vivo* from bone marrow samples or from tissues disaggregated into single cell components and resuspended in culture medium. Cells may be seeded in plates or flasks at different concentrations with culture media such as modified Eagle medium (α-MEM) or Dulbecco's modified Eagle medium (D-MEM), enriched with 5-15% foetal bovine serum and antibiotics, and cultured under appropriate conditions[1-5,24,37,38]. After a few days, adherent cells form some proliferating clusters with at least 50 cells (CFU-F) that are counted after 10 days and put in relation with the initial seeded cell population to quantify the clonogenic potential of that tissue[1,6,37,38]. Adherent cell clusters grow very quickly and become confluent, so that cells have to be re-plated periodically for the further expansion. A homogeneous, adherent cell population is generally achieved after 3-5 weeks of culture and keeps proliferating for up to 40 doublings without differentiating spontaneously[2-5,16-19,24,37,38,44,45].

Using specific media, MSC can be induced to differentiate *in vitro* into different lineages of mesodermal origin, such as adipogenic, osteogenic, chondrogenic, and myogenic lineages[2-5,16-19,24,37,38,44-46] (Figure 1). Bone marrow MSC normally express low levels of neural markers[47]. By conditioning MSC with different cytokines, such as bFGF and EGF, some dramatic changes of MSC morphology resembling neural cells may be rapidly achieved together with the strong expression of specific neural markers such as nestin, neurofilaments, MAP-2, β-tubulin and Neu-N. On the other hand, MSC-mature neural cell co-culture as well as MSC injection inside animal brains lead to further cell maturation, with the acquisition of mature glial and neural features and neuronal-like excitability[19,47-54]. Bone marrow MSC may be induced to differentiate into neurones by co-culturing them with Schwann cells[55]. In addition, even more mature neural or astroglial morphology may be obtained by co-culturing neural-primed MSC with astrocytes[5,19,47-55] or Schwann cells[37].

Immunophenotype

So far, there are still no specific markers for recognising MSC. MSC may be identified by the lack of expression of haematopoietic (i.e. CD45 and CD34) and endothelial (CD31/PECAM-1) markers, as well as by the expression of combinations of surface molecules such as CD105 (SH2 or endoglin), CD73 (SH3 and SH4), CD106 (VCAM-1), CD44 (hyaluronic acid receptor), CD90 (Thy 1.1), CD29, STRO-1, CD54 (ICAM-1), CD13, CD47, CD146, CD49a, CD164, and CD166[2-5,16-18,23,24,37,38,44,45,28-35,56-59]. Many other markers may be expressed by MSC, e.g. adhesion molecules, chemokines, cytokine receptors (even of epithelial origin), such as epidermal growth factor receptor (EGFR or HER-1)[24], and molecules involved in immune responses (MHC

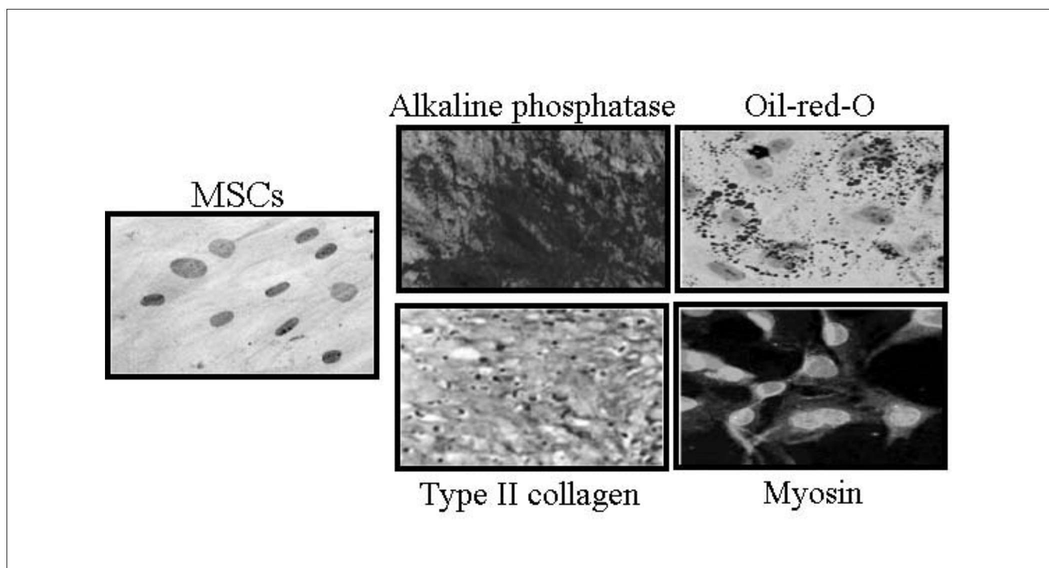

Figure 1 - MSC multilineage differentiation *in vitro* following culture with specific media. Alkaline phosphatase, Oil-red-O, Type II collagen, and Myosin: staining for osteocyte, adipocyte, condrocyte, and myocyte differentiation, respectively.

class I and II, CD119/interferon-g-receptor)[56,57]. Human MSC expanded *in vitro* from the bone marrow of patients with haematological neoplasms may heterogeneously express some molecules, such as CD105, CD90, CD184 and HLA-DR, and this feature inversely correlates with bone marrow angiogenesis[58]. Consequently, it is still difficult to compare precisely the phenotypic pattern of MSC expanded *in vitro* with that really expressed *in vivo* in the tissues. Only *in vitro* and *in vivo* functional studies in animals may aid the assessment of the MSC nature of these cells.

Immune regulation

MSC possess strong immune regulatory properties that are present in different animal species, although with variable and only partially clarified mechanisms. MSC may suppress immune reactions *in vitro* and *in vivo* in a major histocompatibility complex (MHC)-independent manner[56,57,60].

They inhibit T-cell proliferation in response to polyclonal, non-specific stimuli[61], but in a mouse model they can also inhibit antigen-specific immune responses, mediated through both naïve and memory T cells, in a dose-dependent fashion and strictly associated with cell-cell contact[60].

The inhibitory properties of MSC affect practically all kinds of immune effector, including CD4+ and CD8+ T cells[56,57,60-65], B cells[56,66], NK cells[56,67,68], and monocyte-derived dendritic cells[69-72]. The MSC interaction determines lymphocyte[62] and dendritic cell[73] anergy due to early proliferation arrest. Immune regulatory effects are expressed not only by MSC, but also by differentiated cells such as fibroblasts, adipocytes, and osteoblasts[61,74].

In vivo, MSC prolong the survival of MHC-incompatible skin transplants in baboons[63]; in humans they lower the risk of graft-versus-host disease (GvHD) when transplanted together with haematopoietic stem cells[75]; they cure the symptoms of grade IV GvHD, refractory to immunosuppressive therapy[76]; and, in mice, they improve the clinical features of experimentally induced autoimmune encephalomyelitis[77].

Various mechanisms are involved in MSC immune regulatory properties, including the release of soluble factors and cell-cell contact[56,57,60-72]. Unlike in the mouse model[60], in humans the inhibitory effect of MSC persists even in the absence of cell-cell contact[56,65,78,79]. Among various soluble factors, transforming growth factor-b1, hepatocyte growth factor[61,67], prostaglandin E_2, vascular endothelial growth factor[67,72], and indoleamine 2,3-dioxygenase[38,56,64] have been shown to play a role in MSC-mediated immune regulation. Even interferon-gamma, which is a main activation molecule for immune responses, induces MSC immune regulatory effects towards CD4+ and CD8+ T cells, NK cells, and B cells[56].

The expansion of CD4+CD25+ (Foxp3+) regulatory T cells in the target cell population has been shown by some Authors[72], although this evidence is still controversial[56, 60].

Blood Transfus 2007; 5: 120-129 DOI 10.2450/2007.0029-07

The existence of many different mechanisms demonstrates a redundancy of the inhibitory function of MSC, suggesting its relevance also *in vivo*.

Mesenchymal stem cells for clinical use

MSC for clinical use must be collected and expanded *ex vivo* in dedicated facilities, with filtered laminar flow of environmental air and controlled access ('stem cell factory'), in compliance with Good Manufacturing Practice (GMP) rules, which are normally used for industrial production of intravenously-administered drugs. These rules are absolute sterility, specific reagents without autologous proteins and growth factors not authorised for clinical use, and numerous microbiological, virological, immunological, immunophenotypic and functional quality controls to guarantee that the cell product that will be used *in vivo* is safe, qualitatively corresponds to the requirements imposed by law, and possibly effective. Each phase of the *ex vivo* cell production must be standardised and traceable, from sample collection (bone marrow, adipose tissue, cord blood, etc.), to cell seeding and culture (even by using closed culture systems to prevent any kind of contamination), to adherent cell splitting, harvest, qualitative characterisation, cryopreservation, and *in vivo* inoculation[80]. Obviously, the place where cell production is carried out is pivotal. In the facilities dedicated to cell manipulation the 'class cascade', i.e. the presence of different areas compartimentalised according to the GMP rules, is fundamental: the laboratories must have a very low air contamination by particles (class B), contain sterile woods with virtually no particle air contamination (class A, suitable for cell manipulation), and an access filter-zone confined in class B, where the wearing of disposable clothes and access are controlled. Access to the laboratories is obtained through confined areas with higher particle air contamination (class C), which are reached through a wearing room (class D), which, in turn, is connected to the external part of the 'stem cell factory' and has similar particle air contamination. Thus, there is always a one-way access to the laboratories for cell manipulation, from the areas with higher particle air contamination to the virtually sterile areas; in addition, disposable clothes and accurate disinfection are used to prevent any risk to the cell product. Particle contamination below the maximum values approved for each area is achieved through the maintenance of air pressure gradients (about 15 Pa) between the highest and the lowest class area, and through specific systems of air filtering, recycling, and vertical fluxes (for more details see: European cGMP - Annex 1: Manufacture of Sterile Medicinal Products).

Regenerative medicine

Bone regeneration

MSC have been used in several animal models to repair major segmental bone defects[81,82]. In a mouse model of *osteogenesis imperfecta*, a congenital disease of mesenchymal tissues characterised by defective bone formation, bone marrow MSC were infused into irradiated mice, with formation of normally functioning bone and cartilage tissues deriving from the transplanted cells[83]. Three months after their infusion into children with *osteogenesis imperfecta,* MSC caused an increase of the osteoblastic component, formation of new laminar bone, a general improvement in the total mineral content, reduction in the frequency of pathological fractures, and measurable body growth[84].

MSC seeding onto natural or synthetic biomaterials represents the most effective way to induce regeneration and repair of bone, cartilage or tendon tissues[85-87]. In particular, non-porous, biologically inert materials, such as ceramic and titanium, have been replaced by porous biomaterials, which are reabsorbable and osteoconductive, such as hydroxyapatite and tricalcium phosphate[88,89]. Some biodegradable polymers, such as poly-L-lactide (PLA) and poly-L-lactide-co-glycolide (PLGA)[90] are also effective. This approach has been successfully used *in vivo* for the resolution of critical segmental bone defects in which spontaneous local regeneration does not occur and which are unresponsive to the implantation of osteoconductive devices alone[91]. Local implantation of porous biomaterials covered with autologous bone marrow MSC represents the most effective approach to repairing bone defects[92], such as avulsed phalanx[93] and wide mandibular defects[94].

Cartilage regeneration

Up to a few years ago, the only approach to cure joint cartilage defects consisted in the local injection of autologous, *in vitro*-expanded, chondrocyte suspensions, described for the first time in 1994[95]. More recently, bone marrow MSC have been used *in vivo* to repair partial or complete cartilage or meniscus defects in animal models, exploiting several types of biomatrices, especially hyaluronate, as the support[96-100]. In these animal models there has been evidence of meniscus regeneration, reduction in subchondral bone remodelling, less joint cartilage degeneration, and reduced formation of osteophytes as compared with controls treated with hyaluronate only; all these effects were produced without signs of inflammation, thus confirming, *in vivo,* the immune regulatory effect of MSC[99]. Similar results have been

obtained using autologous MSC seeded in a gelatinous matrix of type I collagen or hyaluronate and calcium phosphate, and applied to major osteochondral defects of the knee joint[100,101].

Other types of matrix, based on synthetic polymers such as PLA and PLGA[102], or the addition of factors such as recombinant human bone morphogenetic protein-2[103,104], improve the effectiveness of treatment with MSC. The combined approach of MSC, bioactive matrices, and osteoconductive growth factors is most effective for treating joint cartilage defects[103,104]. Autologous bone marrow MSC have also been used for the treatment of patients with osteoarthritis, exploiting the immune regulatory effect of these cells: arthroscopic and histological improvements have been recorded, although a significant clinical recovery, as compared with controls, has not been observed[105].

Regeneration of tendon, skeletal muscle, and myocardium

The use of MSC to induce tendon repair has been investigated in animal models and humans[102,106]. Autologous MSC, dispersed in type I collagen gel, can produce about 20% recovery of tendon functions, although in a dose-independent way and with heterotopic bone formation in about 30% of cases[107]. A similar approach led to a 37% improvement of the biomechanicaal properties, tissue architecture and functions of Achilles' tendons as compared to those of normal controls at 12 months after transplantation[108]. Some exogenous growth/differentiation factors (GDF), such as GDF-5, GDF-6 and GDF-7, further improve such results[109], as does the use of biomaterials based on PLGA instead of collagen gel[110]. Mechanical stimulation of fibres improves the repair mechanisms[111].

MSC have been used to restore the structure and functions of skeletal muscles, in cases of muscle dystrophy or other congenital myopathies. The inoculation of human adult MSC into *mdx* mice (an animal model of Duchenne's muscle dystrophy) led to the formation of myofibres and long term-acting satellite cells, the restoration of dystrophin expression in the sarcolemma and the production of several muscle growth factors[112], even by using human bone marrow MSC with the entire sequence of dystrophin[113]. These effects are potentially useful in human Duchenne's muscle dystrophy, but so far there is no clear evidence of *de novo* muscle regeneration and clinical improvement mediated by MSC.

Several studies have shown that MSC have a cardiomyogenic potential after myocardial infarction[114-118]. In a randomised clinical study carried out in 69 patients and based on the intracoronary infusion of autologous bone marrow MSC, left ventricular perfusion and heart contractile function improved remarkably after 3 months[119]. However, there was very little formation of new cardiomyocytes derived from the transplanted MSC[120]: it is, therefore, believed that the observed cardiac functional improvement observed is due to other mechanisms, such as the release of soluble trophic factors with a paracrine effect and the stimulation of residual cardiac stem cells[121].

Neural tissue regeneration

Systemically infused bone marrow MSC colonise virtually all organs, where these cells survive only in the presence of local proliferation[122,123]. MSC do not normally seem to pass through the blood-brain barrier: they can survive, migrate, and differentiate into neural-glial cells after *in utero* intraventricular injection inside foetal rat brains[51]. Functional recovery has been shown following *in vivo* transplantation of these cells inside the lesion in animal models of Parkinson's disease, hypoxic-ischaemic neural damage and retinal injury[124]. So far, however, there have been no significant clinical studies unequivocally showing that MSC possess neural regenerative activity in humans.

Gene therapy

MSC may be engineered with genes coding for molecules that are missing in genetic or acquired defects or with therapeutic activity, such as erythropoietin, insulin or coagulation factors; however, preliminary results need to be confirmed with *in vivo* studies to assess whether the correction of the deficiency is long-lasting[5,125].

Immune-modulatory therapy

Acute graft-versus-host disease

MSC can inhibit the immune responses against minor histocompatibility antigens such as HY[60,82], they can prevent the occurrence of GvHD if co-transplanted with haematopoietic stem cells[75], and they can completely modulate grade IV GvHD refractory to immunosuppressive drugs[76]. Similar results have been obtained with adipose tissue-derived MSC[126]. On this background, various clinical trials with autologous or allogeneic MSC are currently in progress, evaluating the effect of these cells on preventing GvHD in MHC-unrelated transplants and treating severe acute GvHD, which is associated with a high mortality due to infectious complications, especially if intestinal mucosa is involved. These trials are based on the collection and expansion of MSC obtained from the same donor of the haematopoietic stem cells.

Autoimmunity

Allogeneic bone marrow MSC may inhibit T- and B-cell proliferation and functions in the BXSB mouse, which is an animal model of human systemic erythematous lupus[127]. MSC-based therapeutic approaches for collagen disorders refractory to conventional immunosuppressive agents are currently under examination[105,128,129]. On the other hand, MSC infusion is clearly associated with a lower incidence and improved clinical features in experimental autoimmune encephalomyelitis, an animal model of human multiple sclerosis[77], similarly to what can be achieved with neural stem cells[130]. Transplantation of MSC could play an important role in inflammatory diseases of the central nervous system, especially if they were able to migrate through the blood-brain barrier, thus coupling their regenerative potential and immune regulatory effects[77,131].

Anti-cancer cell therapy

It has been shown that MSC may support and amplify the proliferation of solid tumours both *in vitro* and *in vivo*, by favouring cancer dissemination and proliferating inside the tumour as fibroblasts of the vascular-stromal axis[38,132,133]. This property must always be considered when large numbers of MSC are infused systemically, even for regenerative purposes. However, MSC transfection with genes coding for molecules with antiproliferative activity, such as interferon-beta, not only inhibits neoplastic growth *in vitro*, but also lowers cancer development *in vivo*[132]. Similar results have been obtained with gliomas[134]. Therefore, cell therapy with MSC engineered to produce anti-proliferative molecules could be an efficient strategy for specific anti-cancer treatments with few side effects.

Conclusions

Since the end of the 1990s a large amount of data concerning MSC biology and differentiation/immune regulatory potential has been published, even though many of these data still remain contradictory. MSC have some advantages in terms of availability, expandability, transplantability, and capability of immune regulation, without the ethical implications associated with the use of embryonic stem cells. Pre-clinical studies in animals have shown that a therapeutic approach involving MSC is feasible in different fields of tissue regenerative medicine and immune-modulating cell therapy, although many potential clinical applications remain to be confirmed.

Key words: mesenchymal stem cells, cell therapy, regenerative medicine, immune regulation.

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Received: 25 June 2007 – Accepted: 22 August 2007
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