Exosomes Mini-Series

A roadmap from research to clinical testing of mesenchymal stromal cell exosomes in the treatment of psoriasis

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ABSTRACT

The most clinically trialed cells, mesenchymal stromal cells (MSCs), are now known to mainly exert their therapeutic activity through paracrine secretions, which include exosomes. To mitigate potential regulatory concerns on the scalability and reproducibility in the preparations of MSC exosomes, MSC exosomes were produced using a highly characterized MYC-immortalized monoclonal cell line. These cells do not form tumors in athymic nude mice or exhibit anchorage-independent growth, and their exosomes do not carry MYC protein or promote tumor growth. Unlike intra-peritoneal injections, topical applications of MSC exosomes in a mouse model of IMQ-induced psoriasis alleviate interleukin (IL)-17, IL-23 and terminal complement complex, C5b9 in psoriatic skin. When applied on human skin explants, fluorescence from covalently labeled fluorescent MSC exosomes permeated in the stratum corneum for about 24 hours with negligible exit out of the stratum corneum into the underlying epidermis. As psoriatic stratum corneums are uniquely characterized by activated complements and Munro microabssesses, we postulated that topically applied exosomes permeate the psoriatic stratum corneum to inhibit C5b9 complement complex through CD59, and this inhibition attenuated neutrophil secretion of IL-17. Consistent with this, we demonstrated that assembly of C5b9 on purified human neutrophils induced IL-17 secretion and this induction was abrogated by MSC exosomes, which was in turn abrogated by a neutralizing anti-CD 59 antibody. We thus established the mechanism of action for the alleviation of psoriatic IL-17 by topically applied exosomes.

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Background

Mesenchymal stromal cells (MSCs), like most cells, secrete a variety of different extracellular vesicle (EV) types. Presently, there is no protocol to isolate a pure EV-type preparation. In accordance with the Minimal Information for Studies of Extracellular Vesicles 2018 recommendations to adopt operation terms to describe preparations [1], the term “MSC-sEV” was adopted to describe preparations of small extracellular vesicles (sEV) of 50–200 nm secreted by MSCs [2]. The discovery of MSC-sEVs was fueled in part by an evolving paradigm shift from differentiation- to secretion-based rationale for the reparative potency of MSC. MSC therapeutic potency was initially attributed to the differentiation potential of MSC to differentiate and form new replacement cells in the diseased tissue. However, pre-clinical animal and clinical data were more consistent with a secretion- rather than a differentiation-based rationale where

MSC secretions modulate the microenvironment and cellular response for optimal tissue repair and recovery [3,4]. Indeed, the administration of MSC-conditioned medium reduced infarct size in myocardial infarction models [5–7], Timmers et al. [7], further demonstrated that the active agent was larger than 1000 kDa.

Consistent with this observation, Bruno et al. [8] and Lai et al. [9] reported that the active agent was an EV, namely, the 80– to 1000-nm microvesicles and 100- to 130-nm exosomes, respectively. Bruno et al. [10] subsequently reported that smaller microvesicles with an average diameter of ~160 nm were renal protective but not the larger ones, with an average diameter of ~215 nm. It is now widely accepted that the active MSC EVs are those in the 50- to 200-nm size range [11]. In head-to-head comparisons, the therapeutic activity of these vesicles was comparable with that of their parental MSCs [8,12,13]. In addition, our laboratory has shown that our MSC-EV preparations produced by a single monoclonal MSC line could recapitulate many of the activities that have been reported for MSCs, such as ameliorating myocardial reperfusion injury [14], senescence in aging [15] and acute radiation syndrome [16]; enhancing osteochondral repair [17] and alleviating immune reactivity in graft-versus-host disease (GVHD) [18], osteoarthritis [19], psoriasis [20] and

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Regulatory Challenges in Using Primary MSCs as EV Producer Cells

MSC-EV preparations have been found to be efficacious in many pre-clinical animal models of disease/injury indications. In fact, the efficacy of MSC-EV has progressed beyond animal models into some preliminary clinical studies, namely, acute GVHD, chronic kidney disease, cochlear implant and animal models of severe coronavirus disease 2019 [23–26]. However, efficacy is always secondary to safety from a regulatory perspective.

Human MSC-EV preparations like their MSC producer cells are used in fully immune competent pre-clinical animal models and unmatched allogenic human recipients without immune suppression. To date, no adverse effect has been reported with the use of human MSC-EV in immunocompetent animals or humans [27,28]. A recent study of minipigs treated with intra-articular injections of MSC-EV for repair of critical-size osteochondral defects reported that MSC-EV–treated pigs had functional repair of the defects while retaining normal liver functions [17]. The level of blood urea nitrogen, creatinine, albumin, total bilirubin, alanine aminotransferase and aspartate aminotransferase in the treated animals were within the healthy reference value range for pigs. Generally, MSC-EV studies suggest that MSC-EVs are as safe or potentially safer than their producer MSCs, which have been tested in >1000 clinical trials [ClinicalTrials.gov]. We had previously noted that although MSCs are generally safe, animal studies have indicated that administration of relatively large living MSCs carries some risks, e.g., occlusion of microvasculature resulting in embolism and death, persistence or amplification of biological activity after an adverse event or the generation of inappropriate and potentially deleterious cell types such as calcified tissues in soft tissues.

Aside from safety, a critical regulatory concern is the reproducibility in MSC-EV manufacture. Most MSC-EVs are presently prepared using primary MSCs. However primary MSCs have a finite lifespan and exhibit donor- and culture-dependent functional heterogeneity. As there is currently no robust identity and potency assay to assess the functionality of MSCs, it will not be possible to sustain long-term large-scale MSC-EV manufacture with functionally identical primary MSCs. This lack of a robust identity and potency assay to assess the functionality of MSC was cited in the recent Food and Drug Administration (FDA) response to Mesoblast biologics license application for remestemcel-L [29]. Specifically, the FDA expressed concern that the International Society for Cell and Gene Therapy position on identity and potency [30,31] is not sufficiently stringent or robust to capture functional heterogeneity among MSC from different donors, or when cultured under different conditions. This FDA concern is highly relevant to the manufacture of MSC-EV using primary MSC, as it was observed that when EVs were prepared using bone marrow–derived MSCs from different donors under similar preparation conditions, they exhibited different functions [32]. Therefore, functional heterogeneity among primary MSC extends to the functionality of secreted EVs and compromises lot-to-lot consistency in quality and performance of the EV.

Mitigating Functional Heterogeneity of Primary MSCs as EV Producer Cells

To address the issue of functional heterogeneity in EV preparations caused by different MSC sources and to enable a robust scalable manufacturing of MSC-EV, we proposed immortalizing MSCs to generate a monoclonal EV-producing MSC line a decade ago [33]. However, this approach introduces a possible risk of tumorigenicity and tumor growth promotion. To evaluate this possibility, the immortalized cells were transplanted in immunodeficient mice; the cells did not engraft and form tumors. The immortalized MSCs also did not acquire anchorage-independent growth. Furthermore, EVs produced by these cells did not promote tumor growth in a mouse model of tumor xenograft [34]. Immortalization also did not compromise the functional activity of the EV. EVs from MSCs before and after immortalization reduced infarct size to the same extent in a mouse model of myocardial ischemia/reperfusion injury [33]. The therapeutic potency of EVs from immortalized MSCs also was evident in other pre-clinical models of diseases such as aging, ischemic heart disease, orthopedic disease, immune disease and radiation injury [14-20,35–41].

The use of an immortalized monoclonal EV-producing MSC line will eliminate the need to replenish cells from different donors, and this greatly reduces heterogeneity in the cell source and will improve the lot-to-lot consistency of EV products. Upon deriving the monoclonal MSC line, it is important to establish a two-tier banking system consisting of a master cell bank and a working cell bank. The latter is generated using cells from the master bank and is used directly in manufacturing. This cell banking system will ensure identical cells are used in each manufacturing run. However, many other parameters in the EV-manufacturing process are known to have profound effects on the consistency of MSC-EV products [2]. The more critical parameters are the culture medium and medium additives, e.g., serum, human platelet lysate, growth factors/cytokines; the culture systems, e.g., two-dimensional vs three-dimensional systems; the culture conditions, e.g., oxygen concentration; and EV-enrichment technologies. Different permutations of these parameters affect the yield and quality of MSC-EV products such that therapeutic efficacy is enhanced for some diseases and diminished for others. Optimizing and controlling these parameters for each disease indication will be challenging unless there are meaningful identity metrics and critical quality attributes (CQAs) that are linked to a clearly defined mechanism of action (MoA) to guide and monitor the optimization and control processes.

Developing Identity and Potency Metrics of MSC EV Preparations

In 2018 and 2019, the International Society for Cell and Gene Therapy, International Society for Extracellular Vesicles and Society for Clinical, Research and Translation of Extracellular Vesicles Singapore (SOCRATES) organized two workshops to discuss development of identity and potency metrics for MSC-sEVs, respectively. In the first workshop [2], participants recommended the use of several metrics that will collectively capture the identity of a preparation as a
biological preparation of small lipid membrane vesicles derived from MSC by measuring:

- the concentration of MSC surface antigen (CD73, CD90, CD105) and non-MSC surface antigen (CD14, CD34, CD11b) as a demonstration of their cellular identity;
- the number of particles per unit weight protein/membrane lipids within a diameter size range of 50–200 nm as a surrogate measurement of sEV;
- the molar or weight ratio of protein to membrane lipids such as cholesterol or phosphatidylcholine as an index of lipid membrane vesicles with a protein cargo; and
- biochemically active components, e.g., enzyme activity of CD73 as being biological in nature

It is anticipated that different manufacturing processes will produce MSC-sEV preparations with different quantitative values. Conversely, a well-controlled and managed manufacturing process will produce MSC-sEV preparations with similar lot-to-lot values for the proposed identity metrics. However, these parameters do not measure the potency of the preparation against a specific disease.

At the second workshop to develop potency metrics to predict the therapeutic potency of an MSC-sEV preparation, there was a consensus that since MSC-sEV preparations have been reported to be efficacious against diseases with very different underlying pathologies, these efficiencies are likely to be mediated by a disease-specific MoA [11]. It was noted at these workshops that MSC-sEV preparations are not pure sEV preparations but rather sEV-enriched, conditioned medium, and depending on the manufacturing processes, may carry excipients that could enhance or promote the therapeutic efficacy of the critical sEV attribute. As such, the MoA and therefore, the sEV attribute, i.e., potency metric, driving the therapeutic action is determined by both the target indication and the manufacturing process. To address these challenging issues, a roadmap was proposed. First, the efficacy against a particular disease should be established in an appropriate clinically relevant animal model using MSC-sEV preparation from a defined manufacturing process. Then sEV-modulated pathological processes that are associated with an improved therapeutic outcome will have to be identified and mapped to biological activities that could be elicited by attributes in the MSC-sEV preparation. Attributes validated as critical to the biological activities and thus therapeutic activity could then serve as CQAs to assess the therapeutic potential of the preparation.

Immunomodulating Activity of MSC Exosome

Our MSC exosome preparations using the MYC-immortalized MSC lineage are highly immunomodulatory and can attenuate inflammation by enhancing secretion of anti-inflammatory cytokines, promoting Treg polarization and inhibiting complement activation [18,40,42]. They have also been shown to inhibit complement activation and the formation of C5b-9 complex, which is a component of the terminal complement complex, through CD59 present in MSC exosomes [42]. They do not express major histocompatibility complex class I and II or co-stimulatory molecules such as CD40, CD80 and CD86 and can polarize THP-1 cells and primary mouse or human monocytes toward T cell-inhibitory molecules such as CD40, CD80 and CD86 antibodies. Infusion of MSC exosomes in a mouse model of human peripheral blood mononuclear cell–induced GVHD improved GVHD symptoms with reduced mortality and increased human CD4+CD25+CD127low/− Tregs [18]. On the basis of these observations, we hypothesize that the signaling pathways (Signal 1 and 2) triggered by anti-CD3 and anti-CD28 antibodies [43] may not be enough to polarize T cells to Tregs by MSC exosomes. Other factors present on APCs may be needed to prime CD4+ T cells for this process to occur. This hypothesis is supported by previous studies showing T-cell stimulation by anti-CD3 and anti-CD28 antibodies could only partially recapitulate APC stimulation and that additional co-stimulatory and adhesion molecules, such as 4-1BB (CD137), CD83, ICAM-1 (CD54) and LFA-3 (CD58), are necessary [44,45].

Rationale for the Use of MSC Exosomes Against Psoriasis

Psoriasis is an inflammatory disease that manifests as localized skin lesions. Both innate and adaptive immune systems have been implicated in the psoriatic immune pathology. The cytokine members in the IL-23/IL-17 axis have been shown to be critical to the pathogenesis of psoriasis and therapeutics targeting major cytokines in this axis have proven to be highly effective in managing the disease [46,47].

Consequently, the immunopathology of psoriasis has focused largely on IL-23/IL-17 cytokines and T cells [46]. However, neutrophils are now increasingly considered a major cell source of IL-17 in psoriasis [49], through the release of neutrophil extracellular traps (NETs) during NETosis [50]. NETosis, which is increased in psoriasis [51], has been shown to induce IL-17 secretion in neutrophils and monocytes [52]. One of the major inducers of NETosis is activated complements [53]. Complements constitute a major first line of immune defense and are pivotal in the psoriatic immunology [54]. Many complement components are synthesized in the skin, and activated complement components such as the highly chemotactic C3a and C5a were found to be elevated in psoriatic plaques relative to non-psoriatic scale [55–58]. The terminal complement complex C5b-9 was also elevated in psoriatic lesional plaques [59]. These terminal C5b-9 complexes likely formed in the skin, as patients with psoriasis with elevated plasma C5b-9 levels do not have elevated intermediate complement complexes in the plasma as would be expected if C5b-9 were formed in the plasma. The activated complement components are highly chemotactic and probably induced neutrophil migration into the stratum corneum to form Munro microabscesses, a characteristic of psoriasis [56–58,60]. Therefore, complement activation of neutrophils could be an early event in psoriatic pathology leading to enhanced NETosis and IL-17 secretion and preceding the activation of IL-17/IL-23 axis in psoriatic immune pathology.

Efficacy of MSC Exosomes in a Mouse Model of Imiquimod-Induced Psoriasis

To investigate whether MSC exosomes is efficacious in reducing C5b-9 formation and IL-17 in psoriasis, MSC exosomes were administered by intraperitoneal injection or topical application in a mouse model of imiquimod-induced psoriasis [20]. In contrast to intraperitoneal injections, topical applications of MSC exosomes reduced the main psoriatic cytokines, IL-17 and IL-23, as well as C5b-9 in the skin of psoriatic mice. When MSC exosomes were covalently tagged with a protein-labeling fluorescent dye and applied topically on human skin explants, the dye was found to localize to the stratum corneum, where it persisted for about 24 h. A negligible amount of the fluorescent dye exited the skin explant to the underlying culture medium. Since the stratum corneum is rich in enzymes such as proteases
[61,62], phospholipases [63], RNases [64] and DNases [65], the loss of fluorescent label by 24 h is likely caused by enzymatic degradation in the stratum corneum. Altogether, these observations strongly suggest that topically applied exosomes are physically confined to the stratum corneum and do not enter systemic circulation. They further suggest that the site of action and targets of action for the therapeutic efficacy of MSC exosomes are in the stratum corneum. As complements and neutrophils are the main mediators of psoriatic pathology that reside in the stratum corneum, they represent the most likely targets of MSC exosomes.

The simplest hypothesis for the reduced IL-17 and C5b-9 levels in MSC exosome-treated psoriatic skin is that MSC exosomes act through CD59 to inhibit formation of the terminal activated C5b-9 complement complex, and this in turn inhibits NETosis and secretion of IL-17 by neutrophils.

Validating the Mechanistic Role of MSC Exosome in Alleviating Psoriatic Immune Pathology

To test this hypothesis, it was observed that upon assembly of the terminal complement complex C5b-9 on the surface of isolated neutrophils, NETs as determined by DAPI-positive extracellular DNA fibers and citrullinated histone H3 were released concomitantly with the secretion of IL-17 [66]. This complement-stimulated release of NETs and IL-17 secretion was inhibited by MSC exosomes, which in turn could be abrogated by a neutralizing antibody against CD59. Together, these observations support the aforementioned hypothesis on the MoA by MSC exosomes in alleviating psoriatic IL-17.

This MoA where exosomes exert their effect extracellularly is consistent with recent observations that internalization of exosomes by cells is inefficient and internalized exosomes may not always be indicative of a functional cellular uptake [67,68].

Summary

The development of MSC exosomes for clinical testing and application follows a similar trajectory as for other therapeutics (Figure 1). A typical first step would be the preparation of characterized MSC exosomes that exhibit efficacy in relevant pre-clinical animal models for the targeted disease indication. At the same time, critical issues in scalability and reproducibility in exosome manufacture should be addressed in anticipation of regulatory compliance in the manufacture of a drug product. For example, we proposed the use of immortalized MSC instead of primary MSC cultures as an exosome-producing cell source to enhance scalability and reproducibility, as described previously. Also, the preparations should be adequately characterized and the MoA for their efficacy against the target disease be elucidated to identify CQAs for potency. Assays to quantify the CQAs have to be developed for use as drug-release parameters to ensure that each product batch meets pre-determined identity, potency and safety parameters. Key critical process parameters in the manufacturing process also will have to be identified to monitor the manufacturing operation and ensure integrity of the process for every manufacturing batch.

Here, we described the trajectory of our development of MSC exosome preparations for topical use in psoriasis. Specifically, we described quantifiable identity and potency metrics that could be used to define the identity and potency of MSC exosomes for psoriasis as outlined in our recent report on the efficacy of exosomes in alleviating complement-activated neutrophils. In the report, the MSC exosome preparation was characterized as having a protein concentration of $1.419 \pm 0.034$ mg/mL, particle concentration of $2.13 \times 10^{11} \pm 5 \times 10^9$ particles/mL, particles with a modal size of $125.1 \pm 6.0$ nm, cholesterol of $8.16 \pm 0.32$ ng cholesterol/mg protein, CD73/ecto-5’-nucleotidase activity of $27.04 \pm 0.79$ mU/mg protein and CD59 concentration of $3.15 \pm 0.43$ ng CD59/mg protein [66]. MSC exosomes preparations using MYC-immortalized MSC were used in a recently completed phase 1 clinical trial (NCT05523011) in a Safety and Tolerability Study of MSC exosome ointment (https://clinicaltrials.gov/).

Author Contributions

Conception and design of the study: RCL, TTT and SKL. Acquisition of data: WKS and BZ. Analysis and interpretation of data: RCL, TTT, WKS and BZ. Drafting or revising the manuscript: RCL, TTT and SKL. All authors have approved the final article.

Figure 1. Schematic representation of development trajectory for MSC exosomes for psoriasis. Key steps depicting the manufacturing considerations taken to ensure consistent batch-to-batch consistency, and a quality control process consisting of exosome characterizations and an established CQA based on the identified MoA in psoriasis treatment.
Declaring of Competing Interest

SKL holds founding shares in Paracrine Therapeutics.

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