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The mesenchymal stem cell secretome: A new paradigm towards cell-free therapeutic mode in regenerative medicine

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ABSTRACT

Mesenchymal Stem Cells (MSCs) have been shown to be a promising candidate for cell-based therapy. The therapeutic potential of MSCs, towards tissue repair and wound healing is essentially based on their paracrine effects. Numerous pre-clinical and clinical studies of MSCs have yielded encouraging results. Further, these cells have been shown to be relatively safe for clinical applications. MSCs harvested from numerous anatomical locations including the bone marrow, adipose tissue, Wharton's jelly of the umbilical cord etc., display similar immunophenotypic profiles. However, there is a large body of evidence showing that MSCs secrete a variety of biologically active molecules such as growth factors, chemokines, and cytokines. Despite the similarity in their immunophenotype, the secretome of MSCs appears to vary significantly, depending on the age of the host and niches where the cells reside. Thus, by implication, proteomics-based profiling suggests that the therapeutic potential of the different MSC populations must also be different. Analysis of the secretome points to its influence on varied biological processes such as angiogenesis, neurogenesis, tissue repair, immunomodulation, wound healing, anti-fibrotic and anti-tumour for tissue maintenance and regeneration. Though MSC based therapy has been shown to be relatively safe, from a clinical standpoint, the use of cell-free infusions can altogether circumvent the administration of viable cells for therapy. Understanding the secretome of *in vitro* cultured MSC populations, by the analysis of the corresponding conditioned medium, will enable us to evaluate its utility as a new therapeutic option. This review will focus on the accumulating evidence that points to the therapeutic potential of the conditioned medium, both from pre-clinical and clinical studies. Finally, this review will emphasize the importance of profiling the conditioned medium for assessing its potential for cell-free therapy therapy.

1. Introduction

Stem cells have been positioned at the apex of developmental hierarchies due to their ability to self-renew and differentiate towards various cell lineages [1]. Owing to these characteristics, stem cells are now at the forefront of new therapeutic approaches for treating a number of incurable diseases that could be either lifestyle-related or primarily genetic. They play a significant role in maintaining tissue homeostasis by replacing cells in response to the requirements of physiological cell turnover in an organism. Besides this, they also play a facilitating role in replacing damaged cells with healthy cells for improving the function of injured tissues. Thus, stem cells enhance the functional capacity of an organ that has been compromised due to substantial cell loss and tissue damage. Stem cells have been majorly classified as embryonic or somatic with the former being obtained from the inner cell mass of blastocysts. Somatic stem cells, on the other hand are obtained from peri-natal or post-natal sources. Somatic stem cells include both hematopoietic stem cells (HSC) and mesenchymal stem cells (MSC) [2]. Use of somatic cells in clinical therapy is neither limited by ethical considerations nor by any safety issues relating to teratomas formation and chromosomal abnormalities [3,4]. MSCs have garnered significant interest due to their immunomodulatory capacity that could enable their use in allogeneic settings [5]. MSCs also display tissue reparative properties apart from anti-tumorigenic, anti-fibrotic,

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anti-apoptotic, anti-inflammatory, pro-angiogenic, neuroprotective, anti-bacterial and chemo-attractive effects [6,7]. All these traits have attracted the interest of clinician scientists and hence the vast number of clinical trials are being evidenced based on the use of MSCs.

The success of MSC transplantation relates to large scale *in vitro* expansion of therapeutically qualified cells under Good Manufacturing Practice (GMP) conditions, although a standard therapeutic cell dose of MSCs, the route of administration and the number of doses are still being optimized. Although culture expansion of MSCs has been widely used, a few issues need to be highlighted. The number of population doublings required for obtaining sufficient numbers of MSCs for therapy would be dependent on the initial number of viable MSCs. Therefore, attaining sufficient numbers could subject to a large number of population doublings with the attendant possibility of stemness attenuation and cellular senescence. Further, to avoid the risk of immunological reactions and eliminate the transmission of zoonotic disease due to the use of fetal bovine serum (FBS), MSCs are now being increasingly propagated in xeno-free media [8]. It is unclear if the data obtained from clinical trials that are based on MSCs cultured with FBS would be comparable with the data obtained with MSCs cultured in xeno-free media. There has also been a constant debate about the decline in the engraftment and homing ability, poor survival rate and impaired differentiation ability of transplanted MSCs *in vivo* limiting their therapeutic potential [9]. Due to the aforementioned issues associated with MSC-based therapy, cell free therapy using the MSC secretome could serve as a better futuristic option in the field of regenerative medicine.

Recently, evidence has accumulated supporting the effectiveness of MSC-conditioned medium (CM) or secretome in studies directed at assessing its therapeutic potential for indications such as osteoarthritis, spinal cord injury, cardiovascular disease, gastric mucosal injury, colitis etc [10–13]. MSC-CM contains a plethora of cytokines and a wide array of bioactive factors that are secreted by MSCs. Characterization of the MSC-CM is important as its therapeutic potential has been attributed to the cytokine mixtures with their attendant paracrine activities [14]. Molecular analyses of the MSC-CM can identify key therapeutically active components that can be further purified and used. Furthermore, there would be an added interest in understanding the mechanisms by which such key components exert their therapeutic effects. Hence, the focus of this review is to summarize some of the experimental, pre-clinical and clinical studies, where the MSC secretome was tested as a treatment option with the larger goal of developing an effective cell-free based therapy.

2. Mesenchymal stem cells

MSCs are non-hematopoietic, multipotent adult stem cells that were initially isolated from the bone marrow and named colony forming unit-fibroblasts (CFU-Fs) [15]. With more than four decades of growing research on MSC populations, they have now been harvested and expanded from numerous adult and peri-natal tissues including the bone marrow (BM), adipose tissue (AT), peripheral blood, menstrual blood, pulp of deciduous teeth, umbilical cord tissue (UCT), Wharton's jelly (WJ), umbilical cord blood, placental tissues (PL), breast milk etc. *via* different protocols [16–18]. Various researchers have successfully obtained clinical grade MSCs using xeno-free media and by utilizing human supplements such as platelet lysate (PL), umbilical cord blood serum (UCBS), human AB serum, etc. to prevent any zoonotic disease transmission and avoid immunological reactions due to the xenogeneic supplement (FBS) [19–21]. However, the majority of the pre-clinical and clinical studies reported till now have been based on the use of MSCs cultured with FBS [22]. Although the vast majority of these studies have shown MSCs to be safe and effective, at least in one MSC-based study, retardation of growth was observed in one of six patients with osteogenesis imperfecta and this was attributed to the immunological attack due to the internalized foreign antigen in the transplanted MSCs [23].

The International Society for Cellular therapy (ISCT) in 2005 had established benchmarks for defining MSCs with the following minimal criteria (a) must be plastic-adherent and fibroblastoid under standard culture conditions (b) must display immunophenotypic expression of CD73, CD90, CD105 and a lack of expression of CD34, CD45, CD14, CD19, CD79a, CD31 and HLA-DR surface markers (c) and lastly, must have the capacity to minimally differentiate into adipocytes, osteocytes, and chondroblasts *in vitro* [24]. Studies have demonstrated that the MSCs derived from disparate tissues exhibit heterogeneity in their biological characteristics and functional features due to their difference in proliferative ability, multi-lineage differentiation potential, pro-angiogenic ability, and immunomodulatory capacity despite their phenotypic similarity [25]. For example, MSCs derived from fetal tissues such as UCT have been shown to exhibit higher proliferative potential along with lower immunogenicity as compared to MSCs from adult tissues such as BM and AT [26]. Additionally, MSCs derived from placental chorionic villi and BM displays superior pro-angiogenic characteristics as compared to the MSCs derived from AT and UCT [27]. Based on these studies the ISCT put forth additional criteria for defining MSCs in 2016 [28]. It was also suggested that additional assays to clarify the biological and functional features of MSCs be carried out so that consistent and reproducible results could be obtained in clinical settings. These assays/analyses could include:

- Potency tests for cellular therapy products
- Analytical test to measure the potency
- Assay Matrix
- Immunomodulation Assays
- Immune plasticity
- Quantitative RNA analysis of selected gene products
- Flow cytometry analysis of functionally relevant surface markers
- Protein-based assay of the secretome

3. Therapeutic characteristics of mesenchymal stem cells

The therapeutic benefits of MSCs have been well demonstrated in numerous experimental, pre-clinical and clinical models using non-clonal populations [29]. MSCs have been considered as an effective tool for tissue repair given their ability to migrate to the site of injury and their capacity to suppress the inflammatory response injury thereby promoting wound repair and healing [30]. The wound healing and tissue reparative properties have also been attributed to bioactive factors secreted by the MSCs contributing to the paracrine activity [31]. It is also worth mentioning that MSCs prevent cell death by decreasing the expression of pro-apoptotic factors (Bax, Caspase-3) while increasing the anti-apoptotic activities (Bcl-2) and restoring the local micro-environment within the damaged tissues [32]. In numerous pre-clinical experiments, MSCs have been transplanted into animal models for generating mesodermal derivatives such as bone, muscle, cartilage *via* differentiation in order to repair the damaged tissues. The self-renewal ability of MSCs along with their differentiation potential contribute to tissue homeostasis. Some of the multifunctional characteristics of MSCs that make them ideal for therapeutic use are their ability to home in to the site of tissue injury, engraftment and an immunosuppressive function as exerted by immunocytes. Such beneficial features led to the transition of MSCs from the bench to the bedside for clinical trials. Due to the low-level expression of MHC class II molecules and the lack of expression of co-stimulatory molecules such as CD80 and CD86, MSCs can interact with and inhibit the proliferation of several immune cell types (T cells, B cells, and natural killer cells) thus rendering them only mildly immunogenic. The low immunogenicity meant that MSCs could be used in allogeneic settings. The immunomodulatory capacity of MSCs, which makes them immune privileged has been substantiated *in vitro* by one-way and two-way mixed lymphocyte reaction (MLR) where MSCs inhibit the proliferation of stimulated allogeneic T cells [33]. Several studies have demonstrated the mediation of the

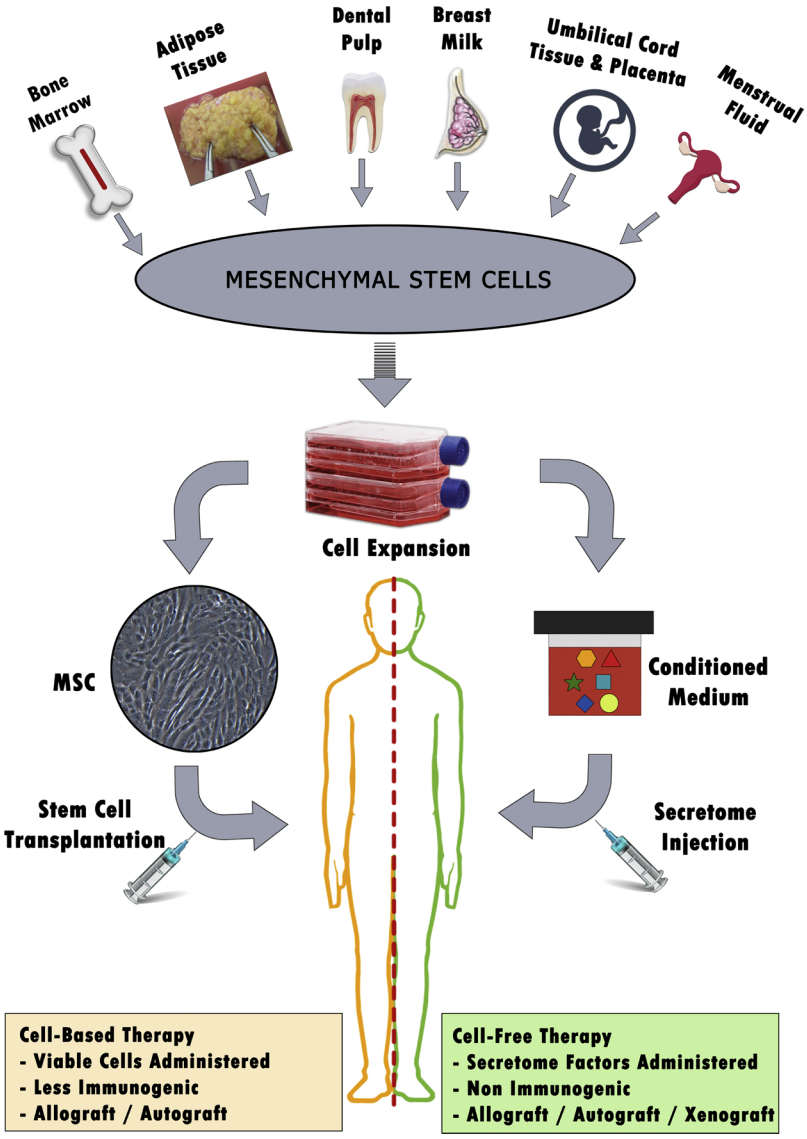

Fig. 1. Pictorial representation of cell-based and cell-free therapy strategies for human clinical use. Harvest and *in vitro* culture expansion of MSC obtained from different anatomical locations yields potentially therapeutic products such as MSC and MSC-CM both of which are rich in growth factors, cytokines, interleukins, etc. Potential therapeutic applications of MSC and MSC-CM would be cell-based and cell-free therapy respectively.

immumodulatory and anti-inflammatory activities of MSCs *via* the release of several cytokines such as prostaglandin E2 (PGE2), HLAG-5, indoleamine 2, 3-dioxygenase (IDO), tumour necrosis factor β1 (TGF-β1) and interleukin (IL)13 [34,35].

4. Cell-based therapy

Autologous and allogeneic MSCs have been used in cell-based therapies to repair and replace damaged tissues and enhance the function of tissues and organs or to exert immunomodulation *via* systemic infusions [36]. The utility of MSCs, harvested from different sources, for cell-based therapies is outlined in Fig. 1. The safety of MSC use in cell-based therapies is strongly supported by the fact that no tumours have been reported in human recipients. This has served to enhance the potential application value of MSCs thus resulting in a number of clinical trials for a wide range of clinical conditions. Ongoing and completed trials have been listed at www.clinicaltrials.gov, a database of the US National Institutes of Health, with the majority of the trials involving the use of BM-MSC [37]. The multifunctional characteristics of MSCs and their availability from a number of sources have led to more than 700 clinical trials being listed as of January 2019 for a myriad of diseases. Many of these trials are founded on the use of MSCs for terminal conditions or as an alternative to conventional therapy in order to improve the quality of life and to prolong the survival of patients. Some of the indications include graft versus host disease (GVHD), systemic sclerosis, liver failure, diabetes, idiopathic Parkinson's disease (PD), spinal cord injury, critical limb ischemia, knee joint osteoarthritis, Duchenne muscular dystrophy, cartilage tissue engineering, cardiovascular, haematological malignancies, autoimmune diseases, etc [38]. Despite encouraging results from these trials, MSC based therapy is still not considered to be the standard of care at the clinic. MSC-based cell therapies have been hobbled by the absence of a standardized protocol for isolation, a lack of clarity on the ideal source for the different indications, absence of a standardized protocol for ex vivo expansion, clonal populations, culture conditions, and the absence of clarity on the mode of infusion, effective delivery route, optimal dosage of infusion, administration frequency, etc [39,40].

5. The mesenchymal stem cell secretome

MSCs are known to secrete a spectrum of protective bio-active factors (secretome) usually classified as cytokines, chemokines, cell adhesion molecules, lipid mediators, IL, growth factors (GFs), hormones, exosomes, microvesicles, etc. These factors have been considered as protagonists to participate in tissue repair and regeneration through their paracrine actions that mediate cell-to-cell signaling [41]. The secreted molecules broadly defined as secretome or CM play a key role in influencing the cross-talk communications between the cells and the surrounding tissues in order to mediate a biological function. This attracted the interest of researchers towards the MSC secretome that could potentially be used in cell-free therapy settings. Critical to the success of such a cell-free therapy would be the identification, analysis and the elucidation of the mechanism of action of each component of the secretome. After the *in vitro* expansion of MSCs under laboratory conditions, the cells tend to release a set of bioactive factors into the culture medium that is now termed as the conditioned medium or the MSC secretome. Such a CM with the bioactive factors can exert beneficial effects on the recipient that could be considered as tissue protective (anti-apoptotic, anti-inflammatory, anti-scarring), immunomodulatory, angiogenic or anti-tumorigenic [42–44].

Interestingly, CM obtained from WJ-MSCs has been shown to be potent cryopreservative along with DMSO and FBS and enhances the free-thaw survival of CD34+ cells by protecting the cell membrane integrity during freezing. It also stimulates mitosis, post-thaw, for the ex-vivo expansion of CD34+ cells [45]. Factors in the MSC-CM can be artificially synthesized and used or the CM itself can be utilized for cell-free therapy with the beneficial effects being obtained *via* paracrine effects on neighbouring cells and tissues in either case. Intravenous administration of recombinant cytokine mixtures that mimic the components of human MSC secretome has revealed enhanced proliferation and osteogenic differentiation of rat BM-MSC [46]. A schematic representation depicting the collection of MSC-CM from the confluent cultures of MSCs and its application in cell-free therapeutic settings is outlined in Fig. 1. Further, it should be emphasized that the artificial synthesis of these bioactive factors could be expensive and may not reflect the profound pleiotropic effects as in the case of MSC-CM. Clearly, given its composition, MSC-CM should better orchestrate a "symphony of signals" rather than what can be effected by the administration of single cytokine [47,48].

5.1. Pre-conditioning strategies to stimulate MSC secretions

Manipulating cultures of MSCs by physiological pre-conditioning, molecular preconditioning using proteins, genetic manipulation, pharmacological preconditioning, serum deprivation, physical preconditioning can enhance the secretion of therapeutic factors into the culture medium [49,50]. Also, serum-deprivation in confluent cultures of MSC enhances the secretion of the prosurvival and angiogenic factors including vascular endothelial growth factor A (VEGF-A), angiopoietins (ANGPTs), insulin-like growth factor-1 (IGF-1) and hepatocyte growth factor (HGF), thereby favoring angiogenesis [51]. Conditioned medium of rat BM-MSC transduced with conditionally expressed GATA-4 gene (G-CM) favored angiogenesis and migration in human umbilical vein endothelial cells (HUVEC) as demonstrated by an increase in capillary-like tube formation and the number of spheroid sprouting structures when compared with the non-transduced or null vector transduced rat MSCs-CM [52]. Quantitative analysis of G-CM by ELISA revealed significantly higher levels of IGF-1 and VEGF-A. Physiological pre-conditioning strategies of subjecting the rat AT-MSC to hypoxic (HPO) conditions for 48 h resulted in an increased secretion of several cytokines such as VEGF, tissue inhibitors of metalloproteinases-1 (TIMP-1), CINC-1, LIX and Chemokine (C-C motif) ligand 20 (CCL-20) compared to normoxic (Nor) conditions. Increase in levels of CCL-20 in HPO-CM accounted for the upregulation of the COX_2-PGE_2 axis mediated by phosphorylation in the ErK1/1-MAPK pathway, thereby promoting the enhancement of angiogenesis and re-epithelization in the *in vitro* and *in vivo* model of gastric mucosal injury [10]. *In vitro* cultured MSC primed with TNF-α and IFN-γ (50 ng/ml each) for 24 h showed a 15- and 60-fold increase in the secretion of immunomodulatory proteins IL-6 and IDO respectively, in comparison with non-stimulated conditions [11].

5.2. Proteomic analysis

Despite displaying similar phenotypic characteristics, it has been reported that MSCs differ significantly in their gene expression patterns and not unexpectedly, demonstrate a heterogeneity in the secretome profile as well, with the differences attributable to the source of the MSCs, the age of the host, and the culture medium in which they are propagated [53,54]. Such differences in phenotypically similar MSC populations have led to a rethink on the basic criteria that define MSCs. Clearly, proteomic profiling of the MSCs is to be added to the criteria that were initially outlined by the ISCT as a means of identifying these cells. Although many questions regarding the complex components of MSC-CM remain unresolved, efforts are ongoing to identify and characterize the secretome from the different sources of MSCs. Characterization of the different MSC secretomes should aid in identifying the utility of each vis-à-vis the different clinical conditions. The process of determining the therapeutic utility of the factors in the MSC secretome based on the identification of the protein components by proteomics followed by data analysis is schematically outlined in Fig. 2. Serum or any growth supplements in the culture medium can overlap with and interfere with the detection and analysis of proteins secreted by the

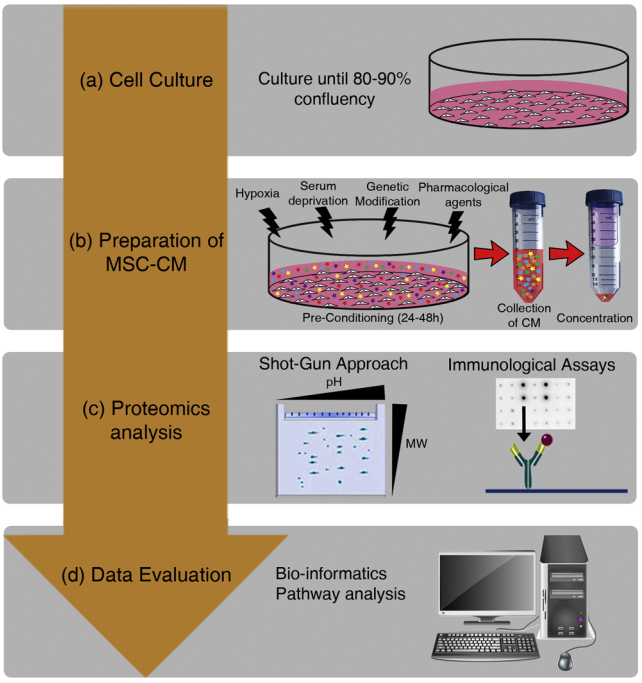

Fig. 2. A schematic diagram depicting the preparation of MSC-CM and the methods of protein identification to assess the therapeutic utility. The various steps include (a) culture of MSC until 70–80% confluency is attained (b) subjecting the cultured MSCs to different pre-conditioning strategies such as hypoxia, genetic manipulation, exposure to pharmacological compounds, serum deprivation for 24–48 h causing the stimulated release of growth factors, cytokines, interleukins, etc. into the medium. Such a conditioned medium containing soluble factors at low concentrations is collected and further concentrated for protein identification (c) Proteomic analysis involving the identification of secreted factors through different approaches such as shot-gun methods and immunological assays (d) determining the functional utility of the secreted molecules by evaluating the data using bioinformatics tools and by pathway analysis for establishing the best therapeutic use.

cultured MSCs. Hence, some components which are secreted at lower concentrations of nanograms to picograms can be masked during proteomic profiling due to the presence of growth supplements in serum. To avoid this problem, it is advisable to culture the cells in serum-free media for 24–48 h on attaining confluency. A number of methods are available to identify the protein components in the MSC-CM or spent media. However, identifying all the secreted proteins is a challenge given that they are present in very low concentrations-ranging from picograms or less to a few nanograms per mL. It is therefore imperative to concentrate the sample either by lyophilization or by ultrafiltration prior to the identification process [55].

Two predominantly available contemporary proteomics approaches for characterizing the MSC-CM are based on shot-gun and immunological assays [56]. Immunological assays offers high specificity, sensitivity, and reproducibility towards a broad range of known proteins. These proteins can be detected and quantified via antibody-based techniques such as enzyme-linked immunosorbent assay (ELISA), Luminex antibody bead-based array, microarray, western blotting, and cytokine antibody array. The shotgun-based proteomics approach is more exploratory in nature but it facilitates in the identification of any unknown and unique secreted proteins. The role of such unique proteins can be determined by accessing publicly available databases and using bioinformatics tools and doing pathway analyses. Different techniques employed under shotgun approaches are gel-based methods like 2-D gel electrophoresis (2-DE), liquid chromatography with tandem mass spectrometry (LC–MS/MS), stable isotope labelling by amino acids in cell culture (SILAC), matrix-assisted laser desorption/ionization - time of flight (MALDI-TOF), MS/MS, quadrupole time-of-flight mass spectrometry (QTOF-MS) etc [57–59]. Some of the well-known proteins with therapeutic utility, that were obtained from different MSC sources and identified using the different approaches are enlisted in Table 1.

6. Cell-free therapy

The presence of a plethora of proteins with therapeutic potential in the MSC-CM has expanded the utility of MSCs to cell-free therapy [60]. This new frontier of research provides several key advantages over cell-based applications: (a) employs the administration of proteins instead of whole cells as a new therapeutic option in regenerative medicine (b) CM can be stored without any toxic cryopreservatives such as DMSO for a relatively long period (c) preparation of CM is more economical as it can be mass-produced from the available MSC populations under cGMP conditions (d) evaluation of CM for safety and efficacy will be much simpler and analogous to conventional pharmaceutical agents [61]. Clinical trials based on administration of a single cytokine have been conducted for the treatment of cardiovascular diseases, degenerative diseases, etc., but the results have not been encouraging [48]. These results led to the suggestion that for optimal clinical outcomes it would be more appropriate to use multiple cytokines/growth factors that can then act synergistically. The above suggestion is very much in line with our contention that the MSC secretome, with its therapeutically effective components, could be a suitable candidate for cell-free therapies. In any event, MSC based cell therapies are still subject to issues such as poor cell survival in the host post-transplantation, a poor differentiation capacity within the host, sequestration at non-target sites and poor long-term engraftment. In sum, there is significant justification for seeking a MSC secretome based therapeutic alternative.

Both the transcriptome and the proteome are significantly different in MSC populations derived from the different anatomical locations and these profiles are also influenced by the age of the individual. Not surprisingly, the secretome profile is also heterogenous [54]. Increased IL6 secretion by MSCs obtained from adult donors leads to a decreased capacity to support hematopoietic stem and progenitor cell (HSPC) quiescence and a disruption of HSPC homeostasis [62]. In such scenarios, it can be argues that MSCs with the appropriate secretome signature can be chosen, keeping the therapeutic end point in mind. ELISA-based studies of the human fetal secretome (HFS) and the human adult secretome (HAS), derived from cultured human fetal tissues and cultured human adult BM-MSC respectively, showed significantly higher levels of Leukaemia inhibitory factor (LIF), a stemness biomarker and Platelet-derived growth factor (PDGFA), a proliferative and osteogenic induction marker in HFS compared to HAS. Furthermore, increased levels of laminB1 (LMNB1) was seen in HFS compared to HAS indicating that the latter was obtained from a more senescent MSC population [63]. A study comparing the CM of bone marrow, adipose tissue and human umbilical cord perivascular (HUCPVC) MSCs by LC–MS/MS indicated the presence of anti-apoptotic agents in the CM derived from the BM and HUCPVC MSCs while the CM obtained from HUCPV and AT MSCs conferred protection against excitotoxicity [53].

Exploitation of the MSC secretome for therapeutic purposes would

Table 1
Therapeutic proteins identified through different analytical approaches from the conditioned medium of mesenchymal stem cells, harvested from varied sources. Ang: Angiopoietin; BMP4: Bone morphogenetic protein; CRH: Corticotropin-releasing hormone; FGF: Fibroblast growth factor; GDF: Growth/differentiating factor; HGF: Hepatocyte growth factor; IDO: Indoleamine 2, 3-dioxygenase; IGF: Insulin-like growth factor; IL: Interleukin; LIF: Leukaemia inhibitory factor; MCP-1: Monocyte chemoattractant protein-1; MFGE8: Milk fat globule-EGF factor 8; NGF: Neurotrophins nerve growth factor; NT3: Neurotrophin 3; PGE2: Prostaglandin E2; TGF: Transforming Growth Factor; TIMP: Tissue inhibitor of metalloproteinases; TNF: Tumour necrosis factor; VEGF: Vascular endothelial growth factor.

Source of MSC-CM	Protein identification method	Identified therapeutic factors	Functional role	References
Human Bone Marrow	Mass spectrometry	VEGF-C, TGF-β1, TGF-β2, GDF6	Haematopoiesis regulation	[75]
Umbilical cord derived MSCs	Nanochip-liquid chromatography/ Quadrupole time-of-flight mass spectrometry	MFGE8	Anti-fibrosis	[69]
Bone marrow MSC	ELISA	HGF, IGF-1, VEGF, Angiogenin	Angiogenesis	[51]
Adipose-derived stem cells	Bio-Plex analysis	IL-6, IL-8, MCP-1 VEGF		[76]
Rat Bone Marrow MSC pre-treated with TNFα and hypoxia and H9 conditioned medium	Western Blotting	Ang-1, Ang-2, FGF-2, FGF-7, VEGF-1, TGF-β	Angiogenesis	[77]
Bone marrow MSC	ELISA	IGF-1, VEGF, TGF-β1	Angiogenesis and bone regeneration	[72]
Human MSC	ELISA	IGF-1, VEGF, TGF-β1, HGF	Osteogenesis	[73]
Bone marrow MSC Placental Chorionic Villi	ELISA	VEGF HGF, PGE2	Angiogenesis	[27]
Human MSC	ELISA	Kynurenine (IDO activity), IL-6, TIMP2, TGF-β1, HGF	Anti-inflammatory Osteoarthritis (OA) therapies	[11]
Mouse MSC	ELISA	IL-10, TNFα	Anti-inflammatory	[68]
Bone marrow MSC [Multilineage-differentiating stress enduring cells (MUSE)]	LC-MS/MS	CRH, LIF, BMP4, FGF18	Anti-apoptotic Control cell commitment	[43]
Human adipose-derived mesenchymal stem cells	Cytokine Array Kit	IL-8, HGF, VEGF, Angiogenin	Angiogenesis	[71]
Human Gingival MSC	Western Blotting	NGF, NT3, IL-10, TGF-β	Neuroprotective	[65]

be based on (a) the understanding of the pharmacokinetics of secretome factors to assess their retention post-transplantation, (b) the production of MSC-CM under cGMP complaint conditions, (c) the determination of the optimal route of administration, (d) establishment of the volume and duration of dosages and (e) positive data from human clinical trials. Data from pre-clinical studies and clinical trials points to the potential utility of MSC-CM obtained from different MSC sources towards different therapeutic endpoints. A subcutaneous injection of HFS suspended in matrigel (50 μg/mL) did not elicit tumour formation during the 1-month observation period in nude mice [63]. This data supports the view that no safety issues would be encountered during the use of MSC-CM. In any case, given that the use of MSCs in clinical trials has been repeatedly shown to be safe, it would be extremely far-fetched to expect the MSC secretome to be tumorigenic. Maximal induction of osteogenesis was observed in the case of rat BM-MSCs that were treated with human fetal MSC-CM (hFMSC-CM) that contained protein at a concentration of 100 μg/μl. Osteogenic induction was clearly demonstrated by the detection of calcium deposits *via* alizarin red S staining [64].

A number of *in vitro* studies have sought to identify the potential therapeutic effects of MSC-CM by treating cell lines with the same. CM from human gingival mesenchymal stem cells (hGMSCs) conferred significant protection against scratch injury induced cell death in the NSC-34 cell line (motor-neuron-like cells). The neuroprotection was attributed to the presence of neurotrophic factors nerve growth factor (NGF), NT3 and the anti-inflammatory cytokine IL-10 and transforming growth factor beta (TGF-β) [65]. Treatment of breast adenocarcinoma (MDA-MB-231), ovarian carcinoma (TOV-112D), and osteosarcoma (MG-63) cell lines with 50% conditioned medium from WJ stem cells for 48–72 h had an inhibitory effect on the growth of these cancer cells which then showed cell shrinkage, blebbing and vacuolations [66]. Interestingly, the secretome of adult MSCs appear to be more immunogenic than the secretome of fetal MSCs. Using the conventional mixed lymphocyte reaction it was observed that lymphocyte proliferation was stimulated by the hAMSC secretome whereas the hFMSC secretome did not elicit a similar response [64].

7. Pre-clinical studies based on the MSC secretome

A number of pre-clinical studies have demonstrated the therapeutic potential of MSC-CM for a variety of ailments such as inflammatory bowel disease (IBD), the antigen-induced model of arthritis (AIA), Parkinson's disease (PD) etc. Nevertheless, clinical trials for such indications need to be conducted to establish the therapeutic efficacy of MSC-CM.

7.1. Anti-inflammatory activity

Many studies have reported the presence of anti-inflammatory factors in the MSC secretome that could be contributing to the beneficial effects seen in animal models of diabetes, acute colitis, inflammatory arthritis, etc [12,67,68]. A single intravenous injection of hAT-CM into streptozotocin (STZ) treated diabetic mice relieved the diabetic neuropathic pain by re-establishing the Th1/Th2 balance with a long-lasting relief of sensory hypersensitivity. Cytokine measurements examined from the dorsal root ganglia, sciatic nerves and spinal cord of treated mice revealed for the restoration of the anti-inflammatory and immunomodulatory cytokines IL-1β, IL-6 and TNF-α to basal levels after 1 week of treatment with hAT-CM. Increase in the level of IL-10 also indicated a switch to an anti-inflammatory environment [67]. Intraperitoneal injection of MSC-CM into the C57BL/6 mice after the induction of colitis led to a significant decrease in colon inflammation and an increase in colon weight and length thereby reducing the disease activity index (DAI) and mortality rate. Furthermore, the mesenteric lymph nodes and spleen of the mice infused with MSC-CM revealed elevated levels of the anti-inflammatory cytokines IL-10 and TGF-β and reduced levels of the pro-inflammatory cytokine IL-17 confirming the anti-inflammatory role of the CM [12]. Similarly, intra-articular injection of murine MSC-CM was shown to be effective in reducing disease severity and cartilage damage in the antigen-induced model of inflammatory arthritis. The high levels of IL10 in the CM again correlate well with an anti-inflammatory response [68].

7.2. Anti-fibrotic activity

The MSC secretome shows anti-fibrotic effects that lead to decreased accumulation of extracellular matrix proteins thereby reducing scar formation. After the induction of hepatic fibrosis in mice either with thioacetamide or with CCI4, injection of the UCMSC secretome resulted in the reduction in fibrotic areas within 3 days and this was accompanied by a decrease in the number of activated hepatic stellate cells expressing α- smooth muscle actin (α-SMA). Analysis of the UCMSC secretome using nano-chip-LC/QTOF-MS revealed the presence of milk fat globule EGF factor 8 (MFGE8), an anti-fibrotic protein known to down-regulate the expression of TGF-βR1 (transforming growth factor β type 1 receptor) at the mRNA and protein level, thereby decreasing the activation of human hepatic stellate cells [69].

7.3. Differentiation potential for functional recovery

It is likely that factors present in MSC-CM could support cell differentiation and thereby repair damaged tissues as a prelude to functional recovery. Grafting of neural stem cells (NSC) pre-treated with MSC-CM into 6-hydroxydopamine (6-OHDA) rat models of PD improved the survival and homing characteristics of the NSC, generated dopaminergic neurons, mediated the repair of the neural damage and resulted in improved learning abilities and memory [70]. Enhanced osteogenic differentiation signifying bone regeneration along with improved bone consolidation was observed in the rat distraction osteogenesis model upon the application of the hFMSC secretome [64]. Systematic infusion of 0.4 ml of hATMSC-CM for 30 days in radiation-induced intra-villi stressed mice promoted intra-villi angiogenesis by recruitment of circulating endothelial progenitor cells from the bone marrow leading to the rapid restoration of the intra-villi microvascular structure [71]. *In vivo* application of MSC-CM containing IGF-1, VEGF and TGF-β1 on the Wistar rat calvarial bone defect model resulted in enhanced bone regeneration along with angiogenesis when evaluated by micro-computed tomography [72].

8. Clinical studies based on the MSC secretome

We performed a thorough search to identify clinical studies based on the use of MSC-CM at www.pubmed.com. To our knowledge, there are just two but more such studies should be initiated shortly to harness the therapeutic benefits of MSC-CM. Both these clinical studies were performed by the same group for assessing alveolar bone regeneration and angiogenesis in newly regenerated bone on administration of the secretome from hMSC. In the first report beta-tricalcium phosphate (β-TCP) or atelocollagen sponge (ACS) acting as a scaffold was soaked in BM-MSC-CM solution for 5 min. and grafted into eight patients (inclusive of three men and five women) requiring bone augmentation after maxillary sinus floor elevation (SFE) and guided bone regeneration (GBR). BM-MSC-CM possessing IGF-1, VEGF, TNF-β1, and HGF in the concentration range of hundreds to thousands of picograms per milliliter demonstrated greater osteogenic potential and resulted in successful alveolar bone regeneration. No safety issues were encountered and there were no local and systemic complications post the engraftment. Additionally, ACS-MSC-CM was resorbed more easily and resulted in denser bone formation compared to β-TCP-MSC-CM as indicated by its tougher mechanical characteristics [73].

The second clinical study was similar to the first one and was focused on assessing bone regeneration and angiogenesis due to MSC-CM.

The experimental group consisted of four patients where β-TCP mixed with BM-MSC-CM was grafted after maxillary sinus floor elevation (SFE). The control group consisted of two patients where β-TCP mixed with saline was implanted. Histological evaluation after 6 months indicated bone regeneration and angiogenesis at a significantly higher level in the experimental group compared to the control group. Further, there were no adverse effects. Therefore, MSC-CM could be a promising alternative to the infusion of recombinant human bone morphogenetic protein-2 (BMP-2), which presently is a widely employed option for the treatment of maxillary SFE. While BMP-2 is infused at high concentrations (1.5 mg/ml), low concentrations of supplemented growth factors were sufficient to synergize with the components within MSC-CM and further promote bone regeneration [74].

9. Summary and future prospects

While results from pre-clinical studies using animal models have supported the utility of MSC-CM, much needs to be done to translate the promise to the clinic. Central to the therapeutic utility of MSC-CM would be the setting up of clinical trials for various diseases to evaluate both the safety and efficacy of MSC-CM. With a large number of MSC-based clinical trials being approved by national agencies, obtaining regulatory approval for cell-free therapy with MSC-CM should be relatively easy. However, at this juncture there are several issues with respect to MSC-CM that need to be sorted out. These include a lack of cGMP complaint protocols for the preparation of the MSC secretome, storage, product shelf-life, product stability, and quality control parameters that are essential to establish the safety and efficacy of MSC-CM. Additional studies focusing on the optimal protein concentration of the dose, administration frequency and the optimum volume of injection could contribute to the success of MSC-CM therapeutically. In conclusion, cell-free therapy employing the use of MSC-CM expands the therapeutic horizons of MSCs, paving the way for an alternative approach in regenerative medicine.

Conflict of interests

The authors declare that they have no competing interests.

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