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Membrane vesicles mediate pro-angiogenic activity of equine adipose-derived mesenchymal stromal cells

Luisa Pascucci [a,*], Giulio Alessandri [b], Cecilia Dall'Aglio [a], Francesca Mercati [a], Paola Coliolo [a], Cinzia Bazzucchi [a], Sara Dante [a], Stefano Petrini [c], Giovanni Curina [c], Piero Ceccarelli [a]

[a] Department of Veterinary Medicine, University of Perugia, Via San Costanzo, 4, 06126 Perugia, Italy
[b] IRCCS Foundation, Neurological Institute 'C. Besta', Cerebrovascular Diseases Unit, Via Celoria 11, 20133 Milan, Italy
[c] Experimental Zooprophylactic Institute of Umbria and Marche, Immunology Unit, Via G.Salvemini 1, 06126 Perugia, Italy

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ABSTRACT

Multipotent mesenchymal stromal cells (MSCs) have attracted a great deal of interest, due to several distinctive features, including the ability to migrate to damaged tissue and to participate in tissue regeneration. There is increasing evidence that membrane vesicles (MVs), comprising exosomes and shedding vesicles, represent a key component, responsible for many of the paracrine effects of MSCs. The aim of the present study was to establish whether equine adipose-derived MSCs (E-AdMSCs) produce MVs that are capable of influencing angiogenesis, a key step in tissue regeneration.

A morphological study was performed using MSC monolayers, prepared for transmission and scanning electron microscopy and on ultracentrifuged MSC supernatants, to identify production of MVs. The ability of MVs to influence angiogenesis was evaluated by means of the rat aortic ring and scratch assays. The results demonstrated that MVs, constitutively produced by E-AdMSCs, are involved in intercellular communication with endothelial cells, stimulating angiogenesis. Although many questions remain regarding their formation, delivery, content and mechanism of action, the present study supports the concept that MVs released by MSCs have the potential to be exploited as a therapeutic tool for regenerative medicine.

Introduction

Use of multipotent mesenchymal stromal cells (MSCs) for regenerative medicine has been proposed in humans and veterinary species (Fortier and Travis, 2011; Sykova and Forostyak, 2013). The beneficial effect of MSCs in enhancing tissue regeneration was initially thought to result from their proliferation and differentiation into tissue-specific mature cells (Kopen et al., 1999; Mezey et al., 2000; Caplan and Bruder, 2001). However, the observation that only a small number of transplanted MSCs survive and integrate into host damaged tissues has highlighted the possibility that alternative mechanisms might exist. In particular, evidence of extensive interaction with the surrounding microenvironment has led researchers to focus more on MSCs as activating agents of regenerative pathways, rather than simply replacing damaged cells (Iso et al., 2007; Horwitz and Prather, 2009; Prockop, 2009; Vrijsen et al., 2010; Baglio et al., 2012).

MSCs produce a plethora of trophic factors, cytokines and signalling molecules, able to influence neoangiogenesis, fibroblast proliferation, inhibition of apoptosis and even recruitment of resident stem cells (Caplan and Dennis, 2006; Caplan, 2007; Gnecchi et al., 2008; Horwitz and Prather, 2009; Boomsma and Geenen, 2012), thereby creating optimal environmental conditions for tissue regeneration via a paracrine mechanism. This complex interaction between MSCs and the tissue microenvironment might involve soluble factors as well as production of membrane vesicles (MVs), containing molecules such as short peptides, proteins, lipids, and various forms of RNAs (Gyorgy et al., 2011).

The term 'exosome' is used to describe a specific subtype of MV, derived from multivesicular bodies (MBs). These represent a type of 'late endosome', containing luminal nanovesicles (30–100 nm), formed by the inward budding of the outer endosomal membrane that are released by fusion of MBs with the plasma membrane (exosomes). The term 'shedding vesicles' refers to another type of vesicle that is released into the extracellular environment by direct budding of the plasma membrane. The size of shedding vesicles has not been clearly defined, but it is estimated that their diameter ranges between 50 and 1000 nm (Gyorgy et al., 2011).

* Corresponding author. Tel.: +39 75 585 7632.
E-mail address: luisa.pascucci@unipg.it (L. Pascucci).

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MVs can be constitutively produced or induced and may act as vehicles of bi-directional information exchange between cells (Camussi et al., 2010). Through their membrane envelope, they are able to reach, interact, and eventually fuse with recipient cells, modulating a variety of physiological and pathological processes (Raposo and Stoorvogel, 2013). MVs derived from MSCs (MSC-MVs) have been isolated, visualised, and their function evaluated in several studies, but their mechanism of action remains relatively unclear (Gatti et al., 2011; Bruno et al., 2013; Kim et al., 2013). Several studies have shown that MSC-MVs can have a similar therapeutic efficacy to donor cells, when used for tissue repair and even in anti-cancer therapy, suggesting that development of innovative 'MV-based therapy' might reduce the difficulties and risks associated with whole cell transplantation (Tetta et al., 2011; Baglio et al., 2012).

In veterinary medicine, equine MSCs have been particularly studied for their beneficial effects on tendon regeneration in sport horses (Godwin et al., 2012). Here, a complex set of mechanisms are likely to be involved, with stimulation of angiogenesis a major contributory factor (Caplan and Dennis, 2006). The aim of the present study was to investigate the capacity of MVs, derived from horse adipose tissue MSCs (E-AdMSC-MVs), to influence angiogenesis.

Materials and methods

MSC culture and MV isolation

Adipose tissue samples were obtained under general anaesthesia, with informed owner consent, from subcutaneous fat of four donor horses, 1–3 years of age, undergoing abdominal surgery. The tissue sampling procedure was approved by the University of Perugia Ethics and Welfare Committee (Protocol number 2012-034; approved 12 September 2012).

Samples were washed with sterile phosphate buffered saline (PBS), supplemented with 200 U/mL penicillin, 200 mg/mL streptomycin, and 12.5 mg/mL amphotericin B (Sigma–Aldrich). Tissues were then finely minced and digested with 0.075% collagenase type I (Worthington Biochemical) at 37 °C for 45 min and centrifuged at 600 *g* for 10 min, to obtain a pellet containing the stromal vascular cell fraction. Cells were incubated at 37 °C with 5% CO_2 for 72 h in tissue culture flasks with complete basal medium, consisting of Dulbecco's modified Eagle Medium (DMEM; Gibco), 10% fetal bovine serum (FBS; Gibco), 100 U/mL penicillin and 100 mg/mL streptomycin. The adherent cells were expanded until they achieved 80% confluence. Cells were recovered using 0.5 mM EDTA/0.05% trypsin (Gibco) and passaged at a density of 5–10,000 cells/cm^2 (passage one). The E-AdMSCs were maintained in complete basal medium until passage three, at which time they were prepared for MV isolation and for observation by electron microscopy.

E-AdMSCs were differentiated along adipogenic, osteogenic or chondrogenic lineages, using specific inductive media, as described previously (Zuk et al., 2002) (see Appendix: Supplementary Fig. S1). Additionally, cells at passage three were evaluated for their expression of CD90 and CD44, two of the main MSC markers in veterinary species (Pascucci et al., 2011) (see Appendix: Supplementary Fig. S2).

Cells at passage three (~20×10^6) from each donor horse were used for recovery of MVs. Cells were incubated for 72 h in FBS-free DMEM, supplemented with 0.5% bovine serum albumin (BSA). The culture supernatant was centrifuged at 2000 *g* for 20 min to remove debris and dead cells. The cell-free supernatant was then centrifuged at 100,000 *g* for 60 min at 4 °C (Beckman-Coulter ultracentrifuge XL-100K), washed in 0.1 M PBS, pH 7.3, and subjected to a second ultracentrifugation step under the same conditions. The MV pellet was finally suspended in 1 mL PBS. In order to quantify and compare the MVs recovered from the four MSC samples, a Bradford assay (BioRad) for protein determination was performed. As a control, an aliquot of FBS-free DMEM with 0.5% BSA was subjected to the same procedure described for conditioned medium. The 'mock pellet' was resuspended in PBS and employed in the formulation of control media used in functional assays, to exclude the possible involvement of trace amounts of BSA in the endothelial cell response.

Electron microscopy of cell monolayers and MVs

For transmission electron microscopy (TEM) of cell monolayers, cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (CB), pH 7.3, for 2 h at room temperature, detached from the well by means of a cell scraper, and centrifuged at 600 *g* for 10 min to remove the fixative. Pellets were subsequently washed twice in CB, post-fixed in 2% osmium tetroxide, dehydrated in a graded series of ethanol up to absolute, pre-infiltrated, and embedded in Epoxy Embedding Medium (Sigma–Aldrich). Ultrathin sections (90 nm) were mounted on 200-mesh copper grids then stained with uranyl acetate and lead citrate. For MVs, approximately 20 μL of each MV suspension, were initially placed on Parafilm. A Formvar-coated copper grid (Electron Microscopy Sciences) was gently placed on the top of each drop for about 60 min in a humidified chamber. Grids were then washed in 0.1 M CB, pH 7.3 and finally fixed with 2.5% glutaraldehyde (Fluka) in CB. After washing in CB, MVs were contrasted with 2% uranyl acetate then air dried. The observation was performed using a Philips EM208 transmission electron microscope equipped with a digital camera (CUME – University Centre of Electron Microscopy).

For scanning electron microscopy (SEM), cells were grown on glass coverslips, fixed with 2.5% glutaraldehyde in 0.1 M CB, pH 7.3, for 2 h at room temperature and dehydrated, in a graded series of ethanol up to absolute. Coverslips were placed on metal stubs and coated with gold to a thickness of 15 nm. MVs suspended in PBS were allowed to adhere to Formvar-coated copper grids and fixed with the same procedure as described for TEM. The preparations were attached to metal stubs and coated with gold to a thickness of 15 nm. SEM was performed using a Philips XL30 scanning electron microscope.

Isolation of equine vascular endothelial cells (E-VECs)

The femoral veins of adult horses, euthanased on clinical grounds unrelated to the study, were removed within 30 min post mortem, with informed owner consent. Subjects with clinical or pathological evidence of vascular disease or endotoxaemia were excluded from the study.

The vessels were rinsed with sterile PBS, pH 7.4, supplemented with 200 U/mL penicillin and 200 mg/mL streptomycin. One end of each vessel was closed with a bowel clamp and 0.1% type I collagenase in PBS was introduced inside the lumen. The open end of the vessel was clamped shut with another sterile bowel clamp and the vessel was incubated at 37 °C for 30 min. After a gentle massage of the vein, the endothelial cell-collagenase suspension was poured off and diluted in a 1:4 ratio with complete ENDOGRO medium (Millipore). The vessel lumen was washed twice with PBS, supplemented with penicillin/streptomycin and the combined cell suspension centrifuged at 500 *g* for 10 min.

The supernatant was discarded and cells were suspended in 20 mL complete ENDOGRO medium, seeded in six-well plates coated with collagen (BD Biosciences), and incubated at 37 °C, with 5% CO_2. E-VECs were subcultured once a week at a 1:3 ratio and were not used for experiments beyond passage four. Due to the absence of antibodies that are specific for equine endothelium, the endothelial cell origin of our cultures was evaluated by morphological examination, whereby endothelial cells form a typical 'cobblestone' monolayer of polygonal cells (Jaffe et al., 1973). Additionally, we evaluated the capacity of E-VECs to form cord structures when seeded on a tridimensional matrix (Matrigel, Sigma–Aldrich) as previously described (Kleinman and Martin, 2005). This is considered a functional feature of endothelial cells and is not retained by fibroblasts (Ponce, 2009).

Scratch assay

A scratch assay was used to assess the angiogenic potential of MSC-MVs. Since protein content, as assessed by the Bradford test, was similar in all MV preparations, the same arbitrary amount of MV suspension was used. Silicone culture inserts (Ibidi) for cell migration assays were gently transferred to collagen-coated 48-well plates. E-VECs (2000 cells at passage three) suspended in 100 μL complete ENDOGRO medium, were seeded into each chamber of the Ibidi insert and incubated at 37 °C, 5% CO_2. After attachment, the cells were allowed to reach confluence, then the insert was gently removed using sterile tweezers and the wells filled with 200 μL MV suspension diluted 1:10 in basal ENDOGRO medium without growth factors or serum. ENDOGRO basal medium was used as negative control. Plates were incubated at 37 °C and observed at 24 and 48 h. The test was conducted in triplicate for each MV sample. E-VECs migrating into the intervening space were counted at 20 × magnification in five random fields. Mean ± SD values are shown and *P*-values calculated using the Student's *t* test, with $P < 0.05$ taken to represent statistical significance.

Rat aortic ring assay

The rat aortic ring assay was carried out as previously described (Aplin et al., 2008) to evaluate the angiogenic potential of E-AdMSC-MVs. The dorsal aorta was excised from 6-week old Sprague-Dawley rats (Charles River Laboratories) and dissected into 1 mm thick rings. Each aortic ring was placed inside a well of a four-well plate with 40 μL collagen solution prepared as previously described (Aplin et al., 2008). The plates were incubated at 37 °C for 30 min to obtain collagen jellification. The wells were filled with 500 μL control medium, composed of 1:1 DMEM and EBM (Lonza) or with 500 μL E-AdMSC-derived MVs diluted 1:1 in EBM. In both cases, FBS was omitted from the medium. The plates were incubated at 37 °C, 5% CO_2 for 7 days. Formation of vascular structures was assayed daily using a Zeiss inverted light microscope and the total number of tubular structures was counted using a 10 × objective.

Results

Preparations obtained by ultracentrifugation of E-AdMSC supernatants revealed the presence of a variety of MVs. By TEM, these appeared to be mainly round in shape and ranged in size from 30 to 200 nm. MVs were observed to be present as isolated vesicles