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Review

Blood/plasma secretome and microvesicles ☆

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ABSTRACT

A major but hitherto overseen component of the blood/plasma secretome is that of extracellular vesicles (EVs) which are shed from all blood cell types. These EVs are made up of microvesicles (MVs) and exosomes. MVs, 100 nm–1 μm in diameter, are released from the cell surface, and are a rich source of non-conventionally secreted proteins lacking a conventional signal peptide, and thus not secreted by the classical secretory pathways. Exosomes are smaller vesicles (≤100 nm) having an endocytic origin and released upon multivesicular body fusion with the plasma membrane. Both vesicle types play major roles in intercellular cross talk and constitute an important component of the secretome especially in the area of biomarkers for cancer. The release of EVs, which are found in all the bodily fluids, is enhanced in cancer and a major focus of cancer proteomics is therefore targeted at EVs. The blood/plasma secretome is also a source of EVs, potentially diagnostic of infectious disease, whether from EVs released from infected cells or from the pathogens themselves. Despite the great excitement in this field, as is stated here and in other parts of this Special issue entitled: An Updated Secretome, much of the EV research, whether proteomic or functional in nature, urgently needs standardisation both in terms of nomenclature and isolation protocols. This article is part of a Special Issue entitled: An Updated Secretome.

1. Introduction

Microvesicles are defined as intact, submicron, phospholipid-rich vesicles that are released from the cell membrane of a diverse range of cells upon activation, with for example growth factors or cytokines and/or during apoptosis [1]. Besides the term microvesicles, numerous other names have been ascribed to them including, microparticles, ectosomes, and plasma membrane derived vesicles. They also come in various sizes ranging from 0.1 to 2.0 μm [2].

Much of the proteomic research looking for biomarkers does not distinguish between the two main types of extracellular vesicle (EV) namely microvesicles and exosomes, or other non-EV 'soluble' proteins. It is important to note that many proteins deemed 'soluble' may in fact be on EVs because they are found from an analysis of acellular body fluids and it may not be readily apparent or researchers may not be aware that this component may have the very small EVs that are not removed at low centrifugation speeds. Galectin-3, for example, was identified as a low abundance protein in the cell culture medium of cells [3]. However we now know, as detailed in Section 3, that this is a protein lacking a signal peptide and is secreted by a non-conventional protein export mechanism [4].

2. Extracellular vesicles: MVs and exosomes

2.1. Biogenesis of microvesicles and exosomes

MVs are released from the plasma membrane following cell activation or during early apoptosis. A range of stimuli such as would be provided by cytokines or endotoxins or physical stimuli such as shear stress [5] can all result in microvesiculation. Upon cell activation there is a rise in the cytosolic calcium concentration leading to the activation of enzymes such as calpain, gelsolin and scramblase as well as certain protein kinases. Simultaneously, enzymes such as translocase, and phosphatases are inhibited, resulting in cytoskeletal reorganisation, loss of membrane asymmetry and membrane blebbing leading to MV formation and release [5]. There is also evidence that MVs are released constitutively as part of their normal physiology [6,7]. During apoptosis, the release of MVs is associated with membrane blebbing, which is a characteristic feature of programmed cell death (Fig. 1). Kinases that have been identified to play a role in membrane blebbing associated with microvesiculation include Rho associated kinase 1 (ROCK-1) [8,9]. The ROCK kinases are activated by GTP bound Rho and are important mediators of cytoskeletal modifications

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such as myosin light chain phosphorylation and actin myosin coupling to the plasma membrane. In experiments using mouse fibroblasts and human epithelial breast cancer cells when they were treated with a ROCK-1 kinase activity inhibitor (Y27632), there was a decrease in MV blebbing [9] suggesting that ROCK kinases do indeed play a role in MV formation during early apoptosis. Cleavage of ROCK-1 and MV release was also blocked by the pan-caspase inhibitor z-VAD-fmk, suggesting a role of caspases (involved in apoptosis) as well in the activation of ROCK-1 [9].

As mentioned earlier, cell activation and apoptosis cause an imbalance in the asymmetry of the cell membrane and as a result the MVs have a rich phospholipid bi-layer consisting of phosphatidyl serine (PS) on the outer leaflet. PS has become a dominant feature for identifying MVs and marker proteins are then generally used to identify from which cell type the MVs are derived [1,5].

In addition to MVs, eukaryotic cells also release another type of extracellular vesicle called exosomes. They are smaller than MVs, ranging in size from 30 to 100 nm in diameter, are coated with a lipid bi-layer and characteristically have a density ranging from 1.13 g ml^{-1} (for B-cell derived exosomes) to 1.19 g ml^{-1} (for intestinal-cell-derived exosomes) [10]. As for MVs, exosomes also carry particular protein markers and express certain surface receptors derived from their parental cells [10]. In terms of exosome biogenesis, a neutral sphingomyelinase (Smase) enzyme is the key controller of this process. Ceramide is produced by activation of neutral Smase which in turn hydrolyses sphingomyelinase on the cytosolic leaflet of the endosomal membrane. This creates a curvature resulting in an inward budding into the endosome (Fig. 1). The intraluminal vesicles resulting from the endosomes are contained inside multivesicular bodies (MVB) and are called exosomes upon their release, when the MVB fuses with the plasma membrane [10].

2.2. Role of extracellular vesicles as mediators of intercellular communication

Exosomes play a significant role in antigen presentation and in mediating death in target cells. Exosomal markers, found in all exosomes originating from various cell types include major histocompatibility complex (MHC) class I and II molecules and tetraspanins such as CD9, CD63, CD81 and CD82, which represent the most abundant protein families present in exosomes [11]. Tetraspanins all have four transmembrane domains and are distinguished by a number of conserved amino acids within the transmembrane domains. The ligands with which these receptors bind have not all been delineated but many are known to interact with integrins and MHC molecules. Exosomes are comprised of amongst other proteins, tubulin, actin, actin-binding proteins (in the cytoskeleton), annexin, and Rab proteins (involved

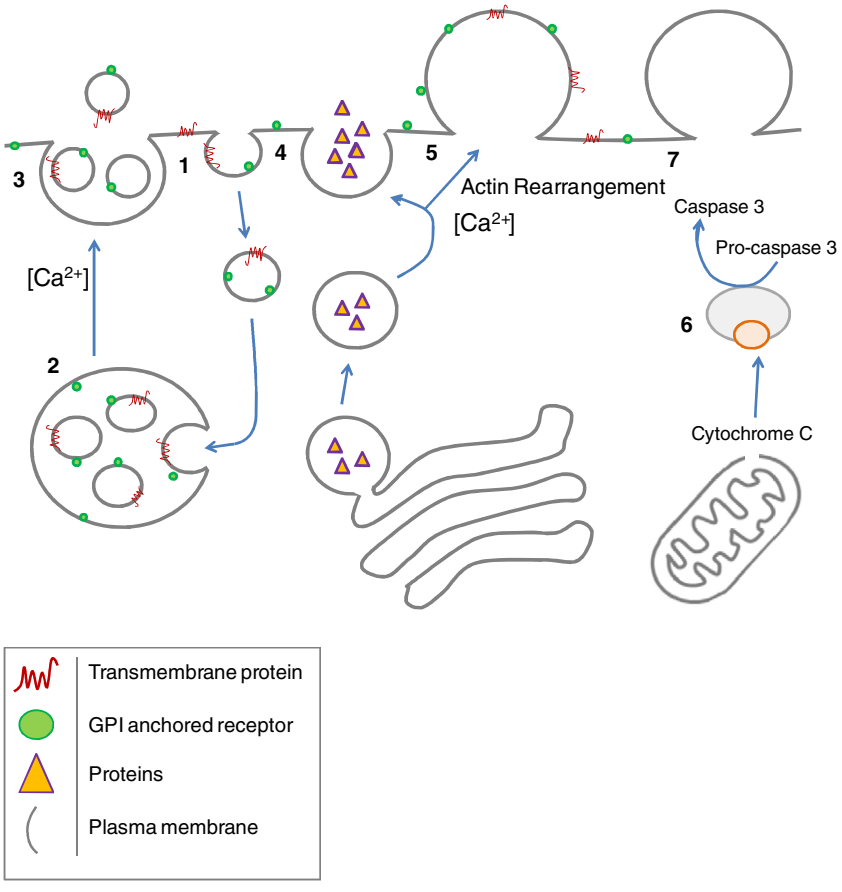


Fig. 1. An overview of extracellular vesicle types and their formation. 1. Endosomes form by an invagination of the plasma membrane. The vesicle thus contains redundant or defunct membrane associated proteins and lipid expressed internally, is either degraded by lysosomes (not shown) or fuses with a multivesicular body. 2. Multivesicular bodies are formed by the fusion of endosomes and other vesicles. They undergo a process of invagination to form a number of 3. Exosomes that express an assortment of membrane proteins (expressed externally), cytoplasmic inclusions such as RNA, peptide hormones and mitochondrial proteins. The MVB migrates to the plasma membrane and fuses, so externally releasing the exosomes into the extracellular space. 4. Vesicles 'pinch off' from the golgi body and traffic cargo (proteins, receptors, carbohydrates or lipid) to the plasma membrane or to organelles. 5. MVs arise from membrane blebs, containing a sample of the cytoplasm and membrane of the parent cell. Due to membrane instabilities, PS is expressed externally, actin rearrangement causing the inclusion of cytoskeletal components. 6. Apoptosome forms when mitochondrial damage leaks cytochrome C and ROS into the cytoplasm, the initiation of apoptosis leading to the formation of caspase 3 from procaspase 3. 7 Apoptotic Bodies are shed expressing external PS, cytoskeletal and cellular components, containing toxic substance and occasionally organelles.

in intracellular membrane fusion and transport) and many others [11–14]. Exosomes also harbour numerous molecules involved in signal transduction, including protein kinases and heterotrimeric G-proteins, various metabolic enzymes (*e.g.* peroxidases, pyruvate, lipid kinases and enolase-1) and heat-shock proteins such as Hsp70 and Hsp90. The latter are ubiquitous chaperone proteins, which are involved in antigen presentation, since they bind to antigenic peptides and participate in peptide loading onto MHC molecules [11].

Many reports have described MV-mediated intercellular cross talk including recent studies that have confirmed that release of MVs is associated with a transfer of antigens and receptors capable of initiating cell signaling [15,16]. MVs induce cell-to-cell communication by direct cell stimulation. This may take the form of either activation of a signaling complex, transfer of surface receptors to local cells or delivery of proteins and mRNA to target cells [17]. It is well documented that cells exchange information amongst themselves through various mechanisms such as release of growth factors, cytokines, chemokines and small molecular mediators such as nucleotides, nitric oxide free radicals and bioactive lipids [18]. It is now becoming apparent that many of these exchanges can be mediated by MVs. For instance, leukocyte MVs activate endothelial cells through the transfer of leukocyte antigens. This passive acquisition of leukocytic phenotypes is associated with changes in phosphorylation of cellular proteins and cell–cell adhesion properties [19]. Platelet-derived MVs modulate monocyte–endothelial cell interactions and stimulate proliferation, survival, adhesion, chemotaxis of haematopoietic cells, and also enhance engraftment of haematopoietic progenitor cells [20,21].

3. Secretomes as novel reservoirs of biomarkers

The term 'secretome' refers to a collection of soluble proteins that are secreted from an organism. They contain an N-terminal, hydrophobic signal peptide and are processed *via* the endoplasmic reticulum and Golgi apparatus before eventual secretion into the extracellular space. These proteins are secreted from the cell *via* the classical, non-classical or exosomal pathways [3]. Secretory proteins play a myriad of functions ranging from roles in the immune system, to acting as neurotransmitters in the nervous system. They may also be involved in building and maintenance of cell walls as well as in inter- and intra-cellular communication and signal transduction [22]. Some secretory proteins, it has been suggested, act as effectors on pathogens and by carrying motifs of host cells are rendered less noticeable by the host immune system [22].

As MVs carry proteins, amongst others, lacking a signal peptide, they mediate an unconventional or non-conventional form of protein export [23] as opposed to the typical protein export in eukaryotic cells, which is mainly *via* the ER/golgi-dependent transport or classical secretory pathway. Proteins which lack the N-terminal, hydrophobic, signal sequence, such as IL-1β and galectin-1, thus do not associate with the ER/golgi membrane translocation machinery and so do not possess ER/golgi-dependent post-translational modifications such as N-linked glycosylation. The proof that such proteins are secreted *via* 'unconventional protein export' was obtained empirically by treating cells with ER/golgi transport inhibitors such as Brefeldin A or nonesin which was found to have no effect on their export into the extracellular space [24].

The majority of unconventionally secreted proteins are normally localised in the cytoplasm [25] and there are four proposed mechanisms of 'unconventional protein export' described in the literature, two of which utilise extracellular vesicles [26]. The first mechanism involves fusion of MVBs with the plasma membrane. MVBs carry exosomes that are packed with cellular content and are released into the extracellular environment as MVBs fuse with the plasma membrane. Another mechanism involves the shedding of MVs into the extracellular space [27]. Export of Macrophage Migration Inhibitory Factor, MIF, Fibroblast Growth Factor, FGF-1 and galectin-3, Gal-3 from human monocytic leukaemia cells employs this mechanism as shown recently [4].

3.1. Proteomic profiling of the secretome

One of the aims of searching for novel biomarkers is to find novel therapeutic targets, not only in cancer but also in infectious disease. Commonly this involves a study of the differential expression of secreted proteins from cancer cells (and infectious agents). To this end there is currently a huge interest in the study of the 'cancer cell secretome,' for example of breast cancer or of gastrointestinal malignancies which shows that secretory proteins mediate intercellular communication leading to neoplasias. Such proteomic profiling of cancer cell secretomes has enabled the detection of novel biomarkers comprising components of the Extracellular Matrix (EM) and other molecules shed by human cell lines; this approach will eventually lead to novel and distinct therapies [3].

3.2. Stem cell secretome

Significant progress has been made recently regarding the analysis of secreted proteins from Amniotic Fluid Stem Cells (AFSC). These proteins have been shown to enhance vasculogenesis which is capable of creating a strong angiogenic response [28]. Proteomic analysis of such secreted proteins enabled the discovery of pro-angiogenic factors such as Vascular Endothelial Growth Factor (VEGF) and anti-angiogenic factors such as interferon-γ. The identification of key antigens, that reflect the phenotype of the parent stem cell, and help to explain their properties will increase their efficient isolation and clinical applications.

Other workers have focussed on the identification of molecules secreted in the early stages of differentiation of human mesenchymal stem cells towards adipocytes and osteoblasts [29]. Human multipotent adipose tissue-derived stem cell secretomes, a cellular model created in the laboratory, have shown a selection of proteins secreted through the non-classical pathway and exosome-derived intracellular proteins. Proteinases, protease inhibitors, metabolic enzymes, heat shock proteins/folding proteins and EM components have all been identified that characterise the structure during the early event of adipogenesis and osteogenesis.

3.3. Tissue secretome

Evaluating the 'tissue secretome' as opposed to the 'cell secretome' can be advantageous as some interleukins and secretory proteins are produced by various cell types of the same tissue, as in adipose tissue where many released proteins are released from cells other than adipocytes, including endothelial cells and macrophages [30]. However, the protein composition of a tissue culture is highly dependent on tissue culture techniques and the presence of peptides, peptide hormones and intracellular protein fractions are known contaminants of a 'tissue secretome' which can severely hamper the detection of secreted proteins. In the case of studies of the secretome from adipose tissue, as the material is put in culture, there is the problem of contamination with tissue-derived serum proteins and intracellular material exposed when tissue is cut and cells damaged [31] or through long periods in culture.

According to the results obtained *via* a 2D gel electrophoresis approach, proteins such as endoplasmin and cathepsin D were secreted by both adipocytes and macrophages [32]. Secretome transcriptomics also enabled the detection of different types of mRNA that are expressed in different stages of the same cell type. For example, the long pentraxin, PTX3, which appears to play a role in the regulation of resistance to pathogens and inflammatory reactions, shows an expression at the mRNA level in the stromal-fraction of adipose tissue but not in fully differentiated adipocytes.

Proteomic studies of the 'tissue secretome' are important because such studies will enable the introduction of novel drug delivery strategies for the treatment of many diseases such as breast cancer [33] or

from visceral adipose tissue which may help with diseases such as type 2 diabetes [30].

3.4. Pathogen-derived secretome

Pathogens, whether they are bacteria, viruses, protozoa or fungi, interact with host cells *via* proteins they secrete. As such, proteins are likely to interface between pathogen and host and are presumed to be involved in pathogenesis. Once more proteomics has advantages over genome sequencing as proteins (that might otherwise be missed) are secreted that lack the predicted amino-terminal signal peptide. The proteomic analysis of secretomes from *Schistosoma mansoni* [34], *Plasmodium falciparum* [35] and *H. pylori* [36] are amongst several studies defining secretome data for several human pathogens [37–44] confirming the importance of this information for understanding the host/pathogen interaction to enable effective vaccine design and development of therapeutic drugs. It should be noted that the secretome from infected cells, which is touched upon in Section 5.1, may also be important in the infection process.

Protein export in fungal species adopts signal peptide dependent and independent pathways. The 'Fungal secretome' plays a role in tip growth, photo reception, conidiation, sexual and asexual reproduction and pathogenesis. Advances in genome sequencing have brought about a rapid increase in sequenced fungal genomes which aid in the study of function and evolution of secretory proteins. As of 2010 there have been 235 genomes from 120 fungal/oomycetes species in the Comparative Fungal Genomic Platform [22]. Fungal genes have a high population of RXLX (EDQ) motifs that act as a host targeting signal and some fungal secretomes contribute to the recognition of pathogen by the host cell [45].

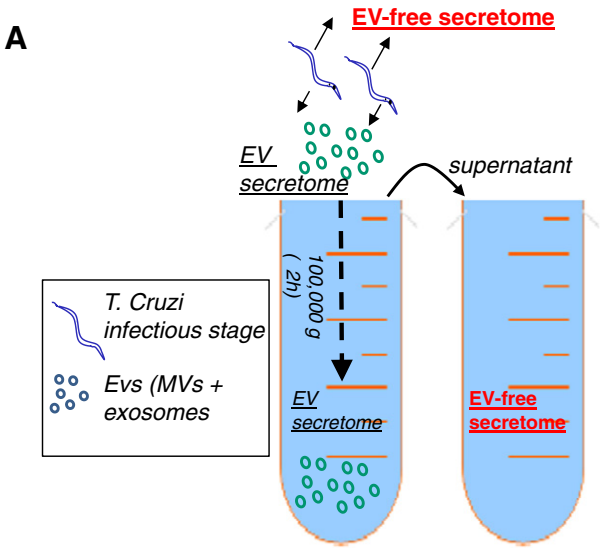
Legionella pneumophilia is an intracellular human pathogen that can cause a severe form of pneumonia but secreted effector molecules may also play a major role in extracellular pathogenicity [46]. Furthermore, secretion of Outer Membrane Vesicles (OMVs) contributes to intracellular pathogenicity which is signified by the inhibition of bacteriophages, altered host membrane traffic and growth of pathogen within phagocytes. Proteomic analysis of vesicle-mediated secretion thus aids in the understanding of virulence of such human pathogens and may lead to the identification of possible new drug targets or diagnostic biomarkers [47].

3.5. Analysis of secretomes must include microvesicles, exosomes and soluble proteins

As mentioned in the introduction, in the proteomic study of secretomes, the distinction between the two types of extracellular vesicle and soluble proteins is not often made, in any one study. However as the microvesicle field gains wider exposure and proteomic researchers have become aware of EVs, more precise analyses are being performed. For example, the recent secretome analysis of *Trypanosoma cruzi* [48] by Igor Almeida's team (summarised in Fig. 2) ensured that a vesicle-free fraction of the secretome (the supernatant from a 100,000 *g* centrifugation for 16 h) from epimastigote and metacyclic stages was analysed, as well as MV and exosomes. Such comprehensive analysis is necessary especially in the area of protozoan parasites where parasite EVs are believed to interact with host cells, even delivering virulence factors [49].

3.6. Exosomes, MVs and secretome

Extracellular vesicles which include MVs, exosomes and apoptotic blebs and which are released into the micro-environment from eukaryotic cells, can be regarded as membranous extracellular organelles [50]. Exosomes, membrane vesicles of endocytic origin that are shed by many cells including platelets, mast, dendritic and various cancer cells upon fusion of MVBs with the plasma membrane are secreted in disease conditions *in vitro* and in bodily fluids such as blood, urine, amniotic fluid and breast milk. They have pleiotropic biological



B

Enrichment on EVs or as soluble protein released from *T. cruzi* infectious forms

	Protein	EV secretome	EV-free secretome
Host-parasite interaction	*Surface protein GP63*	+	/
	Calreticulin	+	/
	Glycoprotein 82 kDa	+	/
	Cyclophlin A	/	+
Proteolysis and peptidolysis	*Aminopeptidase P*	+	/
	Carboxypeptidase	+	/
	calpain-like cysteine peptidase	/	+

Fig. 2. Proteomic study of *Trypanosoma cruzi* secretome [adapted from 48]. A, Schematic diagram showing the procedure for separating out by differential centrifugation *T. cruzi*-released Extracellular Vesicles (EVs) from EV-free supernatant by differential centrifugation. Data from proteins analysed from a second spin at 100,000 *g* for 16 h used to separate out smaller vesicles is not shown. The table in B shows proteins potentially involved in pathogenicity identified that are either enriched for on EVs or found in the EV-free supernatant (truly soluble proteins).

functions including that of antigen presentation, intercellular and intracellular communication, transfer of RNA and proteins and transport and propagation of infectious agents such as prions. Proteomic cataloguing of exosomes has revealed the importance of such vesicles in the diagnosis of tumour progression. and microRNA (miRNA) profiling is used as a diagnostic tool of disease which can thus also aid in the development of novel therapeutics.

MVs are also cell-derived membrane vesicles with a function in intercellular communication mainly in inflammation and cancer. Although several global proteomic studies of MVs have appeared, others have focussed on identifying the protein types found for example on the surface as opposed to within the MV. Such work showed that TGF-β1, that has a signal peptide mechanism for protein export is nevertheless predominantly expressed on the cell surface, whilst proteins such as MIF, FGF-1 and Gal-3 that lack a signal peptide, and so part of the non-conventional secretory process have an intravesicular location [4].

Recent research has suggested that both MVs and exosomes can be used in the drug delivery field as they are capable of delivering

molecules such as mRNA and miRNA that change the phenotype of the recipient cells [51]. Therefore, cell-derived membrane vesicles loaded with therapeutic compounds are engineered to target certain cell types and some of the characteristics of such vesicles are used to design nano-scaled drug delivery systems.

3.7. Secretome analysis

Proteomic analysis is becoming a viable alternative to determining gene expression on a large scale for example by mRNA expression-based measurements. Measuring protein levels enables the direct analysis of extracellular components of cells and is highly attractive in spite of the added experimental complexity.

3.7.1. Secretome sample preparation

The selection of a suitable cell sample is essential as it is the determining factor for the composition of the cell secretome and together with later steps will result in an effective proteomic analysis. Many researchers in proteomics have used different methods to avoid cross contamination with the aim of providing healthy cells grown in a suitable nutrient-enriched culture [52,53]. In short, using appropriate media such as Foetal Bovine Serum (FBS) and introducing extra steps of washing, longer incubation intervals and centrifugation at different speeds, has enabled the collection of healthy cells for secretome analysis.

In an interesting approach for the analysis of secretory proteins, metabolic labeling of proteins was carried out for brief time periods, followed by collection and filtration of cell supernatants and then protein precipitation and subsequent protein analysis [54]. Fluorography and autoradiography are both used as detection methods to increase reliability.

Whilst the wide use of biochemical techniques have contributed to the high output of such studies, confusion about isolation strategies of MVs, exosomes and other generally soluble proteins has led to misinterpretation of results. MVs are found to be denser than exosomes so purification of MVs requires centrifugation at a lower speed than for the isolation of exosomes. Furthermore, most isolation techniques have the inherent flaw of co-purifying EV sub-types.

The study by Inal and co-workers [4] used a MV isolation procedure in which the conditioned medium in which cells were maintained was centrifuged at 160 *g* for 5 min to remove cell debris and for a further 90 min at 25,000 *g* to obtain the MVs. Importantly, this work then confirmed that the isolate consisted of MVs whereas numerous other studies have confused MVs and exosomes. Where serum for example is centrifuged at >100,000 *g* for >1 h, then clearly exosomes (as well as MVs) have been isolated. Such misinterpretations show that the confusion between different strategies of isolation [55] and the use of incorrect terminology [23] can mislead the readers.

Secreted proteins are highly diluted in conditioned medium so to obtain reliable results it is very important to be able to use additional methods to extract secretory proteins such as tc2 reversed phase sorbent [52] to provide significant enrichment of genuinely 'secreted' proteins. Phenol extraction and TCA precipitation of secreted proteins will also lead to more satisfactory results following subsequent analysis by 2D-gel electrophoresis and addition of sodium deoxycholate or sodium lauroryl sarcosinate (from 0.01 to 0.1%) was found to play a key role in the efficiency of this precipitation method [53].

Employing this technique in other studies on bladder cancer has resulted in high precipitation yields and enrichment of low abundant secretory proteins such as cytokines [3]. Sucrose density gradient fractionation followed by centrifugation is used largely in combination with precipitation using chloroform/methanol as a method of isolation of membrane vesicles [56].

3.7.2. Proteomic analysis of the secretome

A variety of proteomic methodologies have been utilised for secretome analysis including 1D, 2D electrophoresis and liquid chromatography coupled with differential mass spectrometry analysis.

Cell-surface biotinylation, and then Western blotting as well as ELISA techniques have been used in an investigation of the role of exosomes in the ectodomain shedding of intracellular proteins in ovarian carcinoma cells [56]. With regard to exosomes and MV isolation and extraction and analysis of proteins, a whole range of different techniques have been adopted to increase the validity of results obtained including Western blotting, Fluorescence-activated cell sorted (FACS) analysis, nano LC-MS-MS, immunoelectron microscopy, 1D and 2D gel electrophoresis and MALDI-TOF-MS.

Each of the methodologies mentioned in this section has its own advantage and disadvantage in relation to quantification, reproducibility, identification of differentially expressed proteins, robustness and cost effectiveness. However, recent studies suggest the need of at least two separate techniques in proteomic analysis which will provide us with unprecedented insight into the structure and function of the secretome.

4. Extracellular vesicles and cancer diagnostics

4.1. Exosomes and microvesicles in developing a malignancy

Extracellular vesicles and cancer diagnostics is a highly topical area because as well as the known secreted and shed biomarker proteins, this area now importantly includes miRNA, many of which are likely to be found in EVs. Exosomes have currently been isolated from bodily fluids such as amniotic fluid, breast milk, malignant ascites, urine and saliva [3]. The exacerbated release of exosomes in tumour cells at later stages of the disease and the over expression of exosomes in different types of malignancies led to the use of such exosomal associated proteins as novel biomarkers in the diagnosis of cancer.

Just as with exosomes, MVs also play an important role in cancer diagnostics, elevated levels of MVs having been found in the blood from cancer patients [57]. Various cells release MVs as a defense mechanism against intracellular stress with apoptotic enzymes such as Caspase 3 [58]. Inhibiting MV release leads to the accumulation of caspase 3 in cells which then undergo apoptosis. Another study also suggests that MVs from chemo-insensitive cells contain high levels of chemotherapeutic drugs thus enabling cells to be resistant to chemotherapy [59]. MVs play various roles in cancer including that of masking cells by facilitating the exchange of surface lipids and proteins such as integrins. Also MVs are capable of inhibiting the complement system. Cells exposed to MAC complex, C5b-9, survived by releasing MVs rich in MAC, thus escaping immuno-surveillance. MVs rich in matrix metallo-proteinases, MMP-2 and MMP-9 and urokinase plasminogen activator (uPA) aid migration of tumour cells by invasion of the Extracellular Matrix [60]. Furthermore, these extracellular vesicles promote angiogenesis and release tissue factor, a procoagulant factor, contributing to fibrin formation which protects the tumour against the immune system. MVs are also able to promote transformation of cells by transmitting proteins from cancerous cells to non-cancerous ones.

4.2. Cancer proteomics

As shown in Table 1, there are many known cancer biomarker proteins that can be isolated from both exosomes and MVs that can potentially aid in cancer diagnosis. Proteins such as CEA and MHC-I have been isolated in many cancer related studies, and are present in both type of vesicle. However, heat shock proteins are unique to exosomes and others unique to MVs could be used as potential biomarkers in the identification and differentiation of both exosomes and MVs.

4.3. Delivery of RNA via exosomes and its role in cancer therapeutics

MicroRNAs (miRNAs) and short interfering RNAs (siRNAs) are transferred through exosomes. These non-coding RNAs regulate a host of cell functions, including proliferation, differentiation, development

Table 1
Expression of known cancer biomarkers and their presence (as potential biomarkers) on exosomes and MVs in different types of malignancies.

Cancer	Cell line	Protein	Cancer biomarker	Exosome[a]	MV[a]	Ref
Bladder	HT1376, RT112, T24	● Gelsolin	Y	Y	?	[61]
		● Integrin α3	N	Y	?	[61]
		● Annexin A4	N	Y	?	[61]
		● Glyceraldehyde-3-phosphate dehydrogenase	N	Y	?	[61]
Breast		● Galectin-1	Y	Y	N	[62]
		● HER-2	Y	Y	N	[62]
		● CEA	Y	N	Y	[62]
		● CA15-3	N	N	Y	[62]
Colorectal	HT-29	● Annexins	N	Y	?	[63]
		● Galectin-4	N	Y	?	[63]
		● Tetraspanin-8	N	Y	?	[63]
		● CEA	Y	Y	?	[63]
		● EGFR	Y	Y	?	[63]
		● Hsc70	N	Y	?	[63]
		● HSP90	N	Y	?	[63]
Colorectal	LM1215	● HSP70	N	Y	?	[63]
		● TSG10	N	Y	?	[63]
Head & Neck	CD8 + Jurkat cells, PCI-13	● FasL	Y	?	Y	[64]
		● sMHC-1	Y	?	Y	[64]
		● MAGE 3/6	Y	?	Y	[64]
Lung	M-BE NCIH226Br	● Cathepsin D	Y	Y	?	[65]
		● LDHB	N	Y	?	[66]
Hepatocellular	MLP-29 (rat)	● ApoE	Y	Y	?	[67]
		● Cytochrome P450	N	Y	?	[67]

[a] Potential biomarker present on exosomes/microvesicles.

and cell death. Their potential role in intercellular communication as cargo in exosomes, enables targeting of specific tissues and cell types, so avoiding non-specific delivery [51]. Whilst the control of gene expression is tightly regulated in healthy cells, it is deregulated in cancerous and viral infected cells. Release of exosomes rich in viral miRNA can result in gene repression in recipient cells [68].

Experiments carried out by Zhu et al. [69] have reported that EBV is present in all Nasopharyngeal Carcinomas (NPC) and that it can transform cells, leading to cell proliferation and metastasis. miRNA profiling enables the identification of novel miRNA genes which could be used as therapeutic targets. miR-BART21 and miR-BART22, for example, are novel and highly abundant in NPC. Non-identification of miRNA from the BHRF1 region of the EBV genome suggests that the presence of mRNA from this region can inhibit pathogenesis. In fact, miR-BART6-5p RNAs suppress the EBNA2 viral oncogene required for the transition from the immunologically less responsive type 1 and 2 latency to the more immunoreactive type 3 latency, thus revealing the regulatory function in EBV infection and latency [70].

To establish a consensus array of miRNAs unique to a particular cancer, comparisons should be made of miRNA expression profiling in exosomes and MVs from patients suffering from various types of cancers. Conducting such studies is vital as it can potentially lead to novel therapies.

5. Extracellular vesicles and pathogens

5.1. Exosomes and intracellular pathogens

Hitherto, the role of exosomes as opposed to MVs in the infection of intracellular pathogens has been largely investigated. Prior to looking at their role in infection, studies focussed on exosomes as a potential source of tumour antigens and on antigen presentation to T-cells. Recent studies have shown that exosomes may promote cell-to-cell spread of infectious agents [71]. Furthermore, exosomes isolated from cells infected with various intracellular pathogens, such as *Mycobacterium tuberculosis* (M. tb) [72] or *Plasmodium yoelii* [73], have been shown to contain microbial components and to stimulate antigen presentation and macrophage activation, suggesting that the function of exosomes may encompass immune surveillance.

5.1.1. Exosomes and Mycobacterium tuberculosis

Exosomes released from macrophages infected by *Mycobacterium tuberculosis* express mycobacterial antigens such as the lipoglycan, lipoarabinomannan. The mycobacterial proteins present in the isolated exosomes, that could promote T-cell activation, were identified in an extensive proteomic analysis of exosomes isolated from *M. tuberculosis* H37Rv-infected macrophages [72]. As a result, they identified 41 mycobacterial proteins including some known antigenic proteins such as ESAT-6 (RV 3875), Ag 85 complex (Rv 3804, Rv 1886c, Rv0129c) MPT 64 (1980c) and MPT 63 (1926c). After incubating J774 cells (a murine macrophage cell line) with M. tb, the group found that exosomes were released possessing a number of highly antigenic M. tb proteins [72]. Furthermore, these released exosomes could stimulate macrophages, activate dendritic cells *in vitro* and activate naïve CD4 and CD8 T-cells *in vivo*. This data suggests a possible use for these exosomes in a Tuberculosis vaccine [73].

5.1.2. Exosomes and Mycobacterium/HIV coinfection

Mycobacterium avium is an opportunistic pathogen that exploits HIV-infected patients. The pathogen expresses glycopeptide lipids (GPLs) on its surface, which constitute a major virulence factor, and recent studies suggest that GPLs play an important role in *M. avium* pathogenesis. Bhatnagar and Schorey [74] showed that macrophages infected with *M. avium* release GPLs that are 'trafficked' from the phagosome through the endocytic pathway to fuse with multivesicular bodies (MVBs). *M. avium* infected macrophages then release exosomes containing GPLs leading to the transfer of GPLs from infected to uninfected macrophages [74]. This proinflammatory response is dependent on Toll-like receptors such as TLR2, TLR4 and MyD88, suggesting that the exosomes released express GPLs derived from *M. avium*.

In a model of HIV-1, the glycome profile and the budding signal of the virion were similar to that of the exosomes which were co-secreted, suggesting that the mechanism for viral propagation was similar to that of the formation of exosomes [75,76]. The secreted exosomes may still potentially contain unpackaged viral genome and other viral proteins necessary for replication in the target cell and this led to the hypothesis that exosomes may be used by viruses as "Trojan Horses" to spread to other cells. Indeed, HIV-1 induces exosomes that "express" CD45, CD80, CD86, as well as MHC-I and II [77], thus, potentially masking themselves as being of "host" origin.

The relationship between host EVs and pathogens is an exciting, emerging field of research. However, a number of technical issues have held up its progress, including the accurate determination of MV size and phenotype, their clear distinction from and relationship to other (pathogen-derived) membrane vesicles and their pure separation for functional experimental purposes.

5.2. Microvesicles and intracellular pathogens

Recently, proteomic studies of pathogen-derived extracellular vesicles have come to the fore [49]. Furthermore, a wide array of proinflammatory cytokines are produced in response to infection [78] and MV production may be triggered by proinflammatory cytokines in the absence of infection [79–81]. MVs for example are induced by *in vitro* stimulation with lipopolysacharide (LPS) and *in vivo* stimulation with intravenous injection of bacterial endotoxin [82–84]. Furthermore, different studies have shown that increased MV levels in blood are associated with systemic infection (by a range of organisms), including in septic patients [85], or those infected with human immunodeficiency virus (HIV) [86]. High plasma MV levels were also observed in Ebola Virus infected macaques [87], as well as in malaria patients, plasmodium infected laboratory animals [81,88,89] and as we found in *Trypanosoma cruzi*-infected mice [58]. In this work by Ramirez and Inal [58], host-derived MVs, released upon parasite stimulation, were found to inhibit complement-mediated lysis of the infectious trypomastigote stage and to increase infection levels through a TGF-β1-mediated pathway.

5.3. Microvesicles and inflammation

MVs are produced by activated immune cells, and not necessarily those infected by pathogens, including monocytes, macrophages, dendritic cells (DC), and lymphocytes. Stimuli for monocytes and macrophages include A23187 [90], LPS [91,92], or serum starvation [83]. DC have also been stimulated with LPS [92,93] and T cells with agonistic CD3 antibodies, Fas ligand, actinomycin-D and staurosporine [94]. Patients with autoimmune disease such as diabetes vascular disease have also been shown to have high levels of MVs, above accepted basal rates [95,96].

The inflammatory conditions created by MVs play a vital role in controlling infection and pathogen clearance [97]. The proinflammatory cytokines induced by MVs may be managing and containing the infection, whilst chemoattractant proteins, possibly present on MVs to recruit leukocytes to the site of infection to remove the pathogen.

Furthermore, MVs are able to upregulate adhesion molecules such as Intercellular Adhesion Molecule-1 (ICAM-I) [98] in target cells and promote monocyte recruitment [99]. The production of TNF renders the endothelium permeable to 'immuno-active' proteins and enables leukocytes to access the site of infection. Although this is an important step in enabling pathogen clearance, it can have adverse consequences, as in certain diseases such as lethal flavivirus encephalitis, in which the immunopathological response, including excessive monocyte infiltration of the central nervous system and the accompanying proinflammatory cytokine response, is one of the main features of the disease, and a major cause of mortality [100,101].

6. Discussion

Secretory proteins also known as 'secretome' are multi-functional, playing various roles as immune regulatory proteins, neurotransmitters, in signal transduction pathways, as building blocks of certain cellular components such as the cell wall and as mediators of intercellular and intracellular communication. Section 3 addressing secretomes as novel reservoirs of biomarkers, gives the reader the concept of different types and functions of secretomes. MVs and exosomes, referred collectively as extracellular vesicles aid in antigen presentation, intercellular communication, transfer of RNA and proteins and propagation of infectious agents and proteomic studies have revealed the importance of these vesicles in cancer diagnosis. Also the introduction of microRNA profiling has opened a new gateway in the development of novel therapeutics. Since MVs play significant roles in homeostasis and inflammation, many studies have looked into their roles in infectious disease. Important questions that need to be addressed include when and in response to what signal(s) in the infection process do cells release MVs [102,103]? Also, what macromolecules do MVs from infected cells harbour and what role(s) do they play in furthering infection? An area that has not been sufficiently addressed in the infectious disease area is that of describing novel biomarkers of infection on both exosomes and MVs. Such information on the composition of MVs and exosomes, particularly over time during an *in vivo* infection and how this correlates with the pathogen's load during different stages of the infection, should now be obtained.

At the 'Microvesiculation and disease' conference held at London Metropolitan University, UK in September 2012 [104], the importance of such areas was debated. The role of stem-cell derived MVs in the paracrine action of stem cells has been stated before which serves to highlight the importance of mRNA and microRNA profiling to distinguish between the donor and recipient cells. For others, it is important to understand and build robust clinical assays to analyse miRNAs as they play vital roles in pathogenesis and therefore will bring forth advances in developing novel therapeutics.

As many have reiterated with reference to many applications of EVs, there is an urgent need for internationally recognised criteria relating to nomenclature and purification [55]. This should be addressed in the future as it is paramount that researchers have a clear understanding about the vesicles they isolate.

As discussed in Section 4, both exosomes and MVs contribute to developing a malignancy. Proteomic studies of these vesicles has shown that the over expression of exosomal or microvesicular proteins can be used as biomarkers in identifying a malignancy which may aid in the development of novel therapeutics. However there are few studies on both exosomes and MVs related to the same type of cancer carried out by the same team of researchers, in order to compare the proteomics efficiently, so in the future, such studies should be encouraged. We hope that much of the work at CMIRC and in the plethora of labs interested in EVs will now contribute to the expansion of the proteomic database and that such initiatives such as the creation of Vesiclepedia [105] the online compendium for such data, will facilitate such comparative studies.

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