Mesenchymal stem cells in regenerative medicine applied to rheumatic diseases: Role of secretome and exosomes.

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ABSTRACT

Over the last decades, mesenchymal stem cells (MSC) have been extensively studied with regard to their potential applications in regenerative medicine. In rheumatic diseases, MSC-based therapy is the subject of great expectations for patients who are refractory to proposed treatments such as rheumatoid arthritis (RA), or display degenerative injuries without possible curative treatment, such as osteoarthritis (OA). The therapeutic potential of MSCs has been demonstrated in several pre-clinical models of OA or RA and both the safety and efficacy of MSC-based therapy is being evaluated in humans. The predominant mechanism by which MSCs participate to tissue repair is through a paracrine activity. Via the production of a multitude of trophic factors with various properties, MSCs can reduce tissue injury, protect tissue from further degradation and/or enhance tissue repair. However, a thorough *in vivo* examination of MSC-derived secretome and strategies to modulate it are still lacking. The present review discusses the current understanding of the MSC secretome as a therapeutic for treatment of inflammatory or degenerative pathologies focusing on rheumatic diseases. We provide insights on and perspectives for future development of the MSC secretome with respect to the release of extracellular vesicles that would have certain advantages over injection of living MSCs or administration of a single therapeutic factor or a combination of factors.

Highlights:

- Mesenchymal stem cell-based therapy generates great interest for regenerative medicine
- Mesenchymal stem cells mainly participate to tissue repair via paracrine activity
- A multitude of trophic and survival factors are secreted by mesenchymal stem cells
- Extracellular vesicles exert similar protective and reparative properties as cells
- Extracellular vesicles represent a promising alternative to stem cell implantation

Keywords: mesenchymal stem cells, trophic factors, exosomes, microparticles, secretome, regenerative medicine, rheumatic diseases

1. Characteristics and functions of mesenchymal stromal cells

Mesenchymal stem or stromal cells (MSCs) are multipotent adult stem cells capable of self-renew and differentiation potential. They are found in large quantities in bone marrow (BM-MSCs) or in adipose tissue (ASCs) but could reside in virtually all post-natal organs and tissues [1]. MSCs are isolated as a heterogeneous cell population characterized by their capacity to adhere to plastic, develop as fibroblast colony-forming-units (CFU-F) and differentiate into three cell lineages of mesodermal origin: osteocytes, chondrocytes and adipocytes. After culture expansion, they are positive for the cell surface markers CD73, CD90 and CD105 and negative for CD11b, CD14, CD34, CD45 and human leukocyte antigen (HLA)-DR [2]. While expanded BM-MSCs are negative for the CD34 marker, recent studies report that freshly isolated BM-MSC are enriched in the $CD34^+$ fraction of BM nucleated cells [3]. Conversely, CD34 is expressed on native ASCs and during the first population doublings but rapidly disappears upon cell proliferation *in vitro* [4, 5].

MSCs produce a large amount of secreted factors, such as cytokines, chemokines or growth factors, which mediate diverse functions via a crosstalk between different cell types [6-8]. In the BM niche, MSCs and osteoblasts constitute the stromal fraction in a complex network formed by hematopoietic stem cells (HSCs), endothelial stem cells and their progeny. Within the niche, MSCs control survival, proliferation and differentiation of stem cells. They also play a role in tissue regeneration either locally or over large distances through the secretion of trophic factors. These soluble mediators may act directly, triggering intracellular mechanisms of injured cells, or indirectly, inducing secretion of functionally active mediators by neighboring cells. Indeed, in case of injury, MSCs attenuate tissue damage, inhibit fibrotic remodeling and apoptosis, promote angiogenesis, stimulate endogenous stem cell recruitment and proliferation, and reduce immune responses (Figure 1).

2. Choice of the best cell source for regenerative medicine

BM-MSCs and ASCs are the best characterized and the most studied sources of adult MSCs. However, new cell sources, in particular from the Wharton jelly, are also interesting for therapeutic applications [1]. Thanks to their differentiation properties, their use in regenerative medicine has been first tested in tissue engineering applications, for bone and cartilage repair. These approaches require defining an optimal combination of scaffold, growth factor and stem cells and, local delivery requiring surgical procedures [9]. More recently, the capacity of MSCs to secrete a variety of trophic factors with diverse functions has motivated the interest of evaluating local or systemic injection of MSCs to stimulate tissue repair in different pathologies. However, the question of the best source of cells for a particular therapeutic application is under evaluation.

Significant differences between BM-MSCs and ASCs have been reported. The cytokine profile of ASCs and BM-MSCs differs [10]. ASCs secrete higher levels of pro-angiogenic factors including vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), angiopoietin (Ang)-1, Ang-2 or platelet derived growth factor (PDGF) to promote angiogenesis [11-13]. Together with angiogenic activity that makes them attractive cells for cardiovascular diseases, ASCs also represent a powerful tool for neurodegenerative medicine. ASCs can stimulate the regeneration of nervous tissues by promoting nerve healing and *de novo* axon growth, via the release of several neurotrophic factors such as brain derived neurotrophic factor (BDNF), nerve growth factor (NGF) or glial derived neurotrophic factor (GDNF) [14]. They protect neurons against apoptosis [15] and slowed disease progression in models of Huntington disease [16].

Regarding the immunomodulation function of stem cells, a first report concluded that ASCs share immunosuppressive properties with BM-MSCs [17]. Since then, the immunomodulatory function of BM-MSCs and ASCs was compared with that of chorionic

placenta- or palatine tonsil-derived MSCs (CP-MSCs) and even some differences exist, comparable effects were observed [18, 19].

ASCs and MSCs exhibit cell specific differences at transcriptional and proteomic levels as well as functional differences in their differentiation processes towards adipocytes, osteoblasts and chondrocytes [20]. MSCs demonstrate higher differentiation potential toward chondrogenic and osteoblastic lineages whereas ASCs possess a better capacity to differentiate into adipocytes [21, 22]. The best strategy for MSC-based therapy has therefore to be determined according to their distinct characteristics associated with their tissue origin for a particular therapeutic application.

3. Secretome-based therapeutic efficacy of mesenchymal stem cells for rheumatic diseases

The therapeutic value of BM-MSCs or ASCs for rheumatic diseases including osteoarthritis (OA) and rheumatoid arthritis (RA) has been evaluated during the last few years. Because the interest of using MSCs for cartilage tissue engineering has been reviewed elsewhere [9], we will focus here on the paracrine effect of MSCs for preventing cartilage degradation or stimulating endogenous cartilage regeneration. Focusing on rheumatic diseases, it is likely that the route of MSC administration will differ according to the pathology. In case of systemic disease, such as rheumatoid arthritis (RA) where several joints may be affected, systemic delivery via the bloodstream should be favored. On the contrary, for lesions that are limited to a single joint, local delivery should be preferred because of better availability of cells and safety.

3.1. Local delivery of mesenchymal stem cells for osteoarthritis treatment

The rationale for using local injection of MSCs for inducing regeneration of OA cartilage is based on a number of *in vitro* studies, when MSCs and chondrocytes are mixed in pellet- or alginate-based co-cultures [23-25]. Whatever the source of MSCs (BM, adipose tissue or synovium), factors secreted by MSCs increased cartilage matrix production by chondrocytes [25]. However, neither the exact mechanism of action when ASCs or BM-MSCs are not in direct contact with chondrocytes, nor the identification of possible mediators, had been investigated. Such paracrine effect was recently demonstrated in our group, where proteins secreted by ASCs were shown to protect OA chondrocytes against apoptosis and degeneration towards hypertrophic or fibrotic phenotypes; HGF being involved in the anti-fibrotic effect observed (Maumus et al., submitted). Although OA is not considered an inflammatory disease, pro-inflammatory mediators, such as cytokines, metalloproteinases (MMP), reactive oxygen species (ROS), are secreted by OA chondrocytes or synoviocytes and, participate to joint tissue alterations. Several pro-inflammatory cytokines are significantly down-regulated in chondrocytes when cultured with ASCs suggesting that ASCs may also be protective through the down-regulation of inflammatory mediators [26]. Interestingly, paracrine factors of BM-MSCs share the same anti-inflammatory effects on OA cartilage and synovial explants *in vitro* [27].

Local injection of BM-MSCs or ASCs in the joint is likely to exert several roles: inhibition of osteophyte formation, decrease of synovial inflammation, reduction of cartilage degeneration with less fibrosis and apoptosis of chondrocytes or stimulation of chondrocyte proliferation and extracellular matrix synthesis (Figure 2). Indeed, using the pre-clinical murine model of collagenase-induced OA, a single ASC injection in the knee joint of mice inhibited synovial activation and formation of chondrophyte/osteophyte in joint ligaments as well as cartilage destruction, probably by suppressing synovial macrophage activation [28]. Intra-articular injection of BM-MSCs can also prevent the development of post-traumatic arthritis [29]. Other pre-clinical studies using larger animal models of OA (rat, rabbit, guinea pig, sheep,

donkey and goat) revealed similar results with cartilage regeneration after injection of MSCs in the damaged joint [30-35].

Finally, an Iranian phase I clinical trial recently reported that intra-articular injection of autologous BM-MSCs in six patients with knee OA was safe and improved pain, functional status of the knee. As important, magnetic resonance imaging (MRI) displayed increased cartilage thickness and decrease of subchondral edemas in three out of six patients [36]. All these data support the trophic action of MSCs for protecting cartilage from degradation and stimulating regeneration.

3.2. Systemic delivery of mesenchymal stem cells for rheumatoid arthritis treatment

The interest of using MSCs to reduce inflammation in various autoimmune and/or inflammatory disorders has been investigated for many years (for review, see [37]). In the collagen-induced arthritis (CIA) murine model, which is representative of RA in humans, contrasted results have been reported (for reviews, see [38, 39]). Injection of primary murine BM-MSCs was shown to inhibit occurrence of arthritis and even partially reverse clinical signs when injected after disease onset [40, 41]. Besides reduced levels of pro-inflammatory cytokines in mouse sera, mechanisms involved in reduction of clinical signs were suggested to be through $CD4^+CD25^+Foxp3^+$ Treg cell induction as well as T-cell anergy [42-45]. The therapeutic benefit of xenogeneic human MSCs, either from adipose tissue or umbilical cord, has also been described [42, 46, 47]. In contrast, other studies failed to demonstrate any improvement with MSC treatment. Systemic infusion of the allogeneic C3H10T1/2 cell line did not decrease the clinical signs of arthritis [48]. Similar results were obtained using primary murine MSCs isolated from different strains of mice suggesting that different genetic backgrounds influence the immunosuppressive effect of BM-MSCs [49]. Alternatively, the immunomodulatory role of BM-MSCs was reported to be dependent on the window of

injection, with therapeutic benefit only when two cell injections on day 18 and 24 were done [41]. More recently, inhibition of TNF-α via infusion of a specific inhibitor resulted in enhanced suppressive activity of MSCs, confirming previous report that exposure of MSCs to TNF-α blocks their suppressive capacity [48, 50]. This hypothesis was further supported by enhanced immunomodulatory activity of BM-MSCs when the anti-inflammatory Bortezomib, a proteasome inhibitor, was injected before MSC infusion [51].

Despite these conflicting results, the safety and efficacy of allogeneic transplantation of MSCs from BM or umbilical cord have been tested in four patients with refractory RA [52]. No serious adverse events were reported but no patient achieved the DAS-28-defined remission in the follow-up period. Nevertheless, larger randomized studies are required to address the interest of using MSCs for RA treatment.

4. Role of extracellular vesicles released by mesenchymal stem cells in the treatment of degenerative diseases

4.1. Biogenesis and characterization of extracellular vesicles

Extracellular vesicles are released in the extracellular space by almost all cells and were considered for long time, as inert cellular debris. They form a variety of complex structures among which exosomes, microparticles (MP) and apoptotic bodies are the best described vesicles. They are surrounded by a phospholipid bilayer and can be distinguished by their size and composition. Exosome size ranges between 30 and 120 nm whereas MP diameter is included between 100 nm and 1µm and apoptotic bodies between 1 and 5 µm. They contain numerous proteins, lipids as well as messenger and micro RNAs responsible for intercellular signaling. Exosomes were first described 30 years ago as being released by sheep reticulocytes [53] and then, by most cell types including immune cells (B cells, dendritic

cells, mast cells or T cells) [54, 55], cancer cells [56] and MSCs [57]. They are also found in physiological fluids such urine, plasma or exsudates [58].

Exosomes originate from internal bud of multivesicular endosomes which fuse with the plasma membrane and are released by exocytosis [59]. They are rich in tetraspanins (CD9, CD63 and CD81), heat-shock proteins (Hsp60, Hsp70, Hsp90) and frequently expose Alix, clathrin, Tsg101 and unique cell type specific proteins that reflect their cellular source. MPs originate from the budding of small cytoplasmic protrusions which then detached from the cell surface through a process dependent on calcium influx, calpain and cytoskeleton reorganization. They expose high amounts of phosphatidylserine, contain protein associated with lipid rafts and are enriched in cholesterol, sphingomyelin and ceramide [60]. Extracellular vesicles from MSCs additionally express the characteristic markers CD13, CD29, CD44, CD73 and CD105 [61-63] and contain proteins, mRNA and microRNA which have been characterized by proteomic or transcriptomic analyses [64, 65].

4.2. Isolation of extracellular vesicles and characterization of proteins

. Most of the protocols rely on the purification of vesicles from supernatants of cells grown in absence of serum [66]. The purification of the exosomal fraction for molecular and functional analyses relies on three different methods which are most frequently used: ultracentrifugation [67], ultrafiltration [68] and immunoprecipitation technologies using antibody loaded magnetic cell beads [69]. Extracellular vesicles can then be efficiently separated from protein aggregates by using their low buoyant velocity and differences in floatation velocity [70]. However, the methods for purification and analysis of the different extracellular vesicle populations have to be improved and standardized

A number of biochemical techniques have been used to identify the protein, RNA species and lipid content of vesicles (for review, see [71]). During the last years, western blot,

fluorescence-activated cell sorting or MS-based proteomic analyses have been performed. More recently, high-throughput studies have been conducted and to date, several thousands of proteins and RNAs have been described in extracellular vesicles purified from various cell types or biological fluids. These studies allowed the identification of a common set of components, mainly associated with the biogenesis or structure of vesicles or, proteins specific for the cell origin or physiopathological status. Quantitative and comparative analyses are still needed to a better understanding the function and role of the extracellular vesicles.

4.3. Biologic function of extracellular vesicles released by mesenchymal stem cells

Extracellular vesicles are an integral component of the cell-to-cell crosstalk contributing to tissue regeneration and likely taking part to the paracrine action of MSCs in regenerative medicine. The paracrine role of MSCs is supported by the beneficial effect of conditioned media (CM) that reproduce benefits reported with the direct injection of MSCs as exemplified for myocardial infarction therapy in swine and hamster models [57, 72-75]. The cardioprotective activity was contained in a >1000 kDa MW fraction and therefore mediated by large complexes with a diameter of 50-100 nm [74]. In addition, extracellular vesicles from MSCs can promote angiogenesis by increasing endothelial cell proliferation and capillary network formation both *in vitro* and *in vivo* [76]. They can improve acute kidney injury by decreasing apoptosis, fibrosis, lymphocyte infiltration, tubular atrophy and by increasing tubular epithelial cell proliferation [63, 77-79]. In some reported cases, pretreatment of extracellular vesicles with RNase abrogated their therapeutic properties highlighting the important role of RNA species [77, 78]. The anti-fibrotic action of MSC-derived exosomes was also shown on liver by the reduction of collagen I and III deposit as well as TGF-β1 expression and Smad2 phosphorylation leading to the inhibition of epithelial-to-mesenchymal transition and protection of hepatocytes [61]. Finally, extracellular vesicles

released by MSCs were shown to inhibit auto-reactive lymphocyte proliferation and promote secretion of the anti-inflammatory cytokines IL-10 and TGF-β [80]. While the role of MSC-derived extracellular vesicles has not been addressed in rheumatic diseases, it is tempting to speculate that they may improve outcomes of OA or RA via anti-fibrotic, anti-apoptotic, anti-inflammatory and pro-regenerative properties.

5. Conclusions

Regenerative medicine is a subject of great expectations and gives rise to enormous hopes for patients who are refractory to proposed treatments, or display severe forms of diseases without possible treatment. MSC-based therapy might therefore be an advantageous alternative to current approaches. In the last decade, the therapeutic potential of MSCs has been demonstrated in numerous pre-clinical models of inflammatory and degenerative pathologies and MSC-based therapy is being evaluated in clinics with promising results. While MSCs are considered relatively safe, the development of therapeutic strategies that may avoid administration of MSCs will attenuate the safety concerns relative to the use of living stem cells. In this respect, extracellular vesicles would have certain advantages over administration of a single factor that cannot mimic the actions of MSCs. Several questions have however to be addressed before clinical use could be considered.

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Legends to figures

Graphical abstract. Role of trophic factors released by mesenchymal stem cells (MSC) in the treatment of rheumatic diseases: possible mechanisms.

Fig. 1. Role of various paracrine factors released by mesenchymal stem cells. Secreted factors may exert different functions on cells via the release of different types of molecules depending on the microenvironment. Angiopoietin (Ang), Basic fibroblast growth factor (bFGF), brain derived neurotrophic factor (BDNF), chemokine ligand (CCL), chemokine (C-X-C motif) ligand (CXCL), erythropoietin (EPO), glial cell line-neurotrophic factor (GDNF), granulocyte macrophage-colony stimulating factor (GM-CSF), heme oxygenase (HO), hepatocyte growth factor (HGF), human leucocyte antigen (HLA), indoleamine 2,3-dioxygenase (IDO), insulin growth factor (IGF), interleukin (IL), keratinocyte growth factor (KGF), leukemia inhibitory factor (LIF), human cathelicidin (LL37), monocyte chemoattractant protein (MCP), metalloproteinase (MMP), nerve growth factor (NGF), nitric oxide (NO), platelet derived growth factor (PDGF), prostaglandin (PGE), placental growth factor (PIGF), stem cell factor (SCF), stromal cell-derived factor (SDF), tissue inhibitor of metalloproteinase (TIMP), transforming growth factor (TGF), thrombopoietin (TPO), TNF-α-stimulated gene/protein (TSG), vascular endothelial growth factor (VEGF)

Fig. 2. Schematic representation of mesenchymal stem cell (MSC)-based therapy after intra-articular injection in the treatment of rheumatic diseases. Through the release of trophic factors, MSCs may act to reduce fibrosis, apoptosis and/or hypertrophic phenotypes associated with cartilage degenerescence and osteophyte formation during osteoarthritis. They may control synovial inflammation occurring during rheumatoid arthritis and/or induce the proliferation of chondrocytes to stimulate regeneration of endogenous cartilage.