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REGULAR ARTICLE

Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury

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Abstract Human ESC-derived mesenchymal stem cell (MSC)-conditioned medium (CM) was previously shown to mediate cardioprotection during myocardial ischemia/reperfusion injury through large complexes of 50–100 nm. Here we show that these MSCs secreted 50- to 100-nm particles. These particles could be visualized by electron microscopy and were shown to be phospholipid vesicles consisting of cholesterol, sphingomyelin, and phosphatidylcholine. They contained coimmunoprecipitating exosome-associated proteins, e.g., CD81, CD9, and Alix. These particles were purified as a homogeneous population of particles with a hydrodynamic radius of 55–65 nm by size-exclusion fractionation on a HPLC. Together these observations indicated that these particles are exosomes. These purified exosomes reduced infarct size in a mouse model of myocardial ischemia/reperfusion injury. Therefore, MSC mediated its cardioprotective paracrine effect by secreting exosomes. This novel role of exosomes highlights a new perspective into intercellular mediation of tissue injury and repair, and engenders novel approaches to the development of biologics for tissue repair.

Abbreviations: AMI, acute myocardial Infarction; CM, conditioned medium; MI/R, myocardial ischemia/reperfusion; MSCs, mesenchymal stem cells; MWCO, molecular weight cut off; NCM, nonconditioned medium.

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Introduction

Mesenchymal stem cells (MSCs) derived from adult bone marrow have emerged as one of the most promising stem cell types for treating cardiovascular disease (Pittenger and Martin, 2004). Although the therapeutic effect of MSCs has been attributed to their differentiation into reparative or replacement cell types (e.g., cardiomyocytes, endothelial cells, and vascular smooth cells) (Minguell and Erices, 2006; Zimmet and Hare, 2005), it remains to be established if the number of differentiated cell types generated is therapeutically relevant. Recent reports have suggested that some of these reparative effects are mediated by paracrine factors secreted by MSCs (Caplan and Dennis, 2006a; Gnecchi et al., 2005, 2006; Schafer and Northoff, 2008). In support of this paracrine hypothesis, many studies have observed that MSCs secrete cytokines, chemokines, and growth factors that could potentially repair injured cardiac tissue mainly through cardiac and vascular tissue growth and regeneration (Caplan and Dennis, 2006b; Liu and Hwang, 2005). This paracrine hypothesis could potentially provide for a non-cell-based alternative for using MSCs in treatment of cardiovascular disease (Pittenger and Martin, 2004). Non-cell-based therapies as opposed to cell-based therapies are generally easier to manufacture and are safer as they are nonviable.

We have previously performed an unbiased proteomic analysis of a chemically defined medium conditioned by highly expandable human ESC-derived MSC cultures (Lian et al., 2007; Sze et al., 2007). We identified >200 proteins in the secretion of these MSCs (Sze et al., 2007). Computational analysis of the secretome predicted that collectively, the secretome has the potential to repair injured tissue such as in myocardial ischemia/reperfusion (MI/R) injury (Sze et al., 2007). MI/R injury refers to cell death and functional deterioration that occurs during reperfusion therapy to restore blood flow and salvage cardiomyocytes at risk of dying from ischemia in an acute MI (AMI) (Cannon et al., 2000; Saraste et al., 1997). Therefore, the effectiveness of reperfusion therapy can be greatly enhanced by preventing reperfusion injury for which there is currently no treatment (Knight, 2007). We tested the computational prediction of tissue salvage during reperfusion injury in a pig and mouse models of MI/R injury. An intravenous bolus administration of MSC-CM just before reperfusion substantially reduced infarct size in both pig and mouse models of MI/R injury by ~60 and ~50%, respectively (Timmers et al., 2008). There was also a significant preservation of cardiac function and reduction of oxidative stress as early as 4 h after reperfusion (Timmers et al., 2008). However, the active component in the secretion and the mechanism by which it mediates this fast-acting effect on MI/R injury have not been elucidated.

It is obvious that the immediacy of this protective effect precludes the relatively lengthy process of gene transcription and tissue regeneration as part of the mechanism. Also, many of the secreted proteins are membrane and intracellular proteins, and are not known to cross plasma membranes readily. This suggests that if these proteins mediate the cardioprotective effect, the mechanism underlying the therapeutic effect of MSC secretion must involve a vehicle that facilitates crossing of membranes, thus representing a radical shift from our present understanding of MSC paracrine secretion which is limited to extracellular signaling by cytokines, chemokines, and growth factors. To better understand the cardioprotective paracrine effects of MSCs, we then systematically fractionated the MSC-CM using membranes with different molecular weight cut off (MWCO). Based on these fractionations, we demonstrated that the cardioprotective activity was in a >1000-kDa MW fraction (Timmers et al., 2008). This suggested that the cardioprotective effect was mediated by large complexes with a diameter of 50–100 nm.

Here we demonstrate that these large complexes are exosomes. By improving our proteomic analysis, we extended our previously reported list of 201 secreted proteins to 739 proteins and observed the presence of many exosome-associated proteins. Some of these proteins were in detergent-sensitive complexes. These proteins can be sedimented by ultracentrifugation together with the membrane phospholipids. Size-exclusion fractionation by HPLC and dynamic light scattering analysis revealed the presence of a population of particles with a hydrodynamic radius (R_h) of 55–65 nm. More importantly, this HPLC fraction reduced infarct size in a mouse model of MI/R injury.

Results

Cardioprotective secretion contains exosome-associated proteins that form multiprotein complexes

To identify the active component, we had previously fractionated the CM by ultrafiltration through membranes with different MWCO. It was shown that CM filtered through a membrane with MWCO of 1000 kDa was not protective in a mouse model of MI/R injury (Timmers et al., 2008). However, CM concentrated by 125 times against a similar membrane was protective. We observed that after filtration through filters with a MWCO smaller than 0.2 mm such as 100, 300, 500, or 1000 kDa, the filtered CM was not cardioprotective (Fig. 1A). In contrast, CM concentrated against a 1000-kDa membrane (Timmers et al., 2008) or a 100-kDa membrane to retain particles >1000 or 100 kDa, respectively, was cardioprotective (Fig. 1). These observations suggested that the active fraction consisted of large complexes of >1000 kDa or had a predicted diameter of 50–100 nm. Consistent with this, visualization of the CM by electron microscopy revealed the presence of spherical structures with a diameter of 50–100 nm and the morphology of a lipid vesicle (Fig. 1B). Based on this size range and morphology, we postulated that the likely candidate was a secreted phospholipid vesicle known as exosome (Fevrier and Raposo, 2004; Keller et al., 2006).

To test this, we first determined if the CM contained the subset of proteins that are commonly found in exosomes such as CD9, CD81, and Alix (Olver and Vidal, 2007). These proteins were not present in our previous proteomic profiling of the secretion (Sze et al., 2007). By making modifications to our proteomics methodology, we extended our list of proteins found in the MSC secretion from 201 to 739 proteins (Supplementary Table 1). The computationally predicted biological activities of this proteome suggested that the secretion will have significant biological effects on cardiac

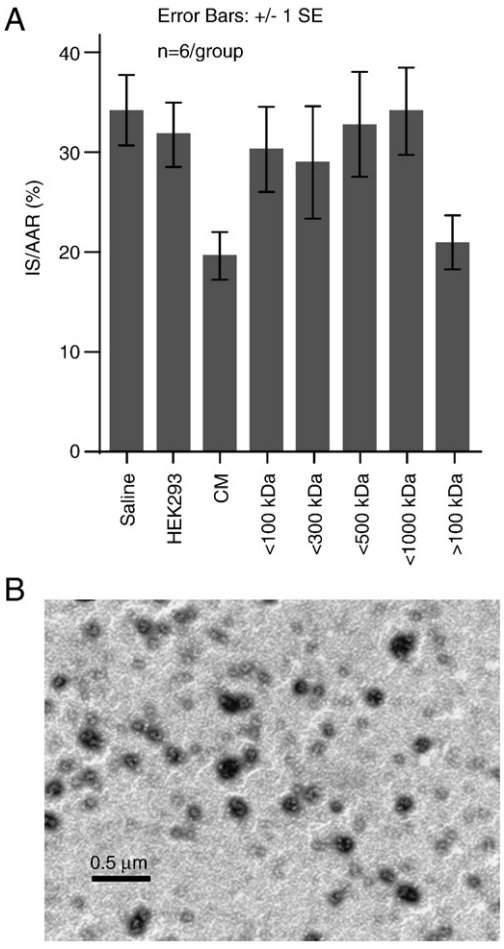

Figure 1 Cardioprotective properties of CM fractions. (A) Saline, HEK293 CM, or different preparations of hESC-MSC CM were administered to a mouse model of MI/R injury as described under Materials and methods. The <1000, <500, <300, and <100 kDa represented CM filtered sequentially with membranes that had MWCO of 1000, 500, 300, and 100 kDa, respectively. The >100 kDa represented CM concentrated 50 times against a TFF membrane with MWCO of 100 kDa. The infarct size (IS) was expressed as a fraction of the area at risk (AAR) in the left ventricle. (B) Transmission electron microscopic picture of CM; scale bar represents 500 nm.

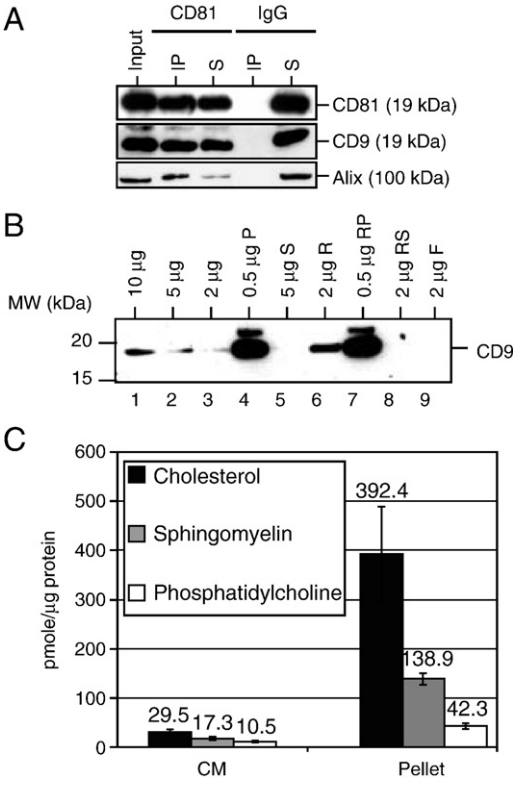

Figure 2 Presence of large lipid complexes in CM. (A) Coimmunoprecipitation of CD81, CD9, and Alix. After immunoprecipitation of hESC-MSC CM with anti-CD81 or mouse IgG, the immunoprecipitate (IP) and supernatant (S) were analyzed by Western blot hybridization using antibody against CD9 and Alix. (B) Size fractionation by ultrafiltration and ultracentrifugation. CM was concentrated 5X using a membrane with MWCO of 500 kDa. The retentate and the unfiltered CM were then ultracentrifuged at 200 000 *g* for 2 h. The supernatant and the pellet were analyzed by Western blotting for the presence of CD9. Lanes 1–3: Different protein amount of CM. Lanes 4 and 5: The pellet (P) and supernatant (S) after ultracentrifugation of unfiltered CM. Lane 6: Retentate (R) after filtration of CM through a membrane with MWCO of 500 kDa. Lanes 7 and 8: The pellet (RP) and supernatant (RS) after ultracentrifugation of retentate. Lane 9: Filtrate (F) after filtration of CM through a membrane with MWCO of 500 kDa. (C) Amount of cholesterol, spingomyelin, and phosphatidylcholine in CM and in the pellet after 200 000 *g* ultracentrifugation of the CM was assayed and quantitated as picomole per microgram protein.

tissue injury and repair (Fig. S1). The subsets of exosome-associated proteins CD9, CD81, and Alix were confirmed to be present in the secretion by Western blot analysis (lane 1, Fig. 2A). The MW of CD9 and CD81 was the expected 25 and 22–26 kDa, respectively. Consistent with our hypothesis that the large complexes are exosomes, we observed that CD9 and Alix coimmunoprecipitated with CD81, suggesting that these proteins were in a single complex (Fig. 2A).

A 24-kDa CD9 sediments at 200 000 *g* and is retained by membrane with 500-kDa MWCO

As exosomes are routinely purified by ultracentrifugation (Thery et al., 2006), we next determined if CD9 was associated with a large complex that can be precipitated by ultracentrifugation. The CM was first fractionated through a membrane (MWCO = 500 kDa) into a >500-kDa retentate fraction and a <500-kDa filtrate fraction followed by ultracentrifugation of both fractions. The 24-kDa CD9 was found in the >500-kDa retentate fraction and could be precipitated by ultracentrifugation (Fig. 2B). CD9 was not detected in the <500-kDa filtrate fraction. Consistent with our exosome hypothesis, major plasma membrane phospholipids such as cholesterol, sphingomyelin, and phosphatidylcholine were also precipitated by ultracentrifugation at 200 000 *g* for 2 h as evidenced by their enrichment in the precipitate (Fig. 2C).

Proteins in the CM are associated with phospholipid membrane

As exosomes are phospholipid vesicles, they are known to have a typical density range of 1.10 to 1.18 g ml^{-1} that could be resolved on sucrose gradients (Raposo et al., 1996; Thery et al., 2006). We therefore postulated that the flotation densities of the putative exosome-associated proteins would be different before and after release from such vesicles by a detergent-based buffer. Therefore, CM or CM pretreated with a detergent-based lysis buffer was fractionated on a sucrose density gradient by equilibrium ultracentrifugation. The fractions were analyzed for the distribution of CD9 and CD81. Both CD9 and CD81 which coimmunoprecipitated (Fig. 2A) had a similar flotation density that was heavier than that expected of proteins in their MW range (Fig. 3). Pretreatment with a detergent-based cell lysis buffer decreased the apparent flotation densities of CD9 and CD81 to that of proteins in a similar MW range (Fig. 3). Our observations demonstrated that the detergent-sensitive flotation densities of proteins were consistent with their location in lipid vesicles.

Exosomal proteins are either membrane bound or encapsulated

As many of the secreted proteins in the CM are known membrane or cytosolic proteins, we investigated if these proteins in the CM were also membrane bound or localized within the lumen of the putative exosomes by limited trypsinization. Membrane-bound proteins would expected to be partially resistant whereas luminal proteins are expected to be resistant to trypsinization. Treatment with

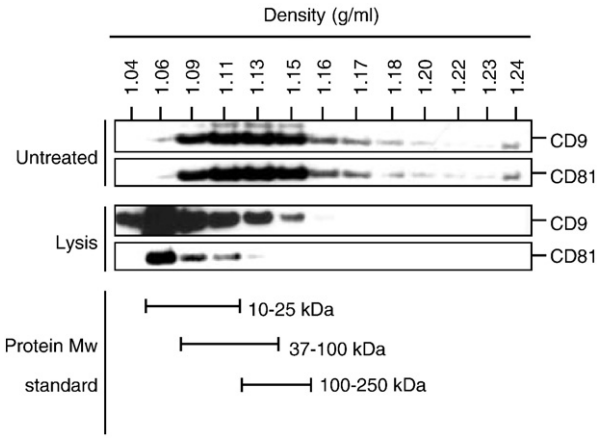

Figure 3 Protein analysis of CM fractionated on a sucrose gradient density. CM or CM pretreated with lysis buffer was loaded on a sucrose density gradient prepared by layering 14 sucrose solutions of concentrations from 22.8 to 60% (w/v) in a SW60Ti centrifuge tube and then ultracentrifuged for 16.5 h at 200 000 *g*, 4 °C, in a SW60Ti rotor. The gradients were removed from the top and the density of each fraction was calculated by weighing a fixed volume of each fraction. The fractions were analyzed by Western blot analysis for CD9 and CD81 in CM (upper panel) and pretreated CM (lower panel). The distribution of a protein standard molecular weight marker set after fractionation in a similar gradient is denoted at the bottom of the figure.

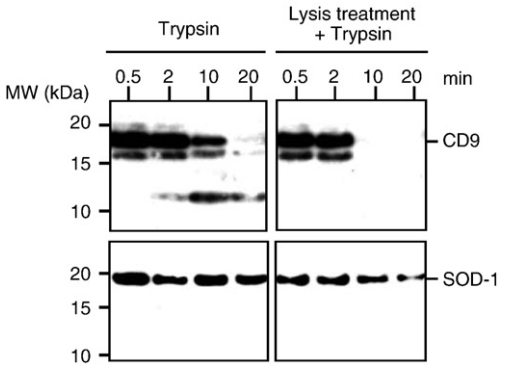

Figure 4 Trypsinization of CM. CM treated with either PBS or lysis buffer was digested with trypsin and an aliquot was removed at 0.5, 2, 10, and 20 min. A trypsin inhibitor, PMSF, was then added to terminate the trypsinization reaction and the aliquots were analyzed for the presence of CD9 and SOD-1 by Western blot hybridization.

a detergent-based lysis buffer would abrogate this resistance. As expected, CD9, a membrane-bound protein was susceptible to trypsin digestion and generated two detectable tryptic peptide intermediates (Fig. 4). In contrast, SOD-1, a cytosolic protein was resistant to trypsin digestion. Pretreatment of CM with a detergent-based cell lysis buffer abolished the resistance of CD9 and SOD-1 to trypsin digestion. The detergent-sensitive partial susceptibility of CD9 and resistance of SOD-1 to trypsin digestion were consistent with their localization in a lipid membrane and lumen of an exosome, respectively.

Purification of a homogeneous population of exosomes by HPLC fractionation

To demonstrate directly that the active cardioprotective component in the secretion is an exosome, CM and nonconditioned medium (NCM) were first fractionated by size exclusion on a HPLC column (Fig. 5A).The eluent was monitored by absorbance at 220 nm and then examined by dynamic light scattering which has a hydrodynamic radius (R_h) detection range of 1 to 1000 nm. The first four eluting fractions in CM (F1–F4) were not present in the NCM and therefore represented secretion from the hESC-MSCs. F1, the fastest eluting fraction with a retention time of 12 min, represented the fraction containing the largest particles in the CM. The particles in F1 were sufficiently homogeneous in size such that they could be determined by dynamic light scattering to have a hydrodynamic radius (R_h) of 55–65 nm. All other peaks contained particles that were too heterogeneous in size to be estimated by dynamic light scattering. F1 contained 4% of total protein input but contained ~50% of the CD9 in the input (Fig. 5B). Proteins were distributed among F2, F3, and F4 fractions according to the principle of size-exclusion fractionation such that larger proteins were eluted first in F2 followed by the smaller proteins in F3 and the smallest in F4 (Fig. 5C). In contrast, proteins in the F1 fraction had a MW distribution that spanned the entire MW spectrum of F2, F3, and F4 (Fig. 5C). The proteins in the F1 fraction despite having a MW range of 20 to 250 kDa

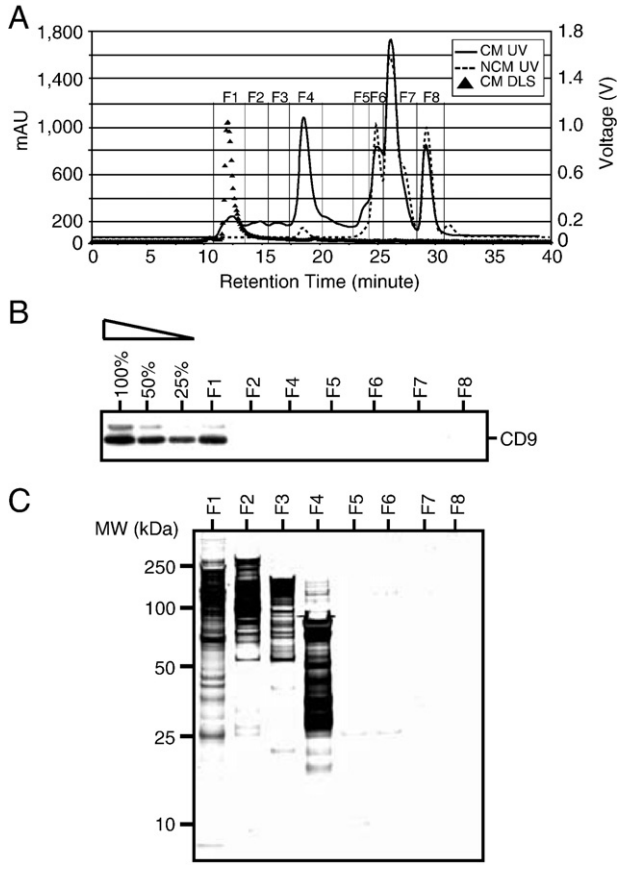

Figure 5 HPLC fractionation of CM. (A) HPLC fractionation and dynamic light scattering of CM and NCM. CM and NCM were fractionated on a HPLC using a BioSep S4000, 7.8 mm×30 cm column. The components in CM or NCM were eluted with 20 mM phosphate buffer with 150 mM NaCl at pH 7.2. The elution mode was isocratic and the run time was 40 min. The eluent was monitored for UV absorbance at 220 nm. Each eluting peak was then analyzed by light scattering and signals as measured in voltage are represented by solid triangles. The eluted fractions, F1 to F8, were collected, their volumes were adjusted to 50% of the input volume of CM, and an equal volume of each fraction was analyzed for (B) the presence of CD9 by Western blot hybridization. Lanes 1–3 were CM loaded at 2X, 1X, or 0.5X of the volume loaded used for each of the fractions, F1 to F8 (lanes 4–11), and therefore represented the equivalent of 100, 50, and 25% input CM. (C) Equal volumes of F1–F8 were separated on a SDS-PAGE and then stained with silver.

sedimented at a similar flotation density of 1.11–1.16 g/ml (Fig. 6B) that was similar to that of CD9 in the CM (Fig. 3). These features of the F1 fraction, i.e., the presence of proteins with a wide spectrum of MW sizes and identical flotation density, the exclusive presence of CD9, and a homogeneous size, indicated that a homogeneous exosome population was purified from the CM by HPLC fractionation. When 0.4 μg of F1 protein was administered to a mouse model of MI/R injury 5 min prior to reperfusion, the F1 fraction reduced infarct size to the same extent as 3 μg CM protein (Fig. 7A). All animals in this study had the same degree of endangered myocardium, as illustrated by similar area at risk within the left ventricle (Fig. 7B).

Paracrine effect was mediated through heart tissues

For elucidating the mechanism of this paracrine effect, an important prerequisite is the identification of the target tissues. Here we determine that the paracrine effect on MR/I injury was a heart autonomous effect and was independent of circulating cells including immune cells. Using an *ex vivo* mouse Langendorff heart model of ischemia/reperfusion injury, we observed that conditioned medium reduced relative infarct size to the same extent as in a mouse model (Fig. 8).

Discussion

The trophic effects of MSC transplantation on ameliorating the deleterious consequences of myocardial ischemia have been implicated in several studies (Caplan and Dennis, 2006a). Transplantation of MSCs into ischemic myocardium has been shown to induce several tissue responses such as an increased

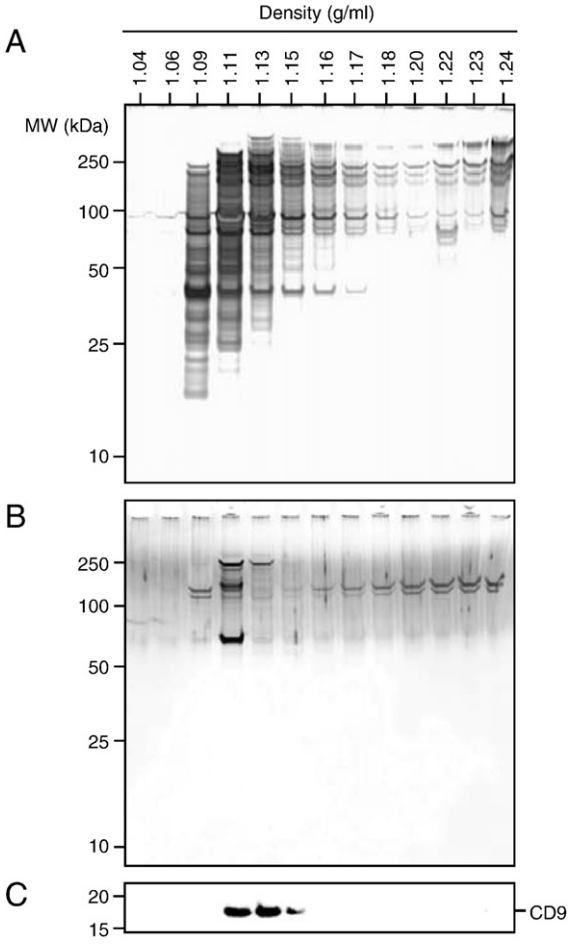

Figure 6 Flotation densities of proteins in CM and HPLC-purified F1 fraction were determined by fractionating CM and F1 onto a sucrose gradient density as described above. The 13 fractions for (A) CM and (B) F1 were separated on a SDS-PAGE and then stained with silver. (C) To evaluate the distribution of smaller proteins, the F1 was also assayed for CD9 (20 kDa) by Western blot hybridization. Proteins in the >1.20 g/ml density fractions were denatured proteins.

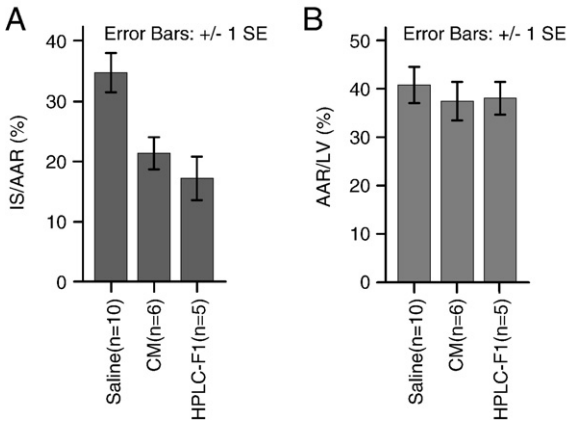

Figure 7 Cardioprotective exosomes. A 0.4 μg F1 protein was administered intravenously to a mouse model of MI/R injury 5 min before reperfusion. Infarct sizes (IS) as a percentage of the area at risk (AAR) on treatment with saline ($n=10$), conditioned medium from hESC-MSCs ($n=6$), and HPLC fraction ($n=5$) were measured. Saline treatment resulted in 34.5±3.3% infarction. CM treatment resulted in 21.2±2.6% infarction ($P=0.022$ compared to saline) and F1 fraction treatment resulted in 17.0±3.6% infarction ($P=0.004$ compared to saline). (B) AAR as a percentage of the left ventricle (LV), showing the amount of endangered myocardium after MI/R injury. All animals were affected to the same extent by the operative procedure, resulting in 39.1±2.2% of AAR among the groups. Each bar represents mean±SEM.

production of angiogenic factors and decreased apoptosis (Tang et al., 2005). It was postulated that these responses were better explained by secretion of paracrine factors than by differentiation of MSCs. In this context, MSCs were shown to secrete many growth factors and cytokines that have

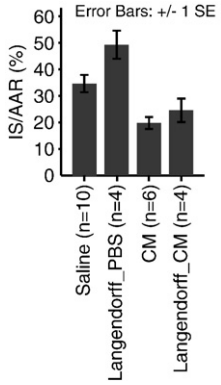

Figure 8 Secretion reduced myocardial ischemia-reperfusion injury *ex vivo*. Perfusion buffer containing 3.5 μg/ml CM was used to perfuse mouse heart in an *ex vivo* mouse Langendorff heart model of MI/R injury 5 min before reperfusion. Infarct sizes (IS) as a percentage of the area at risk (AAR) on treatment with PBS ($n=4$) and CM ($n=4$) were measured after 3 h reperfusion. Langendorff_PBS treatment resulted in 49.3±5.3% infarction. Langendorff_CM treatment resulted in 24.6±4.4% infarction ($P<0.001$ compared to Langendorff_PBS). As a reference for comparison, the *in vivo* effects of saline and CM on IS/AAR as described in Fig. 7 are also included.

effects on cells in their vicinity. To date, many of these studies have focused exclusively on proteins that are known to be secreted. These proteins generally included cytokines, chemokines, and other growth factors (Caplan and Dennis, 2006a). However, our unbiased proteomic profiling of proteins in the secretion of MSCs revealed an abundance of membrane and cytosolic proteins (Sze et al., 2007). This suggests that the trophic effects of MSCs may not be mediated by soluble growth factors and cytokines alone. This was underscored by our observation that the cardioprotective effects of CM were mediated by 50- to 100-nm complexes of >1000 kDa (Timmers et al., 2008) and not small soluble proteins.

Based on the size of the complex, we postulated that the cardioprotective complex in the CM was likely to be an exosome. Exosomes are formed from multivesicular bodies with a bilipid membrane (Fevrier and Raposo, 2004; Keller et al., 2006). They have a diameter of 40–100 nm and are known to be secreted by many cell types (Fevrier and Raposo, 2004; Keller et al., 2006). Electron microscopy confirmed that the CM contained lipid-like vesicles of about 50–100 nm in diameter. The functions of exosomes are not known but they are thought to be important in intercellular communications. Although exosomes are known to have a cell-type-specific protein composition, most carry a common subset of proteins that included CD9, CD81, Alix, TSP-1, SOD-1, and pyruvate kinase (Olver and Vidal, 2007). CD9 and CD81 are tetrapannin membrane proteins that are also localized in the membrane of exosomes. Consistent with the presence of exosomes, CM contained coimmunoprecipitating complexes of CD81, CD9, and Alix. Ultracentrifugation precipitated CD9 with phospholipids and cholesterol, suggesting that the CD81, CD9, and Alix complex was associated with a phospholipid vesicle. This was confirmed by the detergent-sensitive flotation densities of these proteins where we demonstrated that the flotation densities of these proteins in the CM were that of phospholipid vesicles and that detergent treatment which dissolved phospholipid membrane altered the flotation densities of the proteins. We further demonstrated that CD9 in the CM was a membrane-bound protein while SOD-1 was localized within a lipid vesicle by their respective partial and complete resistance to trypsin degradation and the abrogation of this resistance by detergent. Taken together, our observations demonstrated that exosomes with a diameter of 50–100 nm are present in the CM and are therefore the likely candidate for the cardioprotective component in the CM. This was confirmed when a HLPC-purified homogeneous population of particles that had an enrichment of CD9 and a R_h of 55–65 nm substantially reduced infarct size in a mouse model of MI/R injury at a reduced protein dosage equivalent to ~10% of the CM dosage. We further demonstrated using an *ex vivo* mouse Langendorff heart model of MI/R injury that this paracrine effect was a heart autonomous effect, and was independent of circulating cells, such as immune cells or platelets.

In summary, we have identified exosome as the cardioprotective component in MSC paracrine secretion. This involvement of exosomes represents a radical shift in our current understanding of the paracrine effect of MSC transplantation on tissue repair which hitherto has been limited to cytokine, chemokine, or growth factor-mediated extracellular signaling. It also highlights for the first time the role of exosome as mediator of tissue repair. As lipid vesicles,

exosomes represent an ideal vehicle to effect an immediate physiological response to repair and recover from injury through the rapid intracellular delivery of functional proteins. Recently, it was demonstrated that in addition to proteins, microvesicles have the potential to mediate intercellular transfer of genetic material (reviewed in Quesenberry and Aliotta, 2008). Several tumor cell types (Rosell et al., 2009; Taylor and Gercel-Taylor, 2008), peripheral blood cells (Hunter et al., 2008; Valadi et al., 2007), endothelial progenitor cells (Deregibus et al., 2007), and embryonic stem cells (Ratajczak et al., 2006) have been shown to secrete RNA-containing microvesicles. More importantly, these microvesicular RNA could be transferred to other cells and translated in the recipient cells (Deregibus et al., 2007; Ratajczak et al., 2006; Valadi et al., 2007). We also recently demonstrated that the MSC-derived exosomes described here also contained miRNAs and these miRNAs were predominantly in the precursor form (Chen et al., 2010). For reperfused ischemic myocardium, this feature of rapid initiation of cellular repair through the intracellular delivery of functional proteins and possibly RNA is particularly critical as the time window for therapeutic intervention is very narrow. We speculate that the involvement of exosomes in cardioprotection may represent a general function of exosomes in tissue repair. It is possible that different cell types produce exosomes that are specific for certain type of cells or injuries. If true, this novel tissue-repair function of exosomes could potentially engender new approaches to the development of biologics.

Materials and methods

Preparation of CM

The culture of HuES9.E1 cells and preparation of HuES9.E1 CM were performed as described previously (Lian et al., 2007; Sze et al., 2007). For the <100-, <300-, <500-, or <1000-kDa preparations in Fig. 1, the CM was first concentrated 25X by tangential flow filtration (TFF) using a membrane with a 10-kDa MWCO (Sartorius, Goettingen, Germany) and then filtered sequentially through membranes with MWCO of 1000 kDa (Sartorius), 500 kDa (Millipore, Billerica, MA), 300 kDa (Sartorius), and finally 100 kDa (Sartorius). All other CM and NCM used were concentrated 25X or 50X by TFF using a membrane with 10- or 100-kDa MWCO (Sartorius). The CM and NCM preparations were filtered with a 0.2-μm filter before storage or use.

Electron microscopy, antibody array assay, protein analysis

Electron microscopy, antibody array assay, and protein analysis were done using standard protocols; for details please refer to Supplementary Materials and Methods.

LC MS/MS analysis

Proteins in 2 ml of dialyzed CM or NCM were analysis by LC MS/MS using standard protocols with some modifications; for details please refer to Supplementary Materials and Methods.

Immunoprecipitation of exosome-associated proteins

Dynabead M-280 sheep anti-mouse IgG (Invitrogen Corporation, Carlsbad, CA) was washed using 0.1% BSA/PBS before incubation with mouse anti-human CD81 antibody for 2 h with gentle shaking at room temperature. The dynabeads were washed twice and incubated with CM with gentle shaking for 2 h at room temperature. The supernatant was then collected, and the dynabeads were gently washed twice before PBS was added. The supernatant and the dynabeads were denatured, resolved on 4–12% SDS-PAGE, and analyzed by Western blotting.

Sucrose gradient density equilibrium centrifugation

To generate the sucrose gradient density for centrifugation, 14 sucrose solutions with concentrations from 22.8 to 60% were prepared and layered sequentially in an ultracentrifuge tube (Beckman Coulter Inc., CA) starting with the most concentrated solution. CM was loaded on top before ultracentrifugation for 16.5 h at 200 000 *g*, 4 °C in a SW60Ti rotor (Beckman Coulter Inc.). After centrifugation, 13 fractions were collected starting from the top of the gradient. The densities of each were determined by weighing a fixed volume. For pretreatment with detergent-based lysis buffer (Cell Extraction Buffer, Biovision, Mountain View, CA), CM was incubated with an equal volume of the lysis buffer containing protease inhibitors (Halt Protease Inhibitor Cocktail, Thermo Fisher Scientific Inc., Waltham, MA) for 30 min at room temperature with gentle shaking. The protein concentration of CM was quantified using the NanoOrange Protein Quantification kit (Invitrogen Corporation) according to the manufacturer's instructions.

Sphingomyelin, phosphatidylcholine, and cholesterol assay

Cholesterol, sphingomyelin, and phosphatidylcholine concentration in CM and pellet from the ultracentrifugation of CM at 200 000 *g* for 2 h at 4 °C was determined using commercially available assay kits. Cholesterol was measured using the Amplex Red Cholesterol Assay kit (Invitrogen Corporation), sphingomyelin, and phosphatidylcholine were measured using the Sphingomyelin Assay Kit and Phosphatidylcholine Assay Kit (Cayman Chemical Company, Ann Arbor, MI) respectively.

Limited trypsinization of CM

CM was incubated with equal volumes of either PBS or lysis buffer (Cell Extraction Buffer, Biovision, Mountain View, CA) for 45 min at 4 °C with gentle shaking. Then 16 μl of 10× trypsin (Invitrogen Corporation) was added and incubated at 37 °C with gentle shaking. An aliquot was removed at 30 s, 1 min, 5 min, and 20 min, and 1 μl of a 100 mM trypsin inhibitor, PMSF (Sigma-Aldrich, St. Louis, MO), was added. The mixture was denatured and analyzed by Western blot analysis.

HPLC dynamic light scattering

The instrument setup consisted of a liquid chromatography system with a binary pump, an auto injector, a thermostated

column oven and a UV-visible detector operated by the Class VP software from Shimadzu Corporation (Kyoto, Japan). The Chromatography columns used were TSK Guard column SWXL, 6×40 mm and TSK gel G4000 SWXL, 7.8×300 mm from Tosoh Corporation (Tokyo, Japan).The following detectors, Dawn 8 (light scattering), Optilab (refractive index), and QELS (dynamic light scattering), were connected in series following the UV-visible detector. The last three detectors were from Wyatt Technology Corporation (CA, USA) and were operated by the ASTRA software. For details please refer to Supplementary Materials and Methods.

MI and surgical procedure

All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Pigs prepared by the Institute of Laboratory Animal Resources and with prior approval by the Animal Experimentation Committee of the Faculty of Medicine, Utrecht University, the Netherlands. The CM and the HPLC fraction 1 (F1) were tested in a mouse model of MI/R injury. MI was induced by 30 min left coronary artery (LCA) occlusion and subsequent reperfusion. Five minutes before reperfusion, mice were intravenously infused with 200 μl saline-diluted CM containing 3 μg protein or HPLC F1 containing 0.4 μg protein via the tail vein. Control animals were infused with 200 μl saline. After 24 h reperfusion, infarct size (IS) as a percentage of the area at risk (AAR) was assessed using Evans' blue dye injection and TTC staining as described previously (Arslan et al., 2010).

Mouse Langendorff heart model of ischemia/reperfusion injury

For the mouse Langendorff heart model of ischemia/reperfusion injury, mice were given heparin 50 IE subcutaneously. The suture was placed *in vivo* without placing the knot. Hereafter, the heart was excised and aortic root was canulated and perfused in the Langendorff setup. After 10 min recovery, the suture was tightened to induce ischemia for 30 min. Just 5 min prior to reperfusion, the perfusion buffer was changed for a second buffer containing 3.5 μg/ml MSC-CM. Reperfusion was allowed for 3 h before Evans' blue dye injection and TTC staining for infarct size assessment, as described previously (Arslan et al., 2009).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.scr.2009.12.003.

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