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Mesenchymal stem cell: An efficient mass producer of exosomes for drug delivery ☆

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ABSTRACT

Advances in biomedical research have generated an unprecedented number of potential targets for therapeutic intervention to treat disease or delay disease progression. Unfortunately, many of these targets are not druggable as they are intracellular, present in many cell types, poorly soluble or rapidly inactivated. Although synthetic drug vehicles have successfully circumvented many of these problems, natural particulates such as exosomes that intrinsically possess many attributes of a drug delivery vehicle are highly attractive as potentially better alternatives. Of the cell types known to produce exosomes, the readily available proliferative, immunosuppressive and clinically tested human mesenchymal stem cell (MSC) is the most prolific producer. Its exosomes are therapeutic in animal model of disease and exhibit immunosuppressive activity. The quality and quantity of exosome production is not compromised by immortalization to create a permanent MSC cell line. Therefore, MSC is well suited for mass production of exosomes that are ideal for drug delivery.
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Contents

1. Introduction

The rapid advances in biomedical research have led to a better understanding of the molecular pathology underlying many diseases and with this, an unprecedented number of potential targets for therapeutic intervention. However, many of the most promising targets are

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intracellular and can only be accessed by drugs capable of traversing the cell membrane. Many of these targets are also either present ubiquitously in many cell types or are present in non-diseased cell types such that therapeutic intervention against these targets could affect non-diseased cells and inflict severe adverse side effects. The bioavailability of many drugs is also often compromised by their poor solubility in aqueous solution, premature metabolic inactivation and excretion. Many of these problems could potentially be resolved by loading the drugs into drug delivery vehicles.

1.1. Drug delivery vehicles

1.1.1. Synthetic drug delivery vehicles

Most of the drug delivery vehicles presently in use for clinical or cosmetic applications are chemically synthesized using lipids or lipid-like molecules. Among the synthetic lipid or lipid-like vesicles, the liposome is currently the pharmaceutical vehicle of choice. It has been used for the delivery of anti-cancer drugs [1–11], anti-fungal drugs [12], analgesics [13] etc. Liposomes have many positive attributes that are pivotal in their function as drug delivery vehicles (reviewed in [14]). A major attribute is the ease with which their phospholipid membranes breached the plasma membrane to deliver their drug cargo. They also have a highly versatile capacity to incorporate both hydrophilic and hydrophobic drugs by simply synthesizing liposomes either as unilamellar vesicles with one lipid bilayer and a large aqueous core to encapsulate water-soluble drugs, or multilamellar vesicles with several concentric lipid bilayers to more efficiently entrap lipophilic drugs. An unexpected perquisite of such drug encapsulation or entrapment is a protective barrier against premature transformation and elimination. Liposomal membranes are also highly amenable to modifications to display ligands or antibody fragments that bind to specific cell types and enhance cell type-specific drug delivery. They can be coated with inert polymers such as PEG to reduce liposome recognition by opsonins and clearance of the liposomes.

1.1.2. Natural drug delivery vehicles

Despite the remarkable advances and successes in their design and efficacy of synthetic drug vehicles, there is an increasing recognition that nature has particulates with some of the highly desired attributes of drug delivery vehicles such as evasion of host immune system, a natural tropism that engenders highly selective and efficient entry into target cells, longevity in the host, and varied therapeutic payloads and that such particulates should be exploited as drug delivery vehicles (reviewed [15]). Some of the natural particulate candidates for drug delivery include bacteria, viruses, red blood cells, macrophages, lymphocytes and stem cells. Exosome, a recent addition to this list is rapidly emerging as one of the more exciting candidates [16–18].

2. Exosomes

2.1. Basic properties of exosomes

Originally thought to function as a “garbage bag” for maturing reticulocytes to discard unwanted transferrin receptors [19], exosomes are now recognized as a vehicle to facilitate intercellular communication through the exchange of proteins and genetic materials. Exosomes are one of several types of membrane vesicles known to be secreted by cells and these include microvesicles, apoptotic bodies, ectosomes or exosome-like vesicles. Relative to the other secreted lipid vesicles, exosomes are well characterized. They have a diameter of 40–100 nm, has a bi-lipid membrane with the same orientation as plasma membrane and a cargo that includes both proteins and RNA. Exosomes are formed in the multivesicular body (MVB) by the invagination of the endosomal membrane and are released to the extracellular space when MVB fuses with plasma membrane [20]. The invagination of the endosomal membrane during MVB formation confers on exosomes the same membrane orientation as the host cell membrane. They have a flotation density of 1.1–1.18 g/ml on a sucrose density gradient. Their membranes are enriched in lipid rafts, cholesterol, sphingomyelin and ceramide [21,22].

Despite being discovered more than 30 years ago, exosome biology has just started to garner interest from the research community. The reported biological functions of exosomes have extended far beyond their originally proposed function of disposing obsolete transferring receptors in reticulocytes. Exosomes have been reported to be involved in diverse physiological processes e.g. T cell stimulation [23–25], tumor growth suppression [26], neurotransmission [27,28], myelin membrane biogenesis [29], endothelial cell migration [30] as well as egg and sperm fusion [31]. Interestingly, exosomes could also exert both therapeutic and pathological effects. Exosomes have been shown to reduce myocardial ischemia/reperfusion injury [32] or acute tubular injury [33]. On the other hand, exosomes have also been implicated in the dissemination of diseases such as prion diseases, HIV [34–38] and pathogenesis of cancer [39–41].

Exosomes essentially exert many of their functions as an intercellular shuttle to be loaded with a cargo of protein and RNA by effector cells for off-loading in target cells [42]. As such, the exosome represents an ideal drug delivery vehicle to transiently modulate dysregulated processes in specific target cells. Its potential as a therapeutic delivery vehicle was further enhanced by the recent discovery that exosomes can penetrate the blood–brain-barrier [43], a barrier that have proven to be highly impenetrable to many drugs.

2.2. Exosomes — the natural drug delivery vehicle

Exosomes have many of the highly desired attributes that drug delivery vehicles should have. They are well tolerated as demonstrated by their wide distribution in biological fluids such as the blood, urine, bronchoalveolar lavage fluid, breast milk, amniotic fluid, synovial fluid, malignant pleural effusions and ascites [44]. Exosomes can deliver their cargo across the plasma membranes of target cells into the right cellular compartment to exert a functional response. For example, exosomes derived from dendritic cells (also known as Dex) could modulate immune cell response by transferring peptide loaded MHC class I and II complexes to dendritic cells [45,46]. Exosomes have also been shown to mediate intercellular transfer of mRNAs and miRNAs that resulted in the translation of the transferred mRNA in the recipient cells [47].

Another highly desired attribute of drug delivery vehicles is the ability to home to target tissues. Over the years, there has been circumstantial evidence to suggest that depending on their cell source, exosomes could target specific cell types. Recently, it was demonstrated that melanoma exosomes home to sentinel lymph nodes [48] demonstrating that exosomes do have intrinsic homing capability. While such natural homing capability could be exploited for targeted delivery of therapeutics, the repertoire of tissues that are naturally targeted by exosomes may be limiting. Fortunately, exosomes, like synthetic drug delivery vehicles, are amenable to membrane modification to enhance cell type specific targeting. When a fusion gene consisting of a neuron-specific RVG encoding sequence and LAMP2B encoding an exosomal membrane protein was overexpressed in dendritic cells, the dendritic cells secreted exosomes that displayed the RVG peptide on their surface membrane. These exosomes, after being loaded with siRNA, localized to the brain and crossed the blood–brain barrier after injection into mice. They delivered their exogenously loaded siRNA cargo into neurons, microglia, oligodendrocytes and their precursors, and knockdown >60% of target gene and protein [43].

2.3. Cell sources of exosome

Many different cell types such as B cells [23], dendritic cells [25], mast cells [25], T cells [49], platelets [50], Schwann cells [34], tumor cells [51], mesenchymal stem cells [32], human embryonic kidney cell [52], various

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cancer cell lines [53] and sperm [54] are known to secrete exosomes. While all these exosomes share an evolutionary conserved set of proteins molecules including tetraspanins (CD81, CD63, CD9), Alix and Tsg101 suggesting that they also share similar biological activities, they also have unique tissue/cell type-specific proteins that reflect their cellular source origin and the physiological state of the cells [44,55], and thus unique biological activities. For example, exosomes released from maturing reticulocytes are rich in transferrin receptors that the reticulocytes have to dispose of while those from lymphocytes and dendritic cells have few transferrin receptors [23,56,57]. Similarly, exosomes from epididymis are rich in proteins that are essential for the maturation of male gametes [54] and urinary exosomes secreted by kidney tubules carry aquaporin, a kidney specific protein [58]. The cargo of exosomes has also been found to correlate with the physiological state of its cellular source. For example, tumor-derived exosomes have been shown to contain either tumor antigens [51,59–61] or tumor-specific microRNAs [62]. The proteomic and RNA profile of exosome from different cellular sources have been extensively profiled (www.exocarta.org) [26,47,62–66].

3. Mesenchymal stem cell (MSC) as the source of exosomes for drug delivery

Successful exploitation of exosomes as drug delivery vehicles is likely to leverage on a combination of factors that include their natural intrinsic properties, amenability to modifications to supplement or enhance these properties, and scalability of a GMP manufacture process. A common denominator that underpins these factors is the cell source as it plays a defining role in the intrinsic properties of exosomes, their amenability to modifications and its production.

At present, the only human cell type known to have a scalable capacity to mass produce exosomes is MSC. MSC also has several cellular characteristics that are highly desired attributes of drug delivery vehicles. Therefore, MSC is the ideal cell candidate for the mass production of exosomes for drug delivery.

3.1. Background of MSCs

MSCs are multipotent fibroblast-like cells that reside in many adult tissues such as adipose tissue [67,68], periosteum [69], liver [70], lung [70], spleen [70], muscle connective tissue [71], amniotic fluid [72], placenta [73,74], umbilical cord blood [67], dental pulp [75,76] and aborted fetal tissues [77]. According to the minimal defining criteria by International Society for Cellular Therapy [40], MSCs must be plastic-adherent when maintained in standard culture conditions; they must express CD105, CD73 and CD90 and not express CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR surface molecules. Third, MSCs must be able to differentiate into osteoblasts, adipocytes and chondroblasts *in vitro*. While most MSCs differentiate primarily into osteocytes, chondrocytes and adipocytes, they have also been reported to differentiate into an amazing array of cell types that include nearly every major cell type in the adult body [75].

3.2. Tissue sources of MSC

MSCs are one of the most easily accessible primary human cells and can be easily harvested from a large variety of human tissues including those that are considered medical waste. The ever expanding list of tissues for MSC isolation include adipose tissue [67,68], liver [70], muscle [71], amniotic fluid [72], placenta [73,74], umbilical cord blood [67], dental pulp [75,76], human ESC [78] and other sources [70,77].

3.3. Clinical applications of MSCs

MSCs are currently the most studied stem cells with more than 1000 publications cited in Scopus each year since 2005. This could be attributed to their therapeutic efficacy in an amazingly wide spectrum of injuries and diseases [81], their easy availability in accessible and ethically palatable tissues such as bone marrow aspirate, fat tissue [82], and their large capacity for ex vivo expansion [83]. MSCs are also known to have immunosuppressive properties and therefore could be used in allogenic transplantation [75,79,80,84]. These features were instrumental in driving the intense clinical testing of MSCs. In 2010, there were more than 100 registered clinical trials evaluating the efficacy of MSCs [85] in treating numerous diseases such as cardiovascular diseases [86], graft verse host disease (GvHD) [87], bone/cartilage defect, Crohn's disease [88], acute kidney injury, spinal cord injury [89] and diabetes [90].

3.4. Mechanism for the therapeutic efficacy of MSCs

Transplanted MSCs were hypothesized to home and engraft in injured tissues and then differentiate into cells to replace damaged cells. However, it was observed that <1% of transplanted MSCs were observed to reach their target tissue with most of them trapped in the liver, spleen and lungs [91]. Even in those cases where MSC transplantation showed therapeutic efficacy, MSC engraftment or differentiation at the site of injury was very low and/or transient [91–96]. For example, in a clinical trial to test efficacy of bone marrow-derived MSCs treat to osteogenesis imperfecta, it was observed that despite a very low MSC engraftment of <1%, there was significant improvement in growth velocity and bone mineral density [97]. Differentiation of transplanted MSCs at the site of injury is generally inefficient [98]. This discrepancy between the therapeutic efficacy of MSCs and the lack of MSC engraftment or differentiation at the site of injury was further supported by the observations that the therapeutic effect of MSCs is not dependent on the engraftment [92–95].

To reconcile this discrepancy between the therapeutic efficacy of MSCs and the lack of MSC engraftment or differentiation at the site of injury, it was proposed that MSCs exert their therapeutic effects through secreted trophic mediators [99]. Since then, the paracrine effect of MSCs was observed in many animal models of tissue injury including acute myocardial infarction [100], reperfusion injury [101], ischemic acute renal failure [102], wound healing [103], skeletal tissue repair [104], acute kidney injury [105] and hypoxic pulmonary vasoconstriction [106]. We and others subsequently demonstrated that MSC secretion alone could reduce infarct size in a pig and mouse model of ischemia/reperfusion injury [101,107] and improve cardiac function in a pig model of chronic ischemia [108]. Analysis of this revealed that the therapeutic factor in the secretion is a membrane vesicle known as an exosome [32].

3.5. Qualities of MSCs as exosome producers for drug delivery

MSCs have several features that make them ideal candidates as producers of exosomes for drug delivery. Some of these features that have been discussed above are the ease of isolation from ethically non-controversial human materials and the large ex vivo expansion capacity. There is also an increasingly large body of clinical work demonstrating safe transplantation of MSCs, which suggests that transplanting MSC exosomes would unlikely lead to adverse effects. Infusion of human MSC exosomes into immunocompetent mouse model of acute myocardial ischemia have been shown to be therapeutic and without obvious adverse effects [32,77]. Other features are discussed in the sections that follow.

3.5.1. Immunomodulatory properties of MSC and its exosomes

One of the MSC features that drove intense clinical interest in MSC is its unique ability to exert suppressive and regulatory effects on both adaptive and innate immune cells in an autologous and allogeneic manner [109]. MSCs inhibit proliferation of mitogen-activated T cells [110–114], induce an anti-inflammatory tolerant phenotype in dendritic cells (DCs), naive and effector T cells and natural killer (NK) cells [115] and inhibit B cell proliferation [116]. Preliminary data suggest that some of the

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immunomodulatory properties of MSC were transferred to their exosomes. Like MSCs, their exosomes also inhibited proliferation of Concanavalin A-activated lymphocytes (Fig. 1). This ability to exert suppressive and regulatory effects in an allogeneic or autologous manner would enhance the longevity of MSC exosome-derived drug delivery vehicle and bioavailability of its drug cargo.

3.5.2. MSCs are compatible for the commercially sustainable production of exosomes

Although MSCs are known to have a huge expansion capacity *in vitro*, this expansion is finite. Therefore there will be a need to constantly derive new batches of MSCs from hESCs to replenish the cell source of exosomes with each derivation necessitating recurring costs of derivation, testing and validation. This will also render production of MSC exosomes commercially untenable. We have circumvented this by immortalizing MSCs with the myc oncogene [117]. Although the immortalization compromises the differentiation potential of the MSCs, it did not affect the production or therapeutic efficacy of the exosomes.

3.5.3. Most prolific producer of exosomes

We recently observed that when GFP-labeled exosome-associated protein CD81 was expressed in hESC-MSCs, they exhibited a punctate cytosolic distribution and were secreted [118]. CD81 is a classical tetraspanin membrane protein and in other cell types such as HEK 293 cells, the expression of GFP-labeled CD81 was localized to the plasma membrane. When the level of exosomes in different cell lines were quantitatively assessed by purifying exosomes from the culture medium and measuring the level of CD81, MSCs were found to produce the highest amount of exosomes (Fig. 2).

4. Conclusion

The exploitation of exosomes as promising drug delivery vehicles is highly dependent on a reliable cell source. Of the cell types known to secrete exosomes, the human MSC represents the most promising cell source. MSC is not only an easily accessible cell type that could be derived from almost all human tissues, it is highly proliferative. One of the most attractive attributes is the relative safety of MSCs. MSCs are presently undergoing extensive clinical testing to evaluate their therapeutic efficacy in a wide range of indications. There were more than 100 MSC clinical trials in 2010 alone. It is currently the most prolific known producer of exosomes. MSC exosomes are therapeutically efficacious in animal models and exhibited immunosuppressive activity. This production was not compromised in quantity and quality when the cells were immortalized to create permanent cell lines, thus ensuring a sustainable and reproducible production of exosomes.

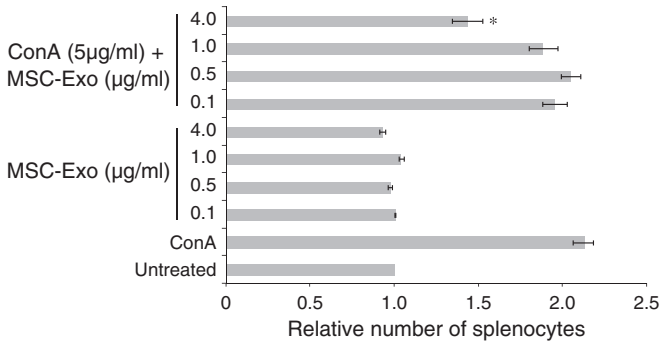

Fig. 1. MSC exosomes inhibit proliferation of mitogen-activated lymphocyte *in vitro*. Mouse splenocytes were isolated and labeled with carboxyfluorescein succinimidyl ester (CFSE). The CFSE-labeled splenocytes were incubated with different concentrations of MSC exosomes (0.1, 0.5, 1 and 4 μg/ml) in the presence and absence of 5 μg/ml Concanavalin A (ConA) for 3 days. MSC exosomes were prepared as described previously [32]. The number of fluorescent cells for each treatment group was quantified by FACS. Data were normalized to the untreated control and presented as mean ± SD of triplicate samples. MSC-Exo did not affect proliferation of splenocytes ($p > 0.05$) and 4.0 μg/ml MSC-Exo significantly inhibited ConA-stimulated splenocytic proliferation (* $p < 0.01$).

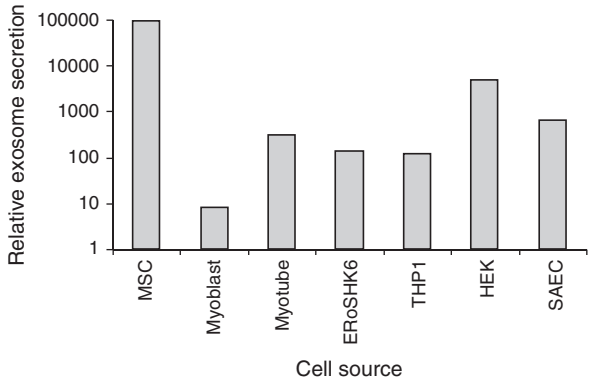

Fig. 2. Relative amount of exosomes secreted by different cell lines. The relative amount of exosomes secreted by five different cells or cell lines was evaluated using culture media conditioned for three days by each of these cell lines. The cells or cell lines used were a cmyc-transformed hESC-MSC (abbreviated as MSC) [117]; a mESC-derived insulin-producing cell line, ERoSHK6 [119]; the human acute monocytic leukemia cell line, THP-1; the human embryonic kidney cell line, HEK, a primary human small airway epithelial cells abbreviated as SAEC (Lonza, Cologne, Germany) and primary human myoblasts and myotubes differentiated from them (Lonza Cologne, Germany). The conditioned media for each cell line was prepared by incubating an approximately 80% confluent culture for 3 days. Conditioned and non-conditioned medium for each cell line were collected, centrifuged at 1000*g* for 10 min followed by filtration through a 0.22 μm filter and then subjected to cholera toxin B-chain (CTB) affinity chromatography for the isolation of exosomes. Exosomes have an affinity for CTB via GM1 gangliosides that are enriched in their membranes. CTB-bound exosomes in the conditioned and non-conditioned media were quantified by ELISA for CD81, an exosome-associated marker. The non-conditioned media served as a background control for their corresponding conditioned media for each cell type. The amount of exosomes secreted by each cell type is represented by the amount of CD81 in the CTB-bound fraction of its conditioned medium, normalized to that of the MSCs.

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