Microreview

Exosomes and other microvesicles in infection biology: organelles with unanticipated phenotypes

Judith Maxwell Silverman\textsuperscript{1,2,3} and Neil E. Reiner\textsuperscript{1,2,3}\textsuperscript{*}

\textsuperscript{1}Departments of Medicine (Division of Infectious Diseases) and \textsuperscript{2}Microbiology and Immunology, University of British Columbia, Vancouver, BC, Canada. \textsuperscript{3}The Immunity and Infection Research Centre, Vancouver Coastal Health Research Institute, Vancouver, BC, Canada.

Summary

The release of exosomes and other microvesicles by diverse prokaryotic and eukaryotic cells and organisms was first appreciated early in the 20th century. The functional properties of these organelles, however, have only recently been the focus of rigorous investigation. In this review, we discuss the release of microvesicles of varying complexity by diverse microbial pathogens. This includes vesicle secretion by Gram-negative bacteria, eukaryotic parasites of the kinetoplast lineage and opportunistic fungal pathogens of both the ascomycetes and basidiomycetes lineages. We also discuss vesicle release from mammalian cells brought about as a result of infection with bacteria, viruses and prions. In addition, we review the evidence showing that in their specific microenvironments, release of these organelles from diverse pathogens contributes to pathogenesis. Germane to this and based upon recent findings with \textit{Leishmania}, we propose a model whereby exosome release by an intracellular pathogen serves as a general mechanism for effector molecule delivery from eukaryotic pathogen to host cell cytosol. These new findings linking exosomes and other microvesicles to infection biology have important implications for understanding the immune response to infection and for the design of research strategies aimed at the development of novel therapeutics and vaccines.

Introduction

Secretion of effector molecules by microbial pathogens has long been theorized to be a keystone of pathogenesis and many examples supporting this argument have been described (Nandan \textit{et al}., 2002; Marti \textit{et al}., 2005; Reiner, 2005; Gomez \textit{et al}., 2009). Recent advances in high throughput mass spectrometry have allowed for detailed secretome analyses of a wide variety of single celled pathogens (recently reviewed in Ranganathan and Garg, 2009). These proteomic studies have contributed greatly to our understanding of strategies used by specific pathogens and have generated a wealth of data from which to explore pathogenesis in greater detail. Moreover, the remarkable diversity of secreted proteins found in these studies highlights the extraordinary complexity of infection. For example, secretomes of various pathogens as well as non-pathogenic mammalian cells (Thery \textit{et al}., 2009) have been found to vary tremendously in the functionality and known subcellular locale of the identified proteins. These include structural proteins, proteins involved in translation, enzymes involved in fatty acid metabolism, and DNA packaging proteins to name just a few. At first the great diversities of the secretomes studied raised doubt about the reliability of the results. Concerns centred around potential contamination by cell lysis during sample collection, and limitations in mass spectrometry were blamed as sources of artefact. However, to date at least 20 independent secretome proteomic analyses of bacterial, fungal and protozoan pathogens have been published, all of which have displayed this wide variation in protein identities and function. While technical limitations should never be completely discounted, it seems unlikely that proteins identified as secreted by multiple approaches in both closely and distantly related organisms, as has been done, can be explained only by artefact (Hiller \textit{et al}., 2004; Galka \textit{et al}., 2008; Silverman \textit{et al}., 2008; Ranganathan and Garg, 2009).

As a result of these studies, we are currently in the exciting position to consider that every identified secreted protein may potentially be involved in pathogenesis. This may involve unanticipated multifunctionality, perhaps influenced by the specific micro-environment dictating
entirely novel functions such as epigenetic inhibition of host defence, scavenging of required nutrients, or activation of pro-pathogen gene transcription. However, identification of secreted proteins alone is not enough to fully understand the infection biology of a pathogen. Extensive work with bacterial pathogens has proven that the mechanism of secretion must also be known before a holistic understanding of the infection can emerge. Moreover, the mechanism of secretion may be even more important from the standpoint of drug design and creation of novel therapeutics.

Unlike the well-defined bacterial secretion systems (types I–IV), general mechanisms of secretion from eukaryotic pathogens have only just begun to be appreciated. In this article we review recent work suggesting that secreted vesicles are a major mechanism of protein secretion from eukaryotic pathogens. Further, we discuss the potential roles played by diverse pathogen-derived or pathogen-controlled vesicle release in infection biology (see Table 1). This includes the roles of these vesicles in effector molecule delivery to host cells, as has been observed for *Leishmania donovani* infected cells and our current understanding of the immunomodulatory effects of pathogen-derived vesicles.

**Secreted membrane vesicle formation by pathogens**

The release of membrane vesicles is now widely considered to be an essential aspect of eukaryotic (reviewed extensively in Simpson *et al.*, 2008 and Thery *et al.*, 2009) and prokaryotic cell biology (reviewed in Ellis and Kuehn, 2010). The release of vesicles by eukaryotic pathogens was recognized first in the middle of the 20th century; however, it was thought that these vesicles found in culture supernatants were merely the result of mechanical cell death or released by apoptotic cells, and only recently has it become clear that microvesicle release is a ‘real’ biological event, which occurs throughout the lifecycle of these organisms. In all cell types, secreted membrane vesicles are formed either at the plasma membrane or in the lumen of various subcellular organelles. Due to the mechanism of their biogenesis, both types of vesicles contain cytosol and display the extracellular plasma membrane leaflet (which is also the luminal leaflet of the intracellular vesicles) on their outer surface. That these vesicles are oriented in the same manner as the cell is undoubtedly important in determining and interpreting their function and the mechanisms involved.

Currently, vesicles secreted by unicellular and lower multicellular eukaryotes fall into two broad categories: exosomes and secreted vesicles. Other categories have been described for mammalian cells (Thery *et al.*, 2009), and whether or not these are also generated by lower eukaryotes remains to be seen.

**Exosomes**

Exosomes are formed within endosomes by invagination of the limiting membrane, resulting in the formation of multi-vesicular bodies (MVB). Exosomes are released into the extracellular space upon fusion of the MVB with the plasma membrane. They are 50–100 nm in diameter and are of a specific density. Only two single-celled eukaryotes have been shown to release *bona fide* exosomes thus far: the pathogenic fungus *Cryptococcus neoformans* (Rodrigues *et al.*, 2008), and the protozoan parasites *Leishmania donovani* and *Leishmania major* (Silverman *et al.*, 2010a). Notably, in the context of infection biology, exosomes have also been shown to be released by mammalian cells infected with bacteria, infectious prion protein and viruses (Nguyen *et al.*, 2003; Bhatnagar *et al.*, 2007). For mammalian cells, various proteins have been shown to be involved in exosome secretion from specific cell types, but as yet very little is known about general exosome secretion. The molecules that have been shown (in specific cell types) to be involved in exosome biogenesis and secretion include Hrs (an ESCRT-0 protein), Rab11, Rab27, ceramide and Rab35 (Savina *et al.*, 2005; Trajkovic *et al.*, 2008; Hsu *et al.*, 2010; Ostrowski *et al.*, 2010; Tamai *et al.*, 2010). Although the role of these proteins in single-celled eukaryote exosome formation and release is unclear, homologues of at least a subset of these proteins (ESCRTs, Rab11 and Rab27) were detected in *C. neoformans* and leishmania exosomes (Rodrigues *et al.*, 2008; Silverman *et al.*, 2010a). Moreover, HSP100 was found to play a role in leishmania exosomal packaging (Silverman *et al.*, 2010b). Similarly, targeting of Sec6 with RNAi resulted in production of

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**Table 1.** Infectious disease where secreted vesicles play a demonstrated role.

| Disease agent | Disease | Function of exosomes | References |
|--------------|---------|----------------------|------------|
| *Leishmania* spp. | Leishmaniasis, AIDS | Delivery of virulence factors to host cells | Silverman *et al.* (2010a) |
| HIV | | Trans-infection of CD4+ T cells, delivery of Nef to bystander cells | Lenassi *et al.* (2010) |
| Prion protein | Transmissible spongiform encephalopathies | Delivery of virulent proteins | Fevrier *et al.* (2004) |
| *Cryptococcus neoformans* | Cryptococcosis | Secretion of virulence factors, generation of polysaccaride capsule | Rodrigues *et al.* (2008); Oliveira *et al.* (2010) |
Exosomes in infection biology

Secreted membrane vesicles

In contrast to the intracellular biogenesis of exosomes, secreted membrane vesicles are derived by budding or shedding from the plasma membrane and are known to be heterogeneous in size and density. Ascomycota opportunistic fungal pathogens, including Histoplasma capsulatum, have been shown to release membrane vesicles of varying size into culture medium (Albuquerque et al., 2008). This study included extensive controls against contamination of the sample by dead and lysed cells, thereby validating the conclusion that fungi secrete membrane vesicles exhibiting size heterogeneity (Albuquerque et al., 2008). Similarly, membrane vesicles containing parasite antigens have been pelleted from conditioned media of Trypanosome cruzi (Goncalves et al., 1991) and ultrastructural analysis demonstrated shedding of vesicles from the flagellum in vitro (Trocoli Torrecilhas et al., 2009). It appears that a fraction of the T. cruzi-secreted vesicles are of the same size and density as exosomes (Geiger et al., 2010), but more in-depth analysis is needed before a definitive identification can be made.

In contrast to trypanosomatids, ascomycetes and basidiomycetes, no evidence supporting exosome or membrane vesicle release into culture filtrate by members of the Apicomplexa (Toxoplasma ssp. and Plasmodium ssp.) or any other eukaryotic pathogens has been presented to date. Determining whether additional eukaryotic pathogens release membrane vesicles, exosomes or other vesicular structures requires further investigation.

Modification of secreted vesicles in response to the environment

In Gram-negative bacteria, outer membrane vesicle (OMV) production is modified by environmental stressors and is in some cases thought to be required for surviving stress. Notably, ultrastructural analysis has shown that vesiculization by bacterial pathogens is upregulated by exposure to host factors and tissues (Ellis and Kuehn, 2010). Elegant work with Legionella has shown that OMVs are regulated along with cell stage development and suggest that legionella OMVs are involved both in inhibiting the fusion of bacteria containing phagosomes with lysosomes and in inducing bacterial differentiation into the intracellular replicative form (Galka et al., 2008). These studies with bacteria suggest that environmental factors may also influence secretion of eukaryotic microvesicles, and our work showing modification of leishmania exosomes in response to conditions mimicking early infection supports this hypothesis.

Leishmania are capable of modifying their exosome release in response to their environment. For example, vesicle release was upregulated threefold per cell in response to heat shock (Silverman et al., 2010a). In addition, the cargo of leishmania exosomes was found to be modified in response to both increased temperature and lowered pH, environmental conditions encountered early in mammalian infection (Silverman et al., 2010a). It is unknown if similar regulation of exosome/microvesicle release occurs with other eukaryotic pathogens. However, considering the conserved mechanisms of biogenesis for these organelles and the similarity of the environmental stimuli, it seems likely that changes in the protein cargo of secreted vesicles occur for other pathogens, depending upon ambient conditions.

Functional properties of secreted vesicles in vitro and in vivo

Vesicle cargo and associated virulence factors

Concentration of virulence factors via packaging in vesicles offers a potentially more potent mechanism for delivery of effectors to host cells, when compared with simple diffusion. In this section we review what is known about the cargo of pathogen-derived vesicles, and speculate as to their potential interactions with host cells. For information concerning the cargo of bacterial OMVs see Ellis and Kuehn (2010) for an excellent review.

So far, more than 300 proteins have been identified as cargo of vesicles secreted by eukaryotic pathogens (Albuquerque et al., 2008; Rodrigues et al., 2008; Silverman et al., 2010a) (Fig. 1). Each of these proteomic analyses described vesicular heat shock proteins (70, 90, 60 and 100) and superoxide dismutase as some of the proteins considered most likely to be involved in virulence. Several unique compounds associated with virulence were identified in C. neoformans exosomes, including laccase, urease, glucosylceramides and the capsular polysaccharide glucuronoxylomannan (Rodrigues et al., 2007; 2008). Leishmania exosomes were found to contain soluble candidate virulence factors elongation factor-1α (EF-1α) (Nandan et al., 2002) and aldolase (Nandan et al., 2007).

All the secreted vesicles analysed so far contained transmembrane small ion channels and transporters (Fig. 1). In addition, C. neoformans exosomes contained Hmp1, a transmembrane protein that may be involved in adhesion to target cells (Rodrigues et al., 2008). Leishmania exosomes were found to contain other well-documented membrane virulence factors, such as

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membrane-bound secreted acid phosphatase (SAcP) and the metalloprotease GP63 (Silverman et al., 2010a). Both of these proteins exist in two forms, either GPI-anchored to the plasma membrane or not, resulting in a soluble version known to be released from the cell. It is not currently known which form exosomal SAcP and GP63 take in exosomes, although it seems likely that these vesicles present both forms of each protein. GP63 has many immune-modulating properties including activation of host negative regulatory pathways (Gomez et al., 2009), direct modulation of host signalling (Halle et al., 2009) and resistance to complement-mediated lysis (Brittingham et al., 1995), to name just a few. Leishmania exosomes also contain kinetoplast membrane protein 11, a known T cell antigen, which has also been shown to have immunosuppressive properties (Carvalho et al., 2005; Peruhype-Magalhaes et al., 2006). Given that leishmania exosomes contain lipophosphoglycan synthesis protein 2, it is also possible that lipophosphoglycans are on the surface of exosomes, although this remains to be examined directly.

To date, no proteomic analysis of trypanosome secreted vesicles has been published; however, the secretome of T. cruzi has been analysed and shown to overlap with the leishmania secretome (Geiger et al., 2010). This suggests that their secreted vesicles may have similar proteomes as well. It is known that trypanosome secreted vesicles display α-galactosyl-containing glycoconjugates and glycoproteins of the gp85/trans-sialidase (TS) superfamily on their surface (Trocoli Torrecillas et al., 2009). These compounds are known to be involved in parasite adhesion to host cells and may play a similar role when located on secreted vesicles (Yoshida and Cortez, 2008).

In the only large-scale lipidomic analysis of eukaryotic pathogen-secreted vesicles published thus far, positive-ion mode analysis of histoplasma secreted vesicles revealed mainly phosphatidylethanolamine, phosphatidylserine (PS) and phosphatidylcholine as the major phospholipid species (Albuquerque et al., 2008). It is clear from these studies that vesicles secreted by eukaryotic pathogens present immunomodulatory compounds on their surface as well as intraluminally; however, sorting out the functional relevance of these compounds will require additional study.

**Potential vesicle interactions with host cells**

There are a number of potential mechanisms by which secreted vesicles might interact with host cells.
mammals, it has been found that exosomal PS can be bound by T cell immunoglobulin domain and mucin domain proteins 1 and 4 (TIM1 and TIM4) expressed, respectively, on activated lymphocytes or phagocytes (Miyanishi et al., 2007). It is tempting to speculate that eukaryotic pathogens mediate uptake of their secreted vesicles by targeting immune cells via binding of vesicular PS to TIM4. Similarly, since the membrane orientation of secreted vesicles is the same as that of whole cells, the vesicles might adhere to host cells through the same molecular interactions that mediate binding of the pathogen itself. For example, leishmania is known to bind to CR3 and mannose receptor to initiate phagocytosis, and it is possible that similar receptor–ligand interactions mediated ingestion of leishmania exosomes. In support of this, it was recently shown that phagocytic cells preferentially phagocytose exosomes (of mammalian origin), despite their small size (Feng et al., 2010). In the case of eukaryotic pathogens, the only evidence in support of vesicle uptake comes from work with fluorescently labelled leishmania exosomes, which were engulfed by macrophages via an apparently actin dependent mechanism (Silverman et al., 2010a). In addition to binding and phagocytosis, it is both possible and likely that pathogen-secreted vesicles fuse with the plasma membrane of their target cells, releasing their contents into the cytosol of the host. To date, there is no evidence for or against the latter hypothesis, but it seems likely that both engulfment and fusion occur in vivo.

**Immunomodulatory activities of pathogen-derived secreted vesicles**

Mammalian exosomes have been shown to have both pro- and anti-inflammatory properties, largely depending on the cell type of origin (Thery et al., 2009). Results with eukaryotic pathogen-derived vesicles have been similar. Vesicles secreted by *C. neoformans* have been shown to enhance the secretion by macrophages of various immune modulators including tumour necrosis factor-α (TNF-α), interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) (Oliveira et al., 2010). Moreover, vesicle-treated macrophages produced significantly greater amounts of nitric oxide and were more efficient at killing *C. neoformans* than were untreated macrophages.

In contrast, studies with leishmania exosomes have shown these vesicles to inhibit pro-inflammatory cytokine responses (TNF-α) while promoting anti-inflammatory cytokine production (IL-10) by human monocytes after infection and activation with interferon-γ. In addition, exosome treatment was generally inhibitory for human monocyte-derived dendritic cells (DCs), both in terms of cytokine production and expression of the maturation marker HLA-DR (Silverman et al., 2010b). These results suggested a model where by leishmania exosomes are anti-inflammatory (Fig. 2). Further investigation using a leishmania mutant that produces exosomes lacking specific cargo showed this anti-inflammatory phenotype to be cargo dependent. Thus, when monocytes and DCs were incubated with mutant exosomes, the vesicles showed a gain-of-function pro-inflammatory phenotype boosting cytokine secretion (Silverman et al., 2010b). In addition, when exosome-treated DCs were co-cultured with naïve T cells, mutant exosome treatment resulted in a 100% increase in the number of IFN-γ producing CD4 T cells as compared with untreated DCs and DCs treated with wild-type leishmania exosomes.

Of special interest is to develop an understanding of the properties of these vesicles in vivo, and a few studies have attempted to address this question. Treatment of resistant C57Bl/6 and susceptible Balb/c mice with, respectively, wild-type *L. donovani* and *L. major* exosomes prior to challenge with homologous organisms resulted in increased parasite load and exacerbated disease progression, suggesting a pro-parasitic role for these vesicles in vivo (Silverman et al., 2010b). Consistent with these findings, treatment of mice with trypanosome-derived secreted vesicles prior to challenge with *T. cruzi* augmented parasite loads in the heart while at the same time inducing a massive inflammatory response (Trocoli Torrecillas et al., 2009). These findings indicate that kinetoplastid-secreted vesicle exosomes are capable of biasing the immune response to make it permissive for infection, perhaps through their generally inhibitory effects on monocytes, macrophages and MoDCs (Fig. 2). Unravelling the role of these secreted vesicles in vivo during infection will require in-depth analysis, but these initial studies are provocative and suggest a path forward.

**Vesiculation observed during infection**

The existence of bacterial OMVs in infected tissues has been well documented (Ellis and Kuehn, 2010). In contrast, observation of vesicle secretion by eukaryotic pathogens during infection has only recently been demonstrated, and is currently limited to exosome release by *L. donovani* (Silverman et al., 2010a) and Maurer’s cleft secretion by *Plasmodium falciparum* (Bhattacharjee et al., 2008). We have shown that leishmania release vesicles into the cytoplasm of infected cells, which accumulate over time (Silverman et al., 2010a). Furthermore, to date all the proteins that have been shown to translocate into the cytosol of leishmania-infected macrophages (Nandan et al., 2002; 2007; Gomez et al., 2009; McCall and Matlashewski, 2010; Silverman et al., 2010a) were shown by to be cargo of leishmania exosomes (Silverman et al., 2010a). Taken together, these findings suggest that
exosomes are a major pathogen-to-host delivery mechanism during leishmania infection.

Despite the lack of data supporting vesicle release by *Plasmodium ssp.* in culture, it has been shown that *P. falciparum* synthesizes and packages Maurer’s clefts within the parasite cytoplasm. These vesicular structures are subsequently exported de novo and are found within the cytoplasm of infected erythrocytes (Bhattacharjee *et al.*, 2008). The data suggest that host-targeting secretion signal-tagged proteins are preferentially exported into host cytosol from Maurer’s clefts, as opposed to direct translocation across the parasitophorous vacuole membrane (Bhattacharjee *et al.*, 2008). Maurer’s clefts therefore may be seen to function as secretory vacuoles. It is tempting to speculate that this sophisticated signal-targeted secretion system is not simply analogous to the leishmania-to-host exosome secretion pathway, but may be an example of higher-order secretion that has evolved from a more primitive exosome-based pathway.

**Exosomes released by infected host cells contain pathogen-derived virulence factors and affect immune responses**

While release of vesicles by eukaryotic pathogens during infection has only recently been described as a potential virulence strategy, release of vesicles from infected host cells has been recognized for some time to be a common phenomenon (Bhatnagar *et al.*, 2007). In some cases, this has been thought to be beneficial to the host and other times beneficial to the pathogen. Work on exosomes released from *Mycobacterium tuberculosis* or *M. bovis* infected macrophages demonstrated that these exosomes contained pathogen-derived antigens and the vesicles induced pro-inflammatory cytokine production by naïve cells (Bhatnagar and Schorey, 2007). Notably, this phenomenon was not limited to infection by bacterial pathogens since *Toxoplasma gondii*-infected macrophages released exosomes with similar properties (Bhatnagar *et al.*, 2007). Furthermore, exosomes released from *M. bovis*-infected macrophages were shown to activate antigen-specific CD4+ and CD8+ T cells (Giri *et al.*, 2010). Together, the results from the mycobacterial system suggest that exosome release from infected macrophages activates pro-inflammatory pathways. Further investigation will be required to determine whether the release of vesicles from infected cells favours control and clearance of the infection by the host.

On the other hand, exosomes released by virally infected cells have been shown to have a number of deleterious effects on the host immune response and to favour spread of infection. For example, HIV-infected cells...
Exosomes as prophylactic or therapeutic vaccines

Pro-inflammatory exosomes released by DCs are potent antigen-presenting organelles, capable of activating T cells, with potential functionality as cell-free vaccines (Escudier et al., 2005; Morse et al., 2005). Exosomes secreted by DCs pulsed with Toxoplasma gondii and L. major antigens were found to be protective against challenge infection with, respectively, T. gondii and L. major (Aline et al., 2004; Beavillain et al., 2009; Schnitzer et al., 2010). These findings have ignited interest in ex vivo harvested DC exosomes as potential cell-free vaccines against various eukaryotic pathogens.

Conclusions

Although much is yet to be learned, the findings discussed in this review make it clear that exosomes play an important role in infection biology. This conclusion applies to organisms of varying complexity ranging from prions to eukaryotic pathogens. These vesicles may be either directly pathogen-derived or released from infected host cells. In either case, as relatively newly recognized secretory organelles, we are beginning to unravel their complex roles in intercellular communication, immune modulation and immune surveillance. Furthermore, we hope to have communicated our view that exosomes and other microvesicles are a common emerging paradigm during infection. This applies to a remarkably diverse array of organisms and vesicle dynamics including secretion, capture, engulfment and cargo delivery, which constitutes a new focus in infection biology. This new perspective has potential to inform infection disease research across kingdoms and may guide the development of novel therapeutics for a group of challenging and difficult-to-treat pathogens.

Acknowledgements

This work was supported by Canadian Institutes of Health Research Grants MOP-84582 and by a Small Projects Health Research Grant from the British Columbia Proteomics Network.

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