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Therapeutic Strategies of Secretome of Mesenchymal Stem Cell

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Abstract

Great progress has been made in the therapeutic strategies of multiple diseases that lack curative treatments with the transplantation of mesenchymal stem cells (MSC), such as in onco-hematological diseases, myocardial infarction (MI), cerebrovascular diseases, degenerative diseases of the nervous system (multiple sclerosis, Alzheimer's disease), and diseases of the immune system, among others. Stem cells (SC) participate in the biological processes of tissue regeneration and repair through cell replication. Recently, the beneficial therapeutic effects of SCs that are generated by the release of proteins with paracrine actions and not by cell differentiation are more well known, and 80% of the therapeutic effect of SC is attributed to paracrine actions. The MSCs release large amounts of proteins and growth factors (GF), nucleic acids, proteasomes, exosomes, and microRNA, and membrane vesicles known as the secretome are released into the extracellular space, regulating multiple biological processes. Currently, the therapeutic strategies in tissue engineering (TE) and regenerative medicine (RM) are focused on the management of products derived from cells that act, both locally and remotely, in the affected tissue or organ, achieving regenerative actions. The application of new knowledge of the secretome initiates a change in the paradigm of regenerative therapy by knowing more about and using cell products derived from cells as a "factory" for biological drugs.

Keywords: stem cell, mesenchymal stem cells, cell therapy, paracrine activity, exosomes, extracellular vesicle, microvesicles, microRNA, miRNA, regenerative medicine, tissue engineering, growth factors, extra-cellular matrix, epidermal growth factor, endothelial cell, fibroblast growth factor, granulocyte colony stimulating factor, granulocyte-macrophage colony stimulating factor, interleukin, IL-1 receptor

antagonist, interferon- γ , low-density lipoprotein, monocyte chemotactic protein-1, nuclear factor κ B, nitric oxide, NO synthase, endothelial NOS, NYHA

1. Introduction

The mesenchymal stem cell (MSC) therapies offer new opportunities for confronting diseases that lack curative treatments through the properties of multipotentiality, self-renewal, and the secretion of paracrine factors derived from exosomes (cytokines, growth factors, microRNAs, and proteases), which act as mediators of intracellular communication and induce the repair and regeneration of organs and tissues [1].

Cell therapy with MSCs is safe and effective in the treatment of degenerative and traumatic diseases; they are found *in vivo* in minimal quantities throughout the body and they have the ability to differentiate into bone, cartilage, and adipose tissue through stimuli and in culture. The MSCs are located in the perivascular environment, activating and creating a regenerative microenvironment, with the secretion of molecules to regulate the immune response; however, the therapeutic effects through paracrine interactions of the MSCs are of short duration. The response to changes in the environment is attributed to MSCs through the transcriptional regulation of mediators that control inflammation, remodeling, repair, and cellular recruitment. The repair process involves the regulation of extracellular matrix (ECM) deposition, collagen synthesis, fibroblast proliferation, platelet activation, fibrinolysis, and angiogenesis; the immune process suppresses T-cells, activates macrophages, and recruits neutrophils [2].

Cell differentiation and replacement is attributed to cellular secretions that function as therapeutic inducers. The secretions of extracellular vesicles (EV) are both local and systemic. To determine the functions of the factors secreted by the MSCs in regeneration, it is necessary to identify precisely the molecular profile of the secretome of the MSC constituted by growth factors (GF), cytokines and chemokines, proteases, ECM, hormones, and lipid mediators, and so on [3].

The secretome of MSCs contains multiple overlapping elements that make it difficult to identify them. The *in vivo* examination of the secretome of MSCs and the strategies to modulate it and the result of the analysis are essential for the design of the next generation of regenerative therapies without cells. In this way, questions arise about the regulatory function of the secretome of the MSC, such as (i) what are the most effective approaches to study the secretome both *in vitro* and *in vivo* and are new technologies necessary to achieve it? (ii) how do the properties of the secretome change or become manageable, and after the transplant how does it evolve in the local microenvironment? (iii) what are the best methods to achieve the sustainability of the secretome and the control in the transplant? [4].

2. Stem cells and mesenchymal stem cells

More than 200 different types of cells make up embryonic and adult tissues and are regulated by local and systemic environmental factors. Embryonic stem cells (ESC) derived from the

internal cell mass of the blastocyst are constituted by ectoderm, endoderm, and mesoderm. Adult stem/progenitor cells, known as somatic SC, are undifferentiated cells located throughout the body. These cells have a high proliferative capacity and a differentiation potential limited to their lineage; they participate in regeneration, cell turnover, and homeostasis. The main function during life is to maintain the number of differentiated cells at a constant level and to replace dead cells or cells lost due to injury or disease [5].

SCs have a great capacity for self-renewal and the potential to produce a differentiated progeny. An SC can have the same phenotype but be less “mature” or less “differentiated” than its descendants and is classified into SCs/progenitors, “somatic”, “adult”, or “tissue” embryonic and nonembryonic cells. ESC are pluripotent, and most populations of progenitor cells arising during embryonic development cannot self-renew and have common properties with adult SCs, such as the potential of differentiation and the capacity for asymmetric cell division [6].

SC can be differentiated into specific cell types. Their ability to self-renew is through indefinite replication, resulting in the creation of two identical SCs, and under appropriate conditions, differentiated into more specialized cells. The MSCs are spindle-shaped adherent plastic cells that can be isolated from the bone marrow (BM), adipose tissue, and other tissues; are multipotent; and have the ability to differentiate. *In vitro* they can differentiate into bone; a subset of the cells have a high proliferative potential colony-forming units (CFU-F) when they are grown in culture. Hematopoietic SCs regulate and maintain hematopoiesis in the microenvironment of BM [7].

The MSCs can produce blood cells, although they are derived from a different population called hematopoietic SCs. The MSCs are classified as nonhematopoietic multipotential SCs and have the ability to differentiate into mesenchymal as well as nonmesenchymal lineages. The MSCs have the capacity for self-renewal, colony formation, phenotypic expression pattern, and differentiation potential; they interact with cells of the innate and adaptive immune system in the modulation of immune response. They participate in physiological processes, such as tissue homeostasis and hematopoiesis, and in pathological processes such as diseases of aging, tissue damage, and degenerative, inflammatory, and autoimmune diseases. After administration *in vivo*, MSCs induce tolerance and migrate to injured tissues where they inhibit the release of proinflammatory cytokines and promote the survival of damaged cells [8].

The International Society of Cell Therapy has established the following minimum criteria to define multipotent MSCs: first, they must be adherent to the plastic, under standard culture conditions (minimal essential medium, plus 20% fetal bovine serum). Second, MSCs should express CD105, CD73, and CD90 and should not express surface molecules such as CD45, CD34, CD14 or CD11b, CD79 α or CD19, and HLA-DR. Third, they must be differentiated into osteoblasts, adipocytes, and chondroblasts *in vitro*. They can be isolated from many adult tissues, BM, and adipose tissue. They have the ability to differentiate and trans-differentiate into cells of different lineages and immunomodulation capacity. The term “mesenchymal stem cell” is used to refer to the subset of mesenchymal cells that demonstrate SC activity and meet these criteria [9, 10].

The main characteristics of MSCs are the potential for self-renewal, differentiation, and multipotency. Under appropriate microenvironmental conditions, they can proliferate and give

rise to other types of cells, they can be trans-differentiated in cells of other lineages, and exert proregenerative, immunomodulatory, and antiinflammatory functions. Because of these characteristics, they can be an ideal therapeutic strategy for the treatment of inflammatory and systemic autoimmune diseases and are essential in the tissue regeneration of congenital, degenerative, and traumatic diseases [5].

The origin of MSCs *in vivo* is controversial. They are located in the perivascular area of the adventitia from almost all vessels (arteries and veins). They are pericytes, which are in intimate contact with the basement membrane and the surrounding endothelial cells, forming the extensive network of the microvasculature. Phenotypic similarities are evident among microvessels, and pericytes can be isolated from any vascularized tissue, near smooth muscle cells of arterioles, venules, and larger vessels, and preserve the expression of pericyte markers such as NG2 and CD146 [11].

The immunomodulatory activity of MSCs is mediated by paracrine factors. Among these, the exosomes participate in the communication between the MSCs and the target tissue. To demonstrate this, one study investigated the effect of the exosomes derived from MSCs on peripheral blood mononuclear cells (PBMC), especially on T-cells. It was shown that the MSC-derived exosomes extracted from the BM of healthy donors suppressed the secretion of the proinflammatory factor TNF- α and IL-1 β and, conversely, increased the concentration of the antiinflammatory factor TGF- β *in vitro*. Exosomes can induce the conversion of T helper type 1 (Th1) into T helper type 2 (Th2) cells and reduce the potential of the T-cells to differentiate into effector T-cells producing interleukin 17 (Th17). In addition, the levels of regulatory T-cells (Treg) and protein 4 associated with cytotoxic T lymphocytes were increased. The results suggest that the exosomes derived from MSCs possess immunomodulatory properties [12].

Inflammation is a response of the organism to self-evolutionary harmful stimuli to maintain homeostasis. In the process, MSCs secrete paracrine factors that influence immune cells, dendritic cells, and macrophages, polarizing them toward a tolerogenic phenotype. Regulatory immune cells accumulate and converge in their regulatory pathways and create a tolerogenic environment conducive to immunomodulation [13].

During tissue regeneration, the regulation of the inflammatory process is essential, as is the control of local and systemic inflammatory response without causing damage in the injured tissues. The MSCs possess immunomodulatory properties that facilitate the repair of tissues by releasing exosomes, which generate an appropriate microenvironment to modulate inflammation. The exosomes contain bioactive molecules, which act as a cell-cell communication vehicle and influence the activities of receptor cells. During this process, the horizontal transfer of exosomal microRNA to recipient cells regulates the expression of the target gene and is essential to control inflammation and tissue homeostasis to develop new therapeutic approaches [14].

In MSC therapy, the following points should be kept in mind: (i) arrival at sites of ischemia or injury, when administered systemically and (ii) modulation of the immune responses mediated by T-cells, which express chemokine receptors and ligands in the migration of the cells and the homing process.

The MSCs induce immunomodulatory effects, interact with innate immune cells (dendritic cells, monocytes, natural killer [NK] cells, and neutrophils) and cells of the adaptive immune system

(Th1, cytotoxic T lymphocyte and B lymphocyte), secreting factors such as TGF- β , IL-10, IDO, PGE-2, sHLA-G5. The MSCs are considered immune privileged cells due to the low expression of the major histocompatibility complex class II (MHC-II) and expressing costimulatory molecules on the cell surface and interfering with different pathways of the immune response. *In vitro*, MSCs inhibit cell proliferation of T-cells, B-cells, NK cells, and dendritic cells (DC), producing what is known as “division arrest anergy”. On the other hand, MSCs can inhibit diverse key functions of the immune cells, such as the secretion of cytokines and the cytotoxicity of T-cells and NK cells; B-cell maturation and antibody secretion; DC maturation and activation; as well as antigen presentation. In inflammation, MSCs must be activated to generate immunomodulation by suppression of molecules such as tumor necrosis factor (TNF)- α and interferon (IFN)- γ . On the other hand, MSCs recruit regulatory T lymphocytes (Tregs) from both lymphoid and graft organs [15].

In vivo studies have shown differences with respect to the immunomodulatory properties of MSCs. Currently, the effectiveness of MSC treatment to suppress the abnormal immune response in scenarios such as prevention, treatment of allograft rejection periods, and autoimmune and inflammatory diseases is being investigated. Clinical trials in humans are being developed in the treatment of autoimmune diseases such as Crohn’s disease, ulcerative colitis, multiple sclerosis, diabetes mellitus type 1, prevention of allograft rejection, survival of bone marrow and kidney grafts, and treatment of resistant graft versus host disease [16].

In vitro the MSCs are able to differentiate to osteogenic, chondrogenic, adipogenic, and myogenic lineages, and they express markers of pericytes (CD146+, CD34-, CD45-, CD56). In vascular damage, released pericytes become MSCs, are activated by the lesion, and respond by secreting bioactive molecules that inhibit immune cells that produce tissue damage and prevent the development of autoimmune reactions. The secretion of these bioactive molecules establishes a regenerative microenvironment in the injured tissue [17].

Activated MSCs also locally produce antimicrobial peptides such as LL37, which eliminate bacteria and attract macrophages and hematopoietic cells. Together, these function as therapeutic elements in the affected site and stimulate and increase TH2 and regulatory T-cells through inhibitory effects on the immune system. Thus, MSCs function as “medical signaling cells” with healing actions at sites of injury or inflammation. These trophic and immunomodulatory activities suggest that MSCs can serve as “pharmacies” regulated *in situ*. The MSCs act as “sentinels” in acute and chronic injuries; they function as multidrug dispensaries *in situ*, with “pharmacy” functions that promote natural regeneration [18].

In addition to the secretion of cytokines/chemokines, the MSCs show a great capacity for mitochondrial transfer and microvesicle secretion (exosomes) in response to injury. On the other hand, MSCs are recruited to the lesion to repair damaged tissues, an event intimately associated with tumorigenesis. Tumors are made up of different types of cancer cells that contribute to heterogeneity. Among these populations are the cancer stem cells (CSC) that participate in its onset and progression. A CSC population consists of MSCs that differ in cells with mesodermal characteristics. Resident or migratory MSCs favor angiogenesis and increase tumor aggressiveness. This interaction between MSCs and CSCs is fundamental in the development of carcinogenesis, progression, and metastasis. In cancer, tumor cells aberrantly secrete large amounts of exosomes to transport paracrine signals that contribute to tumor and distance interaction [19–21].

The MSCs represent an opportunity in cell therapy because: (i) they are easily accessible; (ii) the isolation is simple, they can be expanded to clinical scales in a short period; (iii) they can be preserved with a minimum loss of potency and stored for administration; and (iv) so far they have not shown adverse reactions to allogeneic transplantation compared with autotransplantation, and they can expand *in vitro*, without altering their main properties.

3. Therapeutic approach

The control of the growth, division, and differentiation of MSCs in a safe and predictable manner is essential in tissue regeneration. They should be used as bioreactors to achieve specific cell types in conjunction with soluble factors that lead to healing. A therapeutic strategy is the transplantation of differentiated functional cells to replace cells lost or damaged by disease. However, the strategy requires regulation of the differentiation of the SC toward specific cellular destinations, including those that are outside the mesenchymal lineage, by means of trans-differentiation, where genetic manipulation can promote it and the expression of certain transcription factors for cellular reprogramming.

Because of the plasticity of MSCs, in addition to generating bone, adipose tissue, cartilage, and other skeletal structures, differentiation can generate lineages of liver, kidney, muscle, dermal, nerve, and cardiac cells; regenerate damaged tissue; and treat inflammation in the MI, brain, spinal cord, cartilage, and bone lesions, Crohn's disease, graft-versus-host disease (GvHD) and BM transplantation. The mechanisms of orientation and immunomodulation, the potential for multiple differentiations, and paracrine actions contribute to tissue repair. Induced pluripotent stem cells (iPSC) are very promising for discovering new drugs in medicine regenerative (MR), for their ability to differentiate in any type of cell, and iPSC-induced technology will allow the development of new therapies based on cells and their products as new biological drugs [22].

Transcriptional and epigenetic regulations are essential mechanisms underlying pluripotency, are studied in ESCs, allowing them to give rise to lineages of the three germ layers, and are used in basic studies of tissue formation that provided the foundation for regenerative therapy. Continuous self-renewal is an essential requirement to maintain the transcriptional profile and pluripotent state. To differentiate themselves in other cell lineages, ESCs need to change the transcriptional profiles. On the other hand, new regulators of pluripotency and gene expression may emerge with the study of miRNAs [23].

The immunomodulatory properties of MSCs are related to paracrine factors whose expression varies in each pathology. These factors have a direct impact on cells of the adaptive immune system such as T-cells. However, in the inflammatory process, MSCs secrete paracrine factors that influence other subpopulations of immune cells, such as dendritic cells and macrophages, and polarize them toward a tolerogenic phenotype. *In vivo*, these immunomodulatory factors are increased in the serum of animal models with inflammatory diseases treated with MSCs. The manipulation of immune regulatory cells could improve the immunomodulatory therapeutic strategies of MSCs. Regulatory immune cells accumulate and converge in their regulatory pathways to create a tolerogenic environment [24].

The paracrine signals of the extracellular environment influence the microenvironment of MSCs, both in proliferation and in differentiation. Many therapeutic strategies try to increase the effectiveness of regenerative therapies by direct application in the affected tissue or by differentiation in mature tissues. The MSCs have phenotypic plasticity and harbor an arsenal of bioactive molecules that are released by detecting signals in the local environment or packaging in EVs [25, 26].

The rigidity and/or topography of the cellular environment controls the differentiation of the MSCs, the physical signals determining the target, and cellular differentiation, an environment with high rigidity that leads to osteogenic differentiation, while low rigidity induces lipogenic differentiation. These effects are independent of the chemical/biochemical inducers. Physical factors, such as tension, produce a reorganization of the cytoskeleton during the differentiation of the MSCs and affect the expression of the essential gene of the process. Physical signals control the lineage specification of the MSCs, reorganizing and adjusting the cytoskeleton, and the cells perceive physical signals and transform these into biochemical and biological signals. Specifically, biophysical signals can initiate and strengthen biochemical signaling for the determination and differentiation of the destination of MSCs. The physical properties of the cell environment direct the structural adaptation and functional coupling of the cells to their environment [27].

To facilitate the identification of terms that we use in the following section, we present here abbreviations and meaning of the terms:

“Extracellular vesicle” (EV), is synonymous with “membrane vesicle” (suggested for all populations of vesicles derived from cells);

“Exosomes” are vesicles of 50–100 nm in diameter, generated by exocytosis of multivesicular bodies (MVB), and are a macromolecular complex involved in the degradation of RNA;

“Ectosoma” is a microvesicle derived from neutrophils or monocytes;

“Microparticle” (MV) is any small particle, regardless of its origin, and is more appropriate to indicate membrane-bound structures;

“Microvesicles” (ExMV) are larger extracellular membrane vesicles (100–1000 nm in diameter) [28].

The EVs are classified into three main classes:

1. Microvesicles/microparticles/ectosomes: these are produced by the formation of buds and the fusion of the plasma membrane;
2. Exosomes: these form within the endosomal network and are released by fusing the multivesicular bodies with the plasma membrane; and
3. Apoptotic bodies: these are released as blisters of cells that undergo apoptosis.

The current nomenclature classifies the vesicles by their biogenesis. The criteria for classification are according to their origin, function, or biogenesis.

4. Mesenchymal stem cell extracellular microvesicles (ExMV)

Organ regeneration technologies attempt to restore the anatomical structure and original functionality of a damaged organ. Usually, the response is fibrosis and scar tissue formation and no regeneration. The strategies of IT and MR for the repair of organs/tissues allow restoration of normal functioning. The development of new products, derived from MSCs considered as active biological elements, has already started.

The mechanisms of action of therapies with MSC have focused on paracrine actions, for the ability to generate regeneration without the application of cells. The primordial component, which creates a regenerative medium, is exosomes: intraluminal vesicles of 40–100 nm that transfer proteins and nucleic acids between cells and establish intracellular communication. The exosomes participate in organogenesis and regeneration and repeat the bioactivity of the SCs [29].

The extracellular space of multicellular organisms contains metabolites, ions, proteins, and polysaccharides. In the extracellular environment, a large number of mobile vesicles participate, and the term “extracellular vesicles or EVs” is suggested, which includes exosomes, microvesicles, microparticles (MV) and apoptotic bodies. The EVs comprise a heterogeneous population of lipid vesicles derived from cells containing exosomes and microvesicles. They are the mediators of the intercellular information transfer and can be vehicles for the administration of drugs of autologous cellular products. The therapeutic effects of cell therapies are mainly attributed to the EVs secreted by cells and directly involved in tissue regeneration processes [30].

Currently, interest focuses on EVs (exosomes and MV). Vesicles similar to exosomes have a common origin; however, they lack lipid-based microdomains; their size and sedimentation properties distinguish them from exosomes; and the term refers to an extracellular vesicle with a diameter of 40–150 nm and a density of 1.09–1.18 g/ml, proteins, nucleic acids, and membrane vesicles that generate a regenerative environment [31, 32].

Eukaryotic cells communicate with each other through direct interaction (juxtacrine contact dependent signaling) or by the secretion of factors such as hormones, GF, and cytokines, when they act in the cell itself (autocrine signaling) or act in neighboring cells (paracrine signaling) and distant cells (endocrine signaling). Tissue regeneration is related to the release of paracrine and autocrine substances and not only by cellular replication and differentiation. Eighty percent of the therapeutic effect of adult SCs is through paracrine actions. The molecules released by the SCs, the secretome, contain molecules (100), proteins, microRNA, GF, proteasomes, and exosomes, which generate paracrine activities. The composition of the different types of molecules depends on the stage and varies according to cell type, age, and environment. The secretory activity of cell-derived byproducts acts at a distance and is the main regenerative mechanism [33, 34].

In multicellular organisms, cells exchange information with signals from molecules in packages included in the EVs, which contain proteins, lipids, and nucleic acids. When released in the extracellular environment, exosomes interact with receptor cells by adhesion to the cell surface, by lipid-ligand receptor interactions, by endocytic uptake, or by direct fusion of the vesicles to the cell membrane [35].

Preclinical and clinical studies with MSCs propose inducing endogenous repair, and this represents a new paradigm for the treatment of multiple diseases. The factors are produced from activated cells extracted from their physiological niches, aspirated BM or mobilized blood, such as peripheral blood mononuclear cells (PBMC), and these release biologically active paracrine factors that induce regeneration. The apoptotic secreted from PBMCs has been used successfully for the treatment of MI, chronic heart failure, spinal cord injury, stroke, and wound healing [36].

The EVs derived from the MSCs, exosomes, are powerful intercellular communication vehicles; composed of a lipid bilayer that contains transmembrane proteins, cytosolic proteins, and RNA; participate in diverse physiological and pathological functions of both receptor and parental cells; and transfer the information to other cells and influence the function of the recipient cell. The EVs transmit biomolecules (proteins, lipids, nucleic acids, and sugars) as a single information packet or deliver multiple messengers simultaneously to sites distant from the source EVs [37].

The EVs are between 100 and 1000 nm in diameter, and they are microvesicles, ectosomes, or microparticles, which sprout from the cellular plasma membrane. Other types of vesicles are exosomes, which are generated within multivesicular endosomes or multivesicular bodies (MVBs), secreted by fusing with the plasma membrane.) Exosomes are vesicles that are enriched with components derived from endosomes. They are targeted to the recipient cells and, once bound to a target cell, the EVs induce signaling through the receptor-ligand interaction or are internalized by endocytosis and/or phagocytosis, even fusing with the membrane of the target cell to release its cytosol content, which modifies the cellular receptor [38].

Exosomes of endocytic origin transmit different intercellular signals by surface interactions and by the displacement of functional RNA from one cell to another and are released by mast cells, dendritic cells, macrophages, epithelial cells, and tumor cells. Exosomes, released from mast cells exposed to oxidative stress, have the ability to communicate a protective signal to receptor cells exposed to oxidative stress to reduce cell death. Exosomes can influence the response of other cells to oxidative stress by providing resistance to oxidative stress to recipient cells and decreasing the loss of cell viability. The exosomal transfer of RNA is involved in cell-to-cell communication and influences the response of the recipient cells to an external stress stimulus. The mRNA content of the exosomes produced under oxidative stress differs both from the mRNA in the donor cell and in the exosomes produced by cells grown under normal conditions. [39]. Exosomes produced by cells exposed to oxidative stress have the ability to induce tolerance in other cells. This effect is associated with the change in the content of exosomal mRNA that is attenuated by the reduced activity of the RNA by exposure to ultraviolet light. This shows, for the first time, that exosomal RNA transfer can change the biological function of a recipient cell [40]. The encapsulation of biologically active ingredients of regeneration in carriers of nonliving exosomes offers advantages in the processing, manufacturing, and regulation of MSC-based therapies [41].

Exosomes and ExMVVs derived from MSCs influence tissue responses to lesions, infections, and diseases, and the exosomes of MSCs are not static, since they are the product of origin of MSCs, and they have actions with intercellular immediate neighbors. The MSCs, through paracrine action, activate the endogenous repair pathways, and the horizontal transfer of this load induces therapeutic changes; meanwhile, it has been demonstrated that microvesicles/exosomes derived from MSCs repeat the therapeutic effects of the parental MSCs [42].

A new generation of drug delivery systems can be mediated by exosomes and ExMVs; because they have high administration efficiency and low immunogenicity, new therapies can be implemented, and standardization is achieved with isolation techniques, with high functional efficiency, solid performance, scalable production, adequate storage, and efficient loading methods, which do not damage its molecular integrity and the movement of the elements *in vivo* as novel “nano-vehicles” [43]. The elements derived from cells generate an endogenous mechanism for intercellular communication; they are vehicles with the capacity to transfer biological information and the potential use can be as means of drug delivery [44].

5. Exosomes as biomarkers and therapeutic targets

All cells in the body secrete ExMVs, a heterogeneous population of bilayer vesicles with membrane that transport and deliver loads of proteins and nucleic acids to the recipient cells, allowing cell-cell communication. The exosomes, of endosomal origin, regulate normal and pathological processes. Healthy subjects and patients with different diseases release exosomes with different RNA and protein contents into the circulation, which can serve as biomarkers [45]. Compared to conventional biomarkers in serum or urine samples, exosomal biomarkers have greater sensitivity and specificity due to their high stability. They are present in almost all body fluids that harbor molecular components, exosomal proteins, and miRNA, and they are carriers of genetic information, which can be used for diagnosis. Although most RNAs found in exosomes are nucleotide fragments of degraded RNA with a length of <200 nm, some full-length RNA may be present. For example, circulating exosomal miRNAs are equivalent to those of the cancer cells of origin [45].

The power of nanovesicles as biomarkers depends on the enrichment of the exosomal classification markers, which otherwise only represent a very small proportion (<0.01%) of the total proteome of body fluids. The enrichment of the exosomal biomarkers of diagnosis will help in the discovery of new biomarkers to provide more precise information related to the origin of each exosome. A proteomic analysis and characterization of the plasma exosomes is essential, and gel permeation chromatography has been used to purify TNFR1 exosomal-like vesicles from the low-density lipoprotein (LDL) fraction. With this multistage purification scheme, it was possible to identify the 66 proteins of the circulating healthy exosomes, in the plasma, including proteins, both cytosolic and membrane-associated, as extracellular secreted proteins and associated with the cells identified with vesicular trafficking. The advantage of this analysis is that it allows the separation of different populations of vesicles according to their size. This reduces complexity in the identification of proteins in the plasma sample that may contain more than 1 million different intermixed proteins, and the discovery of peroxisome proliferator-activated receptor gamma (PPAR γ) as a component of plasma exosomes will allow identifying a new pathway for the paracrine transfer of nuclear receptors specific in each pathology [46].

5.1. Exosomal proteins as diagnostic biomarkers

The molecular content of exosomes is the fingerprint of the cell type that released it and its current status; most viable cells release extracellular environment, protein secretions of

exosomes, and when fused with the plasma membrane, appear in the blood and urine, so they are easily accessible, and can be used as biomarkers, for the diagnosis and prognosis of malignant tumors and other pathologies [47, 48].

Due to their cellular origin, exosomes express protein markers specific to the endosomal pathway, such as tetraspanins (CD63, CD9 and CD81), heat shock proteins (Hsp70), proteins of the Rab family, Tsg101 and Alix, which are not found in other vesicles of similar size. Their function is to eliminate damaged or aged cellular molecules, to protect cells from the accumulation of waste or drugs, participate in physiological and pathological processes, and have a wide variety of clinical applications, ranging from biomarkers to cancer therapy [49]. Proteomic and biochemical analysis of the purified exosomes revealed that the bilayer membrane of phospholipids is embedded with various proteins and lipids originating in the parental cells. These can serve as surface markers for the characterization and differentiation of exosomes from other types of microvesicles [50]. Exosomes contain various proteins, which express specific cellular functions, so they can serve as biomarkers for the diagnosis of liver, kidney, and cancer diseases [51]. Proteins in urinary exosomes are easily available through nontoxic means, are invasive, and are useful in diagnosis, especially for diseases of the urinary tract [52].

5.2. Exosomal nucleic acids as diagnostic biomarkers

Exosomes contain exosomal RNAs, especially miRNAs that function as diagnostic biomarkers, are protected from RNase-dependent degradation, are detected in circulating plasma, and serve for the diagnosis of ovarian cancer [53, 54]. Nanostructure analysis and study of the transcriptome of exosomes that transport RNA are diagnostic and found in breast milk, saliva, blood, urine, malignant ascites, amniotic fluid, bronchoalveolar secretion, and synovial fluid [55, 56].

Urinary extracellular vesicles (uEV) are released in the nephron of the kidney and urinary tract. Specific proteomic and transcriptomic markers provide information on the cell of origin and are a reservoir for the discovery of biomarkers in kidney diseases. The uEV are a new means of cell signaling, renal tubular cells, and can provide exosomal markers not detectable in urine. Renal biopsy is an invasive technique with complications such as infection and hemorrhage. The analysis of proteomic and transcriptomic changes of uEV in different disease states as a biomarker can be a noninvasive alternative to biopsy [57].

In conclusion, the most important biomedical utility of exosomes is their application as biomarkers in clinical diagnosis. Compared with those detected in conventional samples, such as serum or urine, exosomal biomarkers provide comparable or superior sensitivity and specificity, attributed to their excellent stability, and the exosomal biomarkers of biofluids can be easily obtained. Recent technical advances in the isolation of exosomes will make diagnostics more beneficial.

6. Stem cell therapeutics; exosomes as biomarkers in cardiovascular diseases

The intercellular communication between cardiac, vascular, SCs, and progenitor cells with differentiated cardiovascular cells is a complex process, with a diversity of mechanisms in

cardiovascular disease and therapeutics. The EVs are produced through different pathways and are released and absorbed by most cells including cardiac, vascular, and progenitor stem cells [58].

The conventional treatment in obstructive coronary disease is percutaneous coronary revascularization, angioplasty, stent placement, or coronary revascularization graft. In patients where there was no improvement or these treatments were not indicated, it is necessary to limit the damage produced by MI, to restore blood flow, and supply blood to the ischemic region. Microcirculation is a therapeutic target for the treatment of ischemic disease. Several preclinical studies show that CD34⁺ cells can stimulate neovascularization in ischemic tissue by increasing capillary circulation and improving acute and chronic myocardial ischemia [59]. A double-blind study showed lower rates of amputation in patients with critical ischemia of the lower limb with the administration of CD34⁺ cells [60]. Other studies mention that transplantation of CD34⁺ cells into ischemic myocardium after MI is better than neovascularization with mononuclear cells [61].

Cardiovascular diseases (CVD) have a high prevalence, morbidity, and mortality. The identification of biomarkers with high sensitivity and specificity can evaluate the prognosis of CVD, optimize personalized treatment, and reduce mortality. Biomarkers based on exosomes may reflect the stage and progression of coronary artery obstruction, heart failure, cerebrovascular accidents, arterial hypertension, cardiac arrhythmia, cardiomyopathy, valvular heart disease, and pulmonary arterial hypertension. On the other hand, exosomes as immunomodulators can be used in cardiac ischemia, pulmonary hypertension and many other diseases, including cancer, and also be used as a biomarker of the disease [62, 63]. Some exosomes can inhibit cell apoptosis and increase cell proliferation, contain specific surface proteins and the like, such as CD9, CD63, CD81, and proteins can transfect cardiomyocytes, endothelial cells, SC fibroblasts, and smooth muscle cells, and induce beneficial cellular changes. The release of exosomes from the cell is mainly regulated by Rab GTPase (Rab27a/b and Rab35). After a MI, the exosomes work in local and systemic microcommunications, in the exosomal transport of miRNA, and in the contribution of signals for cardiac repair, the myocardium can secrete exosomes, especially those that emerge in the border area of MI, so control of the quality and quantity of exosomes can serve as biomarkers in the diagnosis and prognosis of MI and as a new therapeutic objective to regulate cardiac remodeling [64].

The EVs secreted by the cardiac progenitor cells (CPC) can improve the cardiac function after the lesion by the content of exosomes with angiogenic factors that generate the ischemic tissue repair and are cardioprotective agents; the exosomes are the active components of the CD34⁺ of the BM. Experimentally, exosomes secreted by MSCs and CPCs have been shown to decrease tissue damage and facilitate ventricular remodeling in animal models of myocardial ischemia and reperfusion injury. The EVs are the active paracrine component of the CPCs [65].

In a study of stability, the exosomes of adult cardiac myocytes were shown to release heat shock protein (HSP) 60 in exosomes. When this protein is not in the exosomes, apoptosis is generated through the activation of the Toll-like receptor 4 and the release of Hsp60 would damage the surrounding cardiac myocytes. On the other hand, fever and a change in the pH

or ethanol consumption increase the permeability of the exosomes, and different inducers of exosomes modify the content of the exosomal protein. The production of reactive oxygen species (ROS) is an underlying mechanism of increased production of exosomes and ethanol at “physiological” concentrations would trigger the release of exosomes. This work, as determined by Western blot analysis and mass spectrometry, mentions that exosomes retain their protein load in different physiological/pathological conditions; the protein content of the exosomes’ cardiac etiologies differed from other types of exosomes due to their content of cytosolic, sarcomeric, and mitochondrial proteins. Ethanol did not affect the stability of the exosomes but increased the production of exosomes in cardiac myocytes; exosomes derived from ethanol and hypoxia/reoxygenation had a different protein content. Finally, inhibition of ROS reduced the production of exosomes [66].

Through circulating blood, circulating exosomes can reach distant tissues, allow direct communication with target cells, and regulate intracellular signals. Circulating exosomes and their exosomal charges participate in the hypertrophy of cardiomyocytes, apoptosis, and angiogenesis. Circulating exosomes enriched with various types of biological molecules can be modified in number and in loads of exosomes in cardiac lesions, such as MI, reperfusion injury, myocardial ischemia, atherosclerosis, hypertension, and cardiomyopathy due to sepsis, and can influence the function of the cardiomyocytes and contribute to the pathogenesis of CVD. A therapeutic strategy based on exosomes can be used to decrease myocardial injury and induce cardiac regeneration [67].

The CD34+ cells are a structural component in the formation of neovasculature in ischemic tissue, and secrete paracrine factors that stimulate the formation of new vessels, an element of proangiogenic paracrine activity associated with CD34+ cells secreted by exosomes, with a potent angiogenic paracrine activity both *in vitro* and *in vivo*. Exosomes stimulate mechanisms mediated by genetic receptors by transferring proteins, RNA, or microRNA directly to the cytoplasm of target cells [68].

All types of cardiac cells are able to secrete ExMVs, which are captured by the recipient cells and can alter gene expression or activate cascades of intracellular signals. A possible therapeutic intervention to reduce the ischemia/reperfusion injury (I/R) is the pre or post-conditioning, which allows the activation of the salvage recovery pathway by reperfusion salvage kinase (RISK), where transcription factors such as factor 1 α -induced hypoxia (HIF-1 α), mediators such as heat shock protein 70 kDa (Hsp70) and inducible nitric oxide synthase (iNOS), demonstrated in an *in vitro* preconditioning model, which does not influence the secretion of EV and its morphology, but it has an effect on EV size and particularly on its charge. EVs derived from fibroblasts enhanced cell migration and the effect was improved by means of *in vitro* preconditioning. An experimental model of *in vitro* preconditioning of cardiac cells concluded that it does not influence the concentration of ExMVs, but regulates their load and affects migration [69].

In conclusion, cardiac cells, such as cardiomyocytes, endothelial cells, and fibroblasts, release exosomes that modulate cellular functions. The exosomes released by CPCs are cardioprotective and improve cardiac function after MI, compared to that achieved by progenitor cells, and they have antiapoptotic, proangiogenic functions.

7. Cancer stem cells (CSC); exosomes

Malignant tumors arise from a small subset of cancer cells, have tumor heterogeneity, and small populations of cells with characteristics equivalent to SCs. These cells, called cancer stem cells (CSC) or cancer-initiating cells (CIC), have been identified in many malignancies and are thought to form the tumor clonogenic nucleus. The CSCs share many characteristics of ES and show activation of one or more signal transduction pathways, which are involved in tissue homeostasis and development, including Notch, Hedgehog (Hh), and Wnt pathways. Notch signaling, similar to the Wnt and Hh pathways, is a pathway for determining the fate of the evolutionarily conserved primordial cell, with great relevance in the biology of cancer, from CSC to angiogenesis and tumor immunity. The CSCs generally have slow growth rates and are resistant to chemotherapy and/or radiation therapy. The new treatment strategies seek to control the replication, survival, and differentiation of the CSCs [70]. These cells originate from a more differentiated cancer cell, with self-renewing properties, probably as a result of epithelial to mesenchymal transmission [71].

The most important and useful property of the CSC is that of self-renewal and characteristic differentiation, which is considered as a one-way specialization process as the cells develop the functions of their final destination and lose their immature characteristics, such as self-renewal. This property shows parallels between SCs and cancer cells. The tumors originate by the transformation of normal SCs by means of similar signaling routes, to which they regulate self-renewal, both of SCs and CSCs; the latter include the undefined potential of self-renewal that starts tumorigenesis. Otherwise, CSCs could be derived from a SC of normal tissue that undergoes a transformation as a result of oncogenic somatic mutations, due to the influence of extrinsic microenvironmental factors [72].

The CSCs are associated with tumor onset, metastasis, progression, invasion, recurrence, and resistance to therapies, and they play a central role in the biology of cancer cells; they interact with their surrounding cells inducing angiogenesis and metastasis. In the tumor microenvironment, multiple types of cells coexist, including adult SCs, CSCs, and stromal cells, and communicate with each other in modulating, tumor progression, functionally release exosomes that can be absorbed by CSCs or adult SCs, and modify their phenotype. Recent studies show that exosomes participate in interactions between cells within the tumor microenvironment by means of exosomal signals, modulating tumor progression [73].

We take Hannafon's approach in questions related to the function of the exosomes involved in the interaction of CSCs, adult SCs, and the surrounding cells within the tumor microenvironment, which are:

Do CSCs or adult SCs secrete exosomes that affect the function of the stromal cell? Are CSCs or adult SCs modified by the exosomes released from CSCs and surrounding stromal cells?

What are the possible molecular mechanisms and the biological consequences of exosome-mediated interactions between CSCs, adult SCs, and the cells that surround them? [74].

The SCs secrete a large number of exosomes, and in the extracellular environment, they function as intercommunicators in the tumor microenvironment and actively in tumorigenesis,

angiogenesis, and tumor metastasis, and the mechanism of interaction between the cancer cells and the tumor cells involves the exchange of biological material through exosomes. On the other hand, exosomes induce the formation of the premetastatic niche, which regulates tumor metastasis. Mechanisms mediated by exosomes contribute to resistance to antitumor therapy. Certain exosomes have an influence on tumors to evade immune surveillance [75].

Exosomes derived from SCs provide information related to the regulation of genes to target cells, for cell growth and angiogenesis by the modulation of various signaling pathways. Exosomes derived from MSCs potentiate the expression of VEGF in tumor cells by activating the kinase 1/2 pathway regulated by extracellular signal regulated kinases (ERK1/2) that promote tumor growth [76].

The ExMV's released by adipose mesenchymal stem cells (ASC) may contribute to angiogenesis induced by ASCs. In CSC, exosomes derived only from CD105+ CSCs conferred an activated angiogenic phenotype to normal human endothelial cells, stimulating their growth and vessel formation. A specific source of the ExMV's derived from CSCs contributes to triggering the angiogenic process and metastatic diffusion during tumor progression. The effects of exosomes of different types of SCs on angiogenesis are similar to those of exosomes derived from SCs in tumor growth [77].

Exosomes released from SCs contribute to tumor metastasis. Several key steps in tumor invasion and metastasis are associated with MSCs, and include the epithelial-mesenchymal transition and the induction of SC-like properties that allow CSCs to increase their survival capacity through circulation [78].

Recently, CSCs have been used in the diagnosis and treatment of cancer. The exosomes released from prostate and breast cancers have specific biomolecular characteristics, including the expression of several exosomal markers such as CD9, CD63, CD81, ALIX, and TSG101. In addition, the exosomes derived from GC-MSC contain miR-221, which is a new biomarker for the diagnosis of several tumors. The finding of tumor biomarkers is a new diagnostic tool. The release of exosome cells provides valuable detailed molecular information about the cell of origin and the tumor characteristics, can be isolated from easily accessible body fluids, and can provide specific information for the predictive diagnosis of multiple tumors [79].

8. Future conclusions and addresses

Due to its complexity, the research and application of cell therapy with cells and cellular products should be considered with a multidisciplinary and translational approach and represents a great therapeutic potential for refractory diseases to conventional treatments such as noncommunicable chronic diseases: diabetes, cardiovascular ischemic diseases, cerebrovascular or renal diseases, degenerative diseases such as cancer, neurodegenerative such as Alzheimer's disease, multiple sclerosis, and aging.

Thus, cell therapy with MSCs emerges as a promising therapeutic tool, the main therapeutic objective of which is healing through trans-differentiation to repair and replace

damaged cells and generate new healthy cells. The rapid progress in MSC research and the primary function in cellular niches under normal and pathological physiological conditions and the management of cellular intercommunication of the microenvironment through the paracrine secretion and their biological products are being incorporated into clinical practice.

The initial paradigm of cellular therapy for tissue and organ repair and regeneration has been modified, with new knowledge from experimental, preclinical, and clinical studies related to the mechanism of action of MSCs both *in vivo* and *in vitro*, which have demonstrated to be processes fundamentally of paracrine action, by means of the generation of exosomes, microvesicles, and the horizontal transfer of proteins, mRNA, and microRNA.

Recently, a group of secreted vesicles, the “exosome”, has been identified as the main mediator of the therapeutic efficacy of MSC. The ExMV participate in intercellular, local, and remote communication, which are translated into pleiotropic actions and generate a therapeutic potential by transferring biologically active molecules and which can be used as new biomarkers and potential regulators of inflammation and immune response to detect immune rejections. Exosome/microvesicle therapy derived from MSCs has potential advantages. First, it prevents the transfer of cells that may have mutated or damaged DNA. Second, the vesicles are small and easily circulated, while the MSCs are too large to easily circulate through the capillaries and many do not even reach the first capillary bed. Third, the dose of MSC decreases rapidly after transplantation, but the administration of the biological products of the cells allows higher therapeutic “doses”. The disadvantage of using vesicles derived from MSCs is that they are static and cannot occur more when they are transplanted. The therapeutic efficacy of MSCs is based on their ability to respond in the microenvironment of the lesion, whereas the isolated exosomes are not expected to do so. The opportunity to exploit the potential therapy of MSCs and their products opens new scenarios for the identification of new molecules for the repair and regeneration of organs and tissues through proteome analysis of the secretome.

In the short term, the exosomes derived from MSCs will progress to clinical studies, and their usefulness and effectiveness will depend on establishing a series of critical parameters such as standardizing reproducible production methods for the manufacture of exosomes/microvesicles with precisely defined content, standardizing storage methods that maintain their potency, and evaluating therapeutic efficacy in controlled clinical trials, of appropriate power, designed with written criteria and with solid research foundations to generate scientific results that allow the translation of basic knowledge to create new regenerative therapies.

Conflict of interest

No conflicts of interest, financial or otherwise, are declared by the authors.

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