REVIEW

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Mesenchymal stromal cells derived exosomes as tools for chronic wound healing therapy

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Abstract

In modern society, the healing of chronic wounds is still a major cause of discomfort for the patients and a financial burden for the care system. Current approaches use either organic tissue-engineered skin substitutes or stem cells based therapy. It has been shown that mesenchymal stem cells (MSCs) are able to improve the wound healing process by secreting factors with anti-inflammatory, anti-fibrotic and pro-angiogenic activities either as soluble molecules (growth factors, cytokines) or encapsulated within membrane vesicles (microparticles, exosomes). It has been shown that exosomes, the small membrane vesicles originating from the endocytic pathway, are the main mediators of MSCs paracrine effect. Their complex cargo (mRNA, microRNA and various anti-apoptotic and pro-angiogenic factors) has been found to induce migration and proliferation of fibroblasts as well as collagen synthesis. Thus, the combination of MSCs derived exosomes and organic biomaterials in order to enhance the healing process represents a novel approach for chronic wounds therapy, involving a cell-free use of MSCs paracrine activity.

Keywords: exosomes, mesenchymal stem cells, wound healing, regenerative medicine.

Introduction

The skin, the largest organ of the body, acts as a waterproof mechanical barrier between the environment and the organism, thus preventing the loss of body constituents and protecting against external aggressive factors [1]. By consequence, it is often subjected to various injuries of chemical, physical or mechanical origin. The wound healing process immediately begins and consists in four overlapping phases, which occur in a well-established sequence, at a specific moment, and continue for a specific duration at an optimal intensity [2]:

(*i*) Hemostasis – the blood vessels constrict and the platelets aggregation and thrombus (fibrin clot) formation take place. The fibrin network restores the function of the skin as a protective barrier, maintaining its integrity. Moreover, it supports cell migration to injury and stimulates fibroblast proliferation [3].

(*ii*) Inflammation – begins immediately after the injury took place and it represents the initiation of the wound healing process. The inflammatory phase lasts 4–6 days, during which time the neutrophils, monocytes and lymphocytes infiltrate into the damaged site. The monocytes differentiate into macrophages, which perform debris phagocytosis, in addition to the production and release of free radicals, cytokines and pro-angiogenic, inflammatory, and fibrogenic factors [4].

(*iii*) Proliferative phase – includes angiogenesis, fibroplasia, and reepithelialization. The granulation tissue fills the wound, the fibroblasts acquire contractile properties in order to contract the wound edges and the closure of the lesion takes place by migration and proliferation of keratinocytes adjacent to the wound margins.

(*iv*) Tissue remodeling – the maximum tensile strength is obtained by reorganization, degradation, and resynthesis of the extracellular matrix [3]. It is the last stage of the healing process and it can last up to one year. The granulation tissue is remodeled: collagen III is replaced by collagen I, while fibronectin and hyaluronic acid are degraded and the scar tissue rich in collagen fibers is formed [5].

In healthy individuals, the wound healing process is highly efficient and the functional epidermal barrier is rapidly restored. However, the normal repair mechanisms can be impaired and the result is either a chronic wound (ulcerative skin damage) or a scar tissue (hypertrophic scar or keloid) [6]. Chronic wounds are caused by a prolonged or exacerbated inflammatory process induced either by the particularities of the wound (such as pressure ulcers resulting after prolonged pressure on the skin) or as a complication of an illness [diabetes, ischemia, immunocompromised conditions such as cancer and acquired immunodeficiency syndrome (AIDS)] [2]. Frequently concealed as a comorbid condition, chronic wounds affect a vast part of the world population, especially elderly people in developed countries. This condition is a significant cause of morbidity and it can lead to disability and decreased quality of life, increasing pain, stress, depression, and social isolation [7, 8]. On the other hand, due to the prolonged necessity of medical care and high rate of reoccurrence, chronic wounds represent a significant burden to the healthcare resources worldwide [6].

Although induced by various causes, chronic wounds share common traits such as high levels of proinflammatory cytokines, proteases, and reactive oxygen species (ROS) [7, 9]. The cells found at the lesion site (keratinocytes, endothelial cells, fibroblasts, and macrophages) are senescent, having low proliferative and secretory properties, unresponsive to typical wound healing signals [10]. Moreover, chronic wounds are often associated with

persistent infection, which is critical for appropriate wound management [11]. Regarding the presence of local stem cells, necessary for a normal process of healing, they are scars or dysfunctional [7].

The standard wound care is critical for a complete healing process and it is focused first of all on the identification and correction of the triggering and perpetuating factors, followed by debridement, offloading (or compression), revascularization of ischemic limbs, use of antibiotics for the management of infection, and appropriate wound bed preparation [12]. If pursuing these established wound care guidelines does not lead to satisfactory results within four weeks of care (50% in are reduction) [13], advanced wound care therapies are envisaged. Recently, several advanced strategies have been developed such as negative pressure wound therapy [14], hyperbaric oxygen therapy [15], biophysical approaches (electrical stimulation, diathermy, pulsed electromagnetic fields, etc.) [16], local application of growth factors [platelet-rich plasma, fibroblast growth factor (FGF), epidermal growth factor (EGF)] [17], acellular matrices (collagen, xenografts) [18], skin grafting [19] and cellular therapy [20]. Regarding the latter, in the last years, mesenchymal stem cells (MSCs) have been considered to be used for wound healing therapy. Here, we discuss the implication of MSCs in the healing process, sources of MSCs and the emergent importance of exosomes as main effectors of the MSC-derived secretome.

MSC in chronic wound healing therapy

During the last decades, since their first description by Friedenstein *et al.* as plastic adherent cells able to differentiate into multiple cell types [21, 22], MSCs have been intensively studied for their regenerative properties. Due to the variation in nomenclature and characterization of these cells, in 2006, the *International Society for Cellular Therapy* indicated the minimum criteria required for their definition: non-hematopoietic cells positive for markers, such as CD90 (Thy-1), CD105 (endoglin), and CD73 (5'-nucleotidase), and lacking expression of CD14 and CD11b (monocytes and macrophages markers), CD34 (hematopoietic progenitors and endothelial cells marker), CD45 (pan-leukocyte marker), CD79a and CD19 (B-cell markers) and human leukocyte antigen – DR isotype (HLA-DR) [23]. In addition, there is also a minimal requirement regarding the differentiation potential of MSCs. Thus, in order to be considered "true stem cells", the differentiation towards adipogenic, osteogenic and chodrogenic is mandatory [23, 24].

Although initially identified in the bone marrow (BM) [21], MSCs have been isolated from many other tissues, such as adipose tissue, peripheral blood, cord blood, Wharton's jelly, dental pulp and menstrual blood [25]. Regardless of the original tissue, MSCs have a strong differentiating potential, being able to generate cells of mesodermal origin (osteocytes, adipocytes, chondrocytes, myoblasts, and tenocytes), and ectodermal origin (neural cells) [26, 27]. Due to these interesting data, obtained mainly *in vitro*, MSCs have been studied intensively in cellular transplantation research, both in animal models and in clinical trials. Furthermore, the use of adult stem cells had also other advantages *versus* embryonic stem cells, such as the avoidance the ethical issues and the lack of tumor induction. Moreover, possibility to use the patient's own cells reduces the graft rejection due to the donor's incompatibility. All of these indicated MSCs as ideal candidates for cellular therapy in various degenerative diseases.

Recently, MSCs have been shown to play an important role in the wound healing process [28]. Like in most, if not all tissues, endogenous MSCs also reside within the skin, in this case – at the base of the hair follicle (dermal papilla cells), in the dermal sheets which surround the hair follicles (dermal sheath cells), in the interfollicular dermis and, most likely, as recently suggested, they could also originate from the perivascular pericytes [6]. The resident skin MSCs are actively implicated during the wound healing process either by differentiating into fibroblasts, which are responsible for the matrix synthesis or through the release of various molecules involved in tissue regeneration, such as anti-scarring [keratinocyte growth factor (KGF)], anti-apoptotic [stanniocalcin-1 (STC-1), secreted frizzled related protein-2 (SFRP-2), transforming growth factor-beta 1 (TGF-β1), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF)], pro-angiogenic [VEGF, stromal cell-derived factor-1 alpha (SDF-1α)] and mitogenic factors [TGF-α, TGF-β, HGF, insulin-like growth factor-1 (IGF-1), FGF-2, EGF)] [27]. It was also observed that some subpopulations of BM cells could differentiate into keratinocytes [29, 30].

In several *in vivo* studies, exogenous MSCs have been applied to wounds in order to make use of their regenerative properties and the results indicated positive effects on both wound healing and scarring. MSCs have a wide differentiation potential, making them attractive treatment options in regenerative medicine and, over the past decade, have rapidly emerged as treatment of acute and chronic wounds.

As revealed by the data summarized in Table 1, the administration of MSCs from both adipose tissue and bone marrow, either directly or *via* a scaffold, has been able to improve the outcome in diabetic ischemic ulcers and burns, including scars.

Table 1 – ***Bone marrow- and adipose tissue-derived MSCs used in several studies on patients with pathologies involving skin and vascular lesions***

Cell type	Method of delivery	Wound type	No. of patients	Phase	Outcomes	Reference
Autologous BM-MSCs	Suspended in fibrin spray	Diabetic/venous ulcer	5	Case study	Acute wounds closed (eight weeks)	[31]
Autologous BM-MSCs	MSC on collagen scaffolds used as wound dressings	Non-healing wounds	20	Interventional prospective study	Wounds healed in 18/20 patients	[32]
Autologous BM-MSCs	Direct/intramuscular administration	Diabetic ulcer, ischemic	24	Case study	Ulcer surface area decreased (12 weeks)	[33]
Autologous BM-MSCs	Intradermal administration	Severe radiation burns	1	Case study	No inflammatory complications during eight months	[34]

Cell type	Method of delivery	Wound type	No. of patients	Phase	Outcomes	Reference
Allogenic BM-MSCs	Intradermal administration	Hypertrophic scar following burn	1	Case study	Reduction of skin graft contracture	[35]
Autologous BM-MSCs	Intramuscular administration	Diabetic limb ischemia and ulcer	41	Randomized controlled study	Healing time and pain-free walking improvement	[36]
Autologous BM-MSCs	Direct administration	Diabetic ulcer	8	Case study	Complete healing (three cases); reduced wound (five cases)	[37]
Allogenic BM-MSCs	Intramuscular administration	Ischemic ulcers	7	Case study	Complete healing in 6/7 patients	[38]
Autologous BM-MSCs	Intra-arterial administration	Ischemic ulcers	4	Case study	Improved wound healing	[39]
Allogenic ADSCs	Intramuscular administration	Ischemic ulcers	9	Case study	Wound healing in 6/9 cases	[40]
Allogenic ADSCs	Intralesionally administration	Perianal fistula, Crohn's disease	24	Multicenter phase I/IIa clinical trial	56% closure of fistula at 24 weeks	[41]
Autologous ADSCs	Direct administration	Ischemic ulcers	10	Case study	Complete wound healing (6/10 cases); decrease in diameter and depth in all cases	[42]
Autologous ADSCs	Intramuscular administration	Ischemic ulcers	6	Case study	Decreased ulcer number and size	[43]

MSCs: Mesenchymal stem cells; BM-MSCs: Bone marrow-derived MSCs; ADSCs: Adipose-derived stem cells (MSCs).

MSCs secreted factors – mediators of regenerative properties

Due to the capacity to generate various cell types *in vitro*, it was proposed that MSCs would participate directly to the regenerative process, by differentiation into the appropriate cell types and incorporation into the regenerated tissue. However, this hypothesis was challenged by the lack of evidence for MSCs engraftment and persistence in time at the injured site after systemic delivery because only a small amount of cells reach the site of the injury, engraft and survive long term [44]. As mentioned above, MSCs are able to secrete a large variety of anti-apoptotic, mitogenic and pro-angiogenic factors, which indicated the possibility of a strong paracrine effect as the main mechanism by which these cells manifest their regenerative properties. Following numerous *in vitro* and *in vivo* studies, the paracrine role is currently considered one of the primary attributes for MSCs-mediated repair and regeneration *in vivo* [45, 46]. The regenerative effect induced by the MSCs secretome, harvested from the cultured cells *in vitro* and called generically "conditioned medium" was demonstrated in various pathologies. Multiple studies showed that MSCs conditioned medium has been successfully assessed in various pathological conditions such as alopecia, acute and chronic hind limb ischemia, acute and chronic wound healing, myocardial infarction, acute liver injury, cerebral and spinal cord injury, lung injury, and bone defect [47]. The most recent studies, in which the efficiency of the conditioned medium harvested from MSCs derived from various sources was shown, are summarized in Table 2.

It can be noticed that the use of MSCs derived products is of great interest in skin pathology, since the factors secreted by these cells can sustain the wound healing process by acting directly on skin cells properties and by enhancing angiogenesis. Thus, Jun *et al.* showed that the conditioned medium harvested from hypoxic MSCs isolated from the amniotic fluid enhanced *in vitro* the proliferation and migration of human dermal fibroblasts by the activation of TGF-β/SMAD2 and phosphatidyl inositol 3-kinase (PI3K)/protein kinase B (AKT) [57].

Table 2 – ***In vivo studies using MSCs condition medium for the treatment of various pathologies***

Pathology	Animal model	MSCs type	Reference
Chronic obstructive pulmonary disease	Mouse	Human iPSC-MSCs	[48]
Inflammatory bowel disease	Mouse – acute colitis	Mouse BM-MSCs	[49]
Inflammatory arthritis	Mouse – antigen-induced model of arthritis	Mouse BM-MSCs	[50]
Brain hypoxia-ischemia	Mouse	Human ES-MSCs	[51]
Multiple sclerosis	Mouse – multiple sclerosis model (experimental autoimmune encephalomyelitis)	Human hypoxic periodontal ligament stem cells	[52]
Myocardial infarction	Rat	BM-MSCs	[53]
Skin wound healing	Mouse – full-thickness wounds	Horse – PB-MSCs	[54]
Skin wound healing	Diabetic rat – full-thickness wounds	Human – BM-MSCs	[55]
Keloid fibroblasts activity	Mouse – keloid implantation model	Human – ADSCs	[56]

MSCs: Mesenchymal stem cells; iPSC-MSCs: Induced pluripotent stem cells-derived MSCs; BM-MSCs: Bone marrow-derived MSCs; ADSCs: Adipose-derived stem cells (MSCs); ES-MSCs: Embryonic stem cells-derived MSCs; PB-MSCs: Peripheral blood-derived MSCs.

Other studies showed that the adipose tissue-derived MSCs conditioned medium was able to stimulate skin keratinocytes proliferation and fibroblast migration [58, 59]. Moreover, MSCs conditioned medium improved the proliferation and migration of keratinocytes in hyperglycemia *via* extracellular-signal-regulated kinase (Erk) signaling pathway in a ROS-dependent manner, suggesting that the use of MSCs-derived soluble factors could be an alternative therapeutic strategy for the diabetic chronic wound healing problem [60]. Several wound healing mediators were identified in MSCs-conditioned medium, such as: TGF-β1, interleukin (IL)-6, IL-8, monocyte chemoattractant protein-1 (MCP-1), regulated on activation,

normal T-cell expressed and secreted (RANTES), type I collagen, fibronectin, secreted protein, acidic and rich in cysteine (SPARC) and insulin-like growth factor-binding protein-7 (IGFBP-7), suggesting that, besides dermal keratinocytes and fibroblasts migration and/or proliferation, MSCs conditioned medium could also play an important role in the formation of extracellular matrix [58]. On the other hand, it was showed that, by releasing factors such as VEGF-α, IGF-1, EGF, KGF, HGF, angiopoietin-1, SDF-1α, macrophage inflammatory protein-1α (MIP-1α) and MIP-1β, MCP-1 and erythropoietin, MSCs not only act on keratinocytes and fibroblasts, but also recruit macrophages and endothelial cells and thus enhance the wound healing process [61, 62].

Therefore, the use of MSCs-derived conditioned medium, containing a large variety of pro-regenerative factors could prevail over the poor engraftment of the transplanted cells. On the other hand, the standardization of the method of soluble factors production would allow the development of an off-the-shelf treatment [63], which would no longer require a long preparation process by isolating and *in vitro* propagating of autologous cells from the patient. By consequence, a novel type of regenerative medicine is emerging by using stem cells secretome (the mixture of secreted factors, which include: soluble proteins, free nucleic acids, lipids and extracellular vesicles), as non-cellular therapeutic approach [64].

Exosomes – important elements of the MSCs secretome

The uncovering of the extracellular vesicles lead to a novel understanding of the intercellular communication [65, 66]. This communication pathway has a high degree of phylogenetic conservation, since it was observed starting from bacteria up to the mammalian cells and it plays major roles in mechanisms such as defense against viral attack, exchange of genetic material and transfer of biological active molecules [24].

Mammalian cells give rise to three different types of extracellular vesicles, having different biogenesis mechanisms and derived from different subcellular organelles [24, 67]:

(*i*) Exosomes (40–150 nm in diameter), originating in the endocytic pathway; the multivesicular bodies fuse with the plasma membrane and the exosomes are released in the extracellular space (Figure 1). They contain proteins (CD9, CD63, Alix, flotillin-1, Tsg101 and clathrin), coding and non-coding RNAs and have low expression of phosphatidylserine on their surface.

(*ii*) Microparticles (50–1000 nm in diameter), formed by the outward blebbing of the plasma membrane are subsequently release after the proteolytic cleavage of the cytoskeleton; microparticles also contain proteins, especially associated with lipid rafts, non-coding RNAs, their membrane is enriched in cholesterol, sphingomyelin and ceramide and expose high amounts of phosphatidyl-serine.

(*iii*) Apoptotic bodies (500–2000 nm in diameter) are released during the process of programmed cell death, originate through membrane blebbing and contain cellular organelles, cytoplasm and even nuclear fragments.

Exosomes have been proposed as the main mediators of MSCs paracrine effect by fractioning the extracellular components [68]. Thus, a population of small vesicles, up to 150 nm, positives for CD9, CD81 and Alix and able to mediate MSCs regenerative effects in a series of pathologies was identified. Exosomes have been used for both *in vitro* and *in vivo* studies, and proved to be efficient for ameliorating pathological conditions, such as: neurological injury [69], kidney injury [70], diabetes [71], myocardial infarction [72], retinal ischemia [73], hepatic injury [74] and wound healing [75, 76].

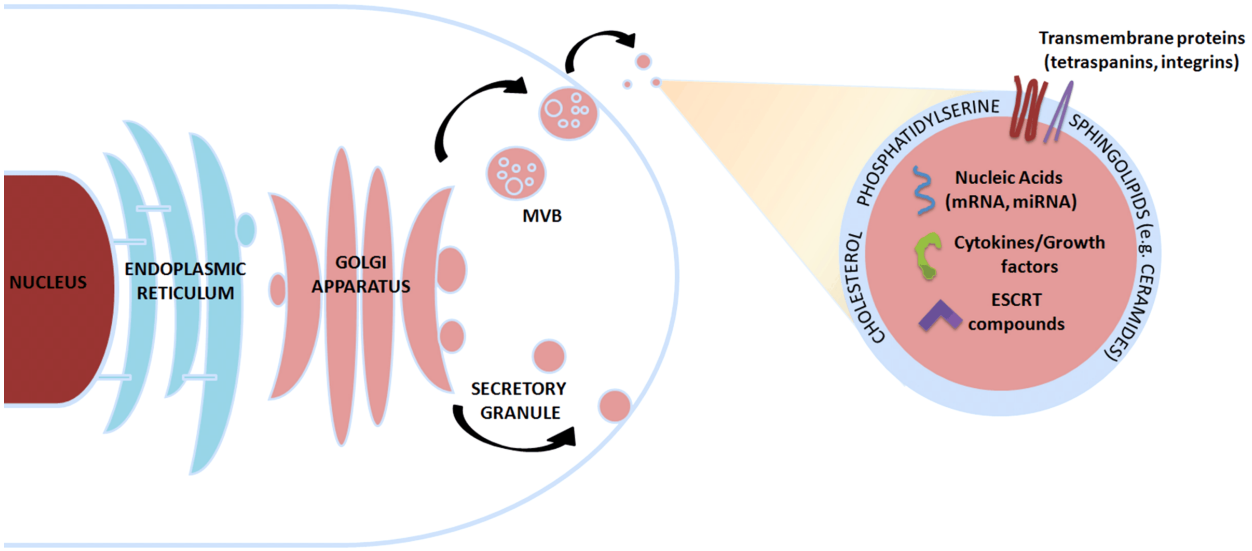

Figure 1 – ***Schematic representation of the release of exosomes from a cell via the fusion of the multivesicular bodies (MVBs) with the plasma membrane and the various contents of the exosome: nucleic acids – mRNA, miRNA, proteins – cytokines, growth factors, endosomal-sorting complex required for transport (ESCRT) proteins like Alix and TSG101, as well as the main components of their membrane: cholesterol, phosphatidylserine, ceramides and transmembrane proteins like tetraspanins (CD9, CD63) and integrins. mRNA: Messenger ribonucleic acid; miRNA: Micro-ribonucleic acid; Alix: Asparagine-linked glycosylation-2 (ALG-2)-interacting protein X; TSG101: Tumor susceptibility gene 101; CD: Cluster of differentiation.***

Several recent papers show the importance of MSCs-derived exosomes in enhancing the skin wound healing process. Thus, the exosomes isolated from bone marrow and adipose tissues are able to promote not only the proliferation and migration of dermal fibroblasts, but also to enhance the angiogenic process [77–79]. These types of extracellular vesicles derived from MSCs impact also the migration of keratinocytes, contributing to the acceleration of the re-epithelialization process *via* the Akt pathway [80]. However, the most important data regarding the potency of exosomes in enhancing the skin wound healing have been acquired by *in vivo* studies. Table 3 gathers the latest information regarding the *in vivo* effect of MSCs derived exosomes on various skin pathological conditions.

Table 3 – *In vivo studies regarding the effect of MSCs-derived exosomes on various skin conditions*

Pathology	Animal model	MSCs type-derived exosomes	Delivery	Reference
Skin wound healing	Rat skin burn model	Human umbilical cord MSCs	Subcutaneous injection	[81]
Skin wound healing	Mouse – full-thickness wounds	Human – ADSCs	Intravenous and subcutaneous injection	[82]
Skin wound healing – anti-scar effect	Mouse – full-thickness wounds	Human – ADSCs	Intravenous injection	[83]
Diabetic wound healing	Diabetic rat skin defect model	Human gingival MSCs	Topical administration by incorporation in chitosan/silk hydrogel sponge	[84]
Diabetic wound healing	Diabetic rat – full-thickness wounds	Human microRNA-126-overexpressing synovium MSCs	Topical administration in chitosan wound dressing	[85]
Atopic dermatitis	Mouse – house dust mite antigens treatment	Human – ADSCs	Intravenous and subcutaneous injection	[86]

MSCs: Mesenchymal stem cells; BM-MSCs: Bone marrow-derived MSCs; ADSCs: Adipose-derived stem cells (MSCs).

Conclusions

The most recent studies indicate that exosomes can promote proliferation and migration of dermal fibroblasts and keratinocytes and enhance the angiogenesis process, leading to a strong regenerative effect on the skin injuries, both in normal and diabetic organisms. The replacement of stem cells with exosomes in the regenerative therapy has some advantages, such as the potential for drug and gene delivery, storage constancy, and stability in the body [87]. Moreover, a perpetual stem cell source can be obtained by immortalization and the nanovesicles isolation protocol can be standardized so that an "off the shelf" medicine would be available for chronic wounds treatment, as well as burns, which require immediate intervention. All these advantages bring light to the use of MSCs-derived exosomes as a new and effective approach in wound healing therapy without the confines that cellular therapy brings.

Conflict of interests

The authors declare no conflict of interests.

Acknowledgments

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