Membrane lipids define small extracellular vesicle subtypes secreted by mesenchymal stromal cells

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Abstract The therapeutic efficacy of mesenchymal stromal cells (MSCs), multipotent progenitor cells, is attributed to small (50–200 nm) extracellular vesicles (EVs). The presence of a lipid membrane differentiates exosomes and EVs from other macromolecules. Analysis of this lipid membrane revealed three distinct small MSC EV subtypes, each with a differential affinity for cholera toxin B chain (CTB), annexin V (AV), and Shiga toxin B chain (ST) that bind GM1 ganglioside, phosphatidyserine, and globotriaosylceramide, respectively. Similar EV subtypes are also found in biologic fluids and are independent sources of disease biomarkers. Here, we compare and contrast these three EV subtypes. All subtypes carry β-actin, but only CTB-binding EVs (CTB-EVs) are true exosomes, enriched with exosome proteins and derived from endosomes. No unique protein has been identified yet in AV-binding EVs (AV-EVs); ST-binding EVs (ST-EVs) carry RNA and a high level of extra domain A-containing fibronectin. Based on the CTB, AV, and ST subcellular binding sites, the origins of CTB-, AV-, and ST-EV biogenesis are the plasma membrane, cytoplasm, and nucleus, respectively. The differentiation of EV subtypes through membrane lipids underlies the importance of membrane lipids in defining EVs and implies an influence on EV biology and functions.—Lai, R. C., and S. K. Lim. Membrane lipids define small extracellular vesicle subtypes secreted by mesenchymal stromal cells. J. Lipid Res. 2019. 60: 318–322.

Supplementary key words cholesterol/trafficking • endocytosis • secretion • exosome • cholera toxin B chain • annexin V • Shiga toxin B chain

Mesenchymal stromal cells (MSCs) are multipotent progenitor cells that are among the most widely used candidate cells in regenerative stem cell trials, with nearly 500 MSC clinical trials (http://www.clinicaltrials.gov/). The therapeutic efficacy of MSCs has been increasingly attributed to a paracrine secretion that ameliorates cellular injury and enhances repair (1). Among the active agents proposed to date were two extracellular vesicle (EV) types, namely 50–1,000 nm microvesicles by Giovanni Camussi’s group (2) and 50–100 nm exosomes by our group (3).

MSC EXOSOMES ARE SMALL LIPID MEMBRANE VESICLES

MSC exosomes were first described in 2010 (3). They are presently classified as a member of the family of small EVs that comprises secreted lipid membrane vesicles of 50–200 nm. The size range of exosomes and small EVs has not been precisely defined, and there are slight variations in the ranges used by different groups. In the early days, EV sizes were often estimated by electron microscopy and the sizes tended to be underestimated, especially if the samples were dehydrated during processing for electron microscopy. On the other hand, dynamic light scattering analysis tends to overestimate the size due to the presence of a hydration shell around the vesicles. In a recent review by Clothide Thery (4), a pioneer in exosome research, she observed that a 2,000 g centrifugation, which is commonly used to remove debris prior to exosome/small EV purification, removed the majority of EVs of diameters larger than 200 nm. For the purpose of this review, small EVs will be defined as those with a size range of 50 to 200 nm.

The presence of a lipid membrane differentiates exosomes and EVs from other biological macromolecules and contributes significantly to the biological potency of...

Abbreviations: AV, annexin V; AV-EV, annexin V-binding extracellular vesicle; CTB, cholera toxin B chain; CTB-EV, cholera toxin B chain-binding extracellular vesicle; EV, extracellular vesicle; MSC, mesenchymal stromal cell; MVB, multivesicular body; ST, Shiga toxin B chain; ST-EV, Shiga toxin B chain-binding extracellular vesicle.†To whom correspondence should be addressed. e-mail: saikiang.lim@imb.a-star.edu.sg

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exosomes and EVs. Disruption of the membrane structure abolishes the therapeutic activity of the exosomes (5). In the first description of MSC exosomes, the presence of a lipid membrane was demonstrated through several lines of evidence. First, the proteins in the preparation had a flotation density of 1.10–1.18 g ml$^{-1}$ that lies between the liposome density of 1.04–1.05 g ml$^{-1}$ (6) and the estimated protein density of 1.35–1.42 g ml$^{-1}$ (7). Second, the tetraspanin proteins CD9 and CD81 were enriched in the exosome preparation and were shown to be partially resistant to trypsin digestion. Limiting trypsic digestion of the exosome preparation generated partial CD9 tryptic peptides (3), and these peptides mapped to intra-membrane and intra-luminal regions (unpublished observations). This partial resistance was abolished by preincubation with detergent. RNA in the exosome preparation also had a much lighter density of $\sim 1.11$ g ml$^{-1}$ than the expected $>1.8$ g ml$^{-1}$ (8). The RNA was also resistant to RNase activity, and this resistance was abolished by pretreatment with phospholipase. Together, these observations demonstrate that the proteins and RNAs are encapsulated with a lipid membrane structure. Third, major plasma membrane phospholipids, such as cholesterol, sphingomyelin, and phosphatidylcholine, were enriched in the preparation.

In contrast to MSC exosomes, the MSC microvesicles, as first described, encompass EVs across a wide size range (2) and are likely to include both large and small EVs. Consistent with the presence of small EVs, one of the two sources of the microvesicles appeared to originate in the multivesicular bodies where exosomes are made. The other is from blebbing at the plasma membrane. Apart from having a much wider size range, this microvesicle preparation has several other features that contrast significantly with those in the exosome preparation. For example, this preparation contains mRNA, and mammalian RNAs have an estimated median length of 1.4 kb (11). This contrasts significantly with the $<300$ nt RNA in MSC exosomes (3). Furthermore, unlike the RNA in MSC exosomes, the RNA in the MSC microvesicles can be degraded by RNase without compromising the integrity or structural morphology of microvesicles (2, 12). The miRNA composition is also significantly different between those present in MSC exosomes and MSC microvesicles. The former is enriched in pre-miRNA (8), while the latter is enriched in mature miRNA (13). A possible reason for the differences in the MSC microvesicle preparation and MSC exosome preparation could be the inclusion of the $>200$ nm EVs whose larger cargo volume may dominate the features of the preparation. Because this review is focused on small MSC EVs, MSC microvesicles will not be included.

MSC EXOSOMES CARRY ENDOCYTOSIS CTB AND TRANSFERRIN

Exosomes are the only EV type known to have an endosomal biogenesis. The biogenesis of exosomes begins when the membrane of endosomes invaginates to form a multivesicular body (MVB) with numerous intraluminal vesicles. When MVBs fuse with the plasma membrane, the intraluminal vesicles in the MVB are released as exosomes. As a result, exosomes are typified by a conserved set of proteins that are associated with endocytosis and endosomal trafficking, such as caveolins, clathrin, transferrin receptors, tetraspanins (CD81, CD63, CD9), Alix, and Tsg101 [reviewed in (14)].

The MSC exosome preparation, as first described in 2010, was uniformly sized with a hydrodynamic radius of 55–65 nm. It should be noted that the estimated size of EVs is influenced by the method of measurement or instrumentation. For instance, size, as estimated by electron microscopy, tends to be smaller due to dehydration during sample preparation. Consistent with the initial characterization of the preparation as an exosome preparation, this preparation was subsequently confirmed to contain bona fide exosomes (i.e., derived from endosomes) by demonstrating through pulse-chase experiments that MSCs secrete a fraction of endocytosed extracellular ligands, such as biotin-conjugated transferrin or biotin-conjugated cholera toxin B chain (CTB), in small EVs (15). Transferrin is the ligand for transferrin receptor, the first exosome marker to be identified (16, 17), and transferrin receptor 2 has been reported to be localized in lipid rafts and released into exosomes with CD81 (18). On the other hand, CTB binds GM1 ganglioside, a lipid that is highly enriched in lipid rafts [reviewed in (19)].

The pulse-chase of transferrin and CTB into MSC exosomes is consistent with the well-documented “lipid raft” characteristic of exosomes. Exosomes have been long known to be enriched in lipid raft proteins (e.g., flotillin-1), raft-associated proteins (e.g., ezrin/villin-2), and raft-associated lipids such as GM1 gangliosides (20, 21). The endocytosed transferrin and CTB on MSC exosomes were found to colocalize with each other and with other exosome markers. Proteome analysis also revealed when the proteins in the proteome of CTB-binding EVs (CTB-EVs) were clustered into pathways where they are known to function. Four of the top 20 pathways were endocytic or exocytic processes, namely, caveolar-mediated endocytosis signaling, virus entry via endocytic pathways, mechanisms of viral exit from host cells, and clathrin-mediated endocytosis signaling (22). Together, the presence of a proteome enriched in proteins associated with endocytosis, endosome-associated activities, or exocytosis, and the colocalization of endocytosed transferrin and CTB with exosome markers confirmed that CTB-EVs from MSCs are bona fide exosomes. This association between CTB and exosomes was further confirmed recently when cholera toxin was shown to be propagated from cell to cell through exosomes (23).

SMALL MSC EVS INCLUDE EXOSOMES AND OTHER SMALL EV SUBTYPES

The presence of nonexosomal EV subtypes in the first described MSC exosome preparation was suspected when CTB was found to extract only a fraction of the total protein (22). Subsequent testing using known membrane
lipid-binding ligands led to the identification of two other EV subtypes, AV-binding EVs (AV-EVs) and Shiga toxin B chain-binding EVs (ST-EVs). AV binds phosphatidylserine, while ST binds globotriaosylceramide. The use of membrane lipid-binding ligands to identify other EVs was rationalized on the importance of the lipid membrane as the defining and physically delimiting feature of EVs. This also ensures that lipid membrane-bound entities and not similarly sized macromolecules were isolated.

Like CTB-EVs, AV- and ST-EVs are ~100–200 nm vesicles that could be visualized by electron microscopy (22). Based on the distribution of proteins in MSC small EVs across a sucrose gradient of 1.09–1.15 g/ml, the density of CTB-, AV-, and ST-EVs is likely to be within this range. A retrospective analysis of published data revealed that CD9 in the MSC exosome preparation had a modal flotation density of 1.13 g/ml (3), while the RNA in the MSC exosome had a modal flotation density of 1.11 g/ml (8). Because CTB-EVs carry almost all the CD9, and only the ST-EVs, but not the CTB- or AV-EVs, carry RNA (22), the CTB-EVs and ST-EVs may have different densities. The density of AV-EVs is presently not known. All three EV types carry β-actin. The size distribution of proteins from each of the three EVs on a 1D protein gel displayed discernible differences. Western blotting revealed that the exosome proteins CD81, CD9, ALIX, and TSG101 were enriched relative to β-actin in the CTB-EVs, present at very low levels in AV-EVs, and not detectable in ST-EVs. On the other hand, ST-EVs carry a high level of extra domain A-containing fibronectin and RNA, both of which are not detectable in CTB- and AV-EVs.

The unique fibronectin- and RNA-enriched cargo of ST-EVs demonstrates that ST-EVs are distinct from CTB- and AV-EVs. However, ST-EVs may not be the only RNA-bearing EVs in the MSC exosome preparation, as RNA sequencing revealed that the RNA in ST-EVs represents only a fraction of the total RNA in the MSC exosome preparation.

At present, there is no unique protein that characterizes AV-EVs. The low level of exosome markers in AV-EVs was specific to AV-EVs and not contamination by CTB-EVs, as the levels of the markers in AV-EVs were similar with or without a prior extraction with CTB (22).

**BIOGENESIS OF MSC EV SUBTYPES**

Aside from the CTB-EVs, which were shown to be bona fide exosomes with an endosomal biogenesis, the biogenesis of AV-EVs and ST-EVs has not been elucidated. Because AV-EVs have exposed phosphatidylserinases, a reasonable assumption is that they were derived from apoptotic cells. However, it was observed that during staurosporine-induced apoptosis, MSCs secrete AV-EVs that have a more elevated level of CD9 relative to β-actin than AV-EVs produced by healthy MSCs, suggesting that AV-EVs produced from healthy MSCs were different from that of apoptotic MSCs.

As the key differentiating feature of CTB-, AV-, and ST-EVs is the presence of specific membrane lipids, it is likely that their biogenesis occurs at sites in the plasma membrane or membrane organelles where the specific membrane lipids are present and also highly enriched to facilitate polyvalent binding. The latter is important because all three lipid binding proteins have multivalent binding activities. Each AV molecule binds four to eight phosphatidylserine molecules (24, 25). As both CTB and ST are pentamers, they each can potentially bind five or more lipid molecules. Each CTB monomer is known to bind one GM1 ganglioside (26), while each ST monomer binds one or more globotriaosylceramides (27). When permeabilized MSCs were stained with fluorescence-labeled CTB, AV, or ST, it was observed that CTB staining localized to CD81 in the plasma membrane or in punctate cytoplasmic distribution, AV staining was concentrated in the cytoplasm at the perinuclear area with little colocalization with CD81, and ST staining was limited to the nucleus. The cellular distribution of CTB-binding activity is consistent with its association with bona fide exosomes that originate from endosomes.

The subcellular binding activities of AV do not coincide with the distribution of phosphatidylserine in the plasma membrane, endosomes, and secretory vesicles [reviewed in (28)]. A possible reason for this discrepancy is that AV binding of phosphatidylserine is context dependent; that is, the presence of other phospholipids, such as phosphatidylethanolamine, could affect the binding of phosphatidylserine by AV (29). Another possible reason is that AV is polyvalent and the other locations of phosphatidylserines may not be enriched enough for polyvalent binding by AV. Although the subcellular distribution of AV binding sites do not coincide with the endosomal pathway, the presence of a low level of exosomal markers in AV-EVs suggests that biogenesis of AV-EVs may involve endosomes, and that AV-EVs may be a subpopulation of exosomes.

In contrast to CTB and AV binding, ST binding sites were localized to the nucleus. While this nuclear localization is consistent with ST-EVs’ cargo of RNA and an enrichment of pre-miRNA (8), the mechanism for the biogenesis of ST-EVs with ST-EVs’ cargo of RNAs and the enrichment of the extracellular matrix protein is at present not known. There is evidence of vesiculation in the nucleus, particularly in the transport of riboprotein complexes and viruses out of the nucleus (30). It is conceivable that the viruses are then subsequently released out of the cell. While it is possible that the biogenesis of ST-EVs follows a similar pathway as nuclear vesiculation of viruses, there is no evidence to support this yet.

The differences in cargo load, membrane lipids, and the subcellular distribution of EV membrane lipids strongly suggest that the three EV types, CTB-, AV-, and ST-EVs, are unique EV types. However, this should be confirmed directly through methods such as single EV analysis of double- or triple-labeled EVs.

**PHYSIOLOGICAL RELEVANCE OF CTB-, AV-, AND ST-EVs**

The physiological relevance of the three EV subtypes to MSC biology has not been elucidated yet. Part of the challenge is the isolation of the EV subtypes away from the
lipid-binding ligand for further characterization. The strong affinity between the lipid and lipid generally precludes the disruption of this bond without breaking the EV. While there is preliminary evidence that other cell types also secrete EVs exhibiting similar affinity for the three lipid binding ligands and have similar subcellular binding sites for the three lipid binding ligands (22), the relatedness of each EV subtype from different cell types has not been investigated yet.

The discovery of three EV subtypes in MSC secretion that could be differentiated from each other by their binding affinity for specific lipid binding proteins subsequently led to the discovery that EVs with similar binding affinities were also present in biological fluids such as plasma and ascites (31–33). The protein cargo is substantially different among the EV subtypes (31). It was also observed that the level of some proteins within each EV subtype differed between those from normal and diseased patients. Thus, each EV subtype from the plasma represents a unique source of disease biomarkers. Recently, it was reported that TIMP metalloproteinase inhibitor 1 (TIMP-1) in plasma CTB-EVs and plasminogen activator inhibitor-1 (PAI-1) in plasma AV-EVs complement plasma placental growth factor (PIGF) in predicting preeclampsia in an 843-patient cohort of a low-risk obstetric population with a strong predictive value, as measured by a combined area under the receiver operating characteristic curve score of 0.96 (32). Matrix metalloproteinase 9 (MMP-9) in the plasma of serous ovarian cancer was reported to preferentially localize in ascites AV-EVs (33). Together, these reports demonstrate that EV subtypes in biological fluids have different cargos, and as such, different EV subtypes may have a different biological function.

CONCLUSIONS

The lipid membrane of an EV not only defines and limits the physical structure of the EV, it is also integral to EV biogenesis and cargo loading. As reviewed here, differences in the membrane lipid composition of similarly sized EVs can denote a difference in biogenesis, cargo content, and functions. As seen in EV subtypes from biological fluids of healthy and sick patients, EV cargo content is also dependent on the physiological state of the producing cells. Therefore, small EVs, including those described as exosomes, are an essentially heterogenous mix of EV types. This has significant implications for exosome/EV research. It is commonly assumed in the field that the components in an exosome/EV preparation are evenly distributed in each of the EVs in the preparation. Given that most EV preparations are inevitably a heterogenous mix of different EV types (4), the components in an exosome/EV preparation are differentially distributed among the different EV types present in the preparations. Therefore, it is imperative that these components must first be properly segregated to the appropriate EV types, as this is most fundamental to any attempts to elucidate the biogenesis or mechanism of action of any exosome/EV preparation. For example, the absence of RNA in MSC CTB-EVs, which are shown to be bona fide exosomes, demonstrates that an RNA cargo may not be a property of exosomes. Therefore, the loading of RNAs into EVs may not involve lipid rafts. Instead, the presence of RNA in ST-EVs suggests that RNA loading into EVs may involve nuclear vesiculation.

However, the elucidation of the biological function of each of these EV types has been technically very challenging, as the binding affinities of CTB, AV, and ST for the EVs are very high such that isolation of each EV type away from the binding ligand for biological investigations or even protein quantitation is impossible. Presently, efforts to analyze these EVs using single EV analytical techniques are underway.

REFERENCES

raft microdomains in the plasma membrane. *J. Extracell. Vesicles.* 2:
doi:10.3402/jev.v2i0.22614.

cytosis of transferrin and recycling of the transferrin receptor in rat

17. Pan, B. T., and R. M. Johnstone. 1983. Fate of the transferrin re-
ceptor during maturation of sheep reticulocytes in vitro: selective

2006. TfR2 localizes in lipid raft domains and is released in exo-
somes to activate signal transduction along the MAPK pathway. *J.


102: 4336–4344.

MUC1-containing lipid rafts from plasma membranes and exosomes

least 3 EV types each with a unique permutation of membrane lipid,

23. Zanetti, C., A. Gallina, A. Fabbri, S. Parisi, A. Palermo, K. Fecchi,
propagation of cholera toxin CTA ADP-ribosylating factor by exo-

ing to phospholipids: stoichiometry, specificity, and the role of neg-

25. Sopkova, J., M. Renouard, and A. Lewit-Bentley. 1993. The crystal
structure of a new high-calcium form of annexin V. *J. Mol. Biol.* 234:
816–825.

26. Merritt, E. A., S. Sarfatty, F. van den Akker, C. L’Hoir, J. A. Martial,
bound to receptor GM1 pentasaccharide. *Protein Sci.* 3: 166–175.

27. Ling, H., A. Boodhoo, B. Hazes, M. D. Cummings, G. D. Armstrong,
J. L. Brunton, and R. J. Read. 1998. Structure of the Shiga-like toxin
I B-pentamer complexed with an analogue of its receptor Gb3.


Bosce, C. Whittle, B. G. Klupp, C. A. Siebert, D. Vasishlan, F. J.
Bauerlein, et al. 2015. Structural basis of vesicle formation at the

2014. Plasma biomarker discovery in preeclampsia using a novel dif-
ferential isolation technology for circulating extracellular vesicles.

32. Tan, K. H., S. S. Tan, M. J. Ng, W. S. Tey, W. K. Sim, J. C. Allen, and
S. K. Lim. 2017. Extracellular vesicles yield predictive pre-eclampsia

33. Reiner, A. T., S. Tan, C. Ageriter, K. Auer, A. Bachmayr-Heyda,
EV-associated MMP9 in high-grade serous ovarian cancer is pref-
entially localized to annexin V-binding EVs. *Dis. Markers.* 2017:
9653194.