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Review

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Exosome platform for diagnosis and monitoring of traumatic brain injury

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We have previously demonstrated the release of membranous structures by cells into their extracellular environment, which are termed exosomes, microvesicles or extracellular vesicles depending on specific characteristics, including size, composition and biogenesis pathway. With activation, injury, stress, transformation or infection, cells express proteins and RNAs associated with the cellular responses to these events. The exosomes released by these cells can exhibit an array of proteins, lipids and nucleic acids linked to these physiologic events. This review focuses on exosomes associated with traumatic brain injury, which may be both diagnostic and a causative factor in the progression of the injury. Based on current data, exosomes play essential roles as conveyers of intercellular communication and mediators of many of the pathological conditions associated with development, progression and therapeutic failures and cellular stress in a variety of pathologic conditions. These extracellular vesicles express components responsible for angiogenesis promotion, stromal remodelling, signal pathway activation through growth factor/receptor transfer, chemoresistance, immunologic activation and genetic exchange. These circulating exosomes not only represent a central mediator of the pro-inflammatory microenvironment linked with secondary brain injury, but their presence in the peripheral circulation may serve as a surrogate for biopsies, enabling real-time diagnosis and monitoring of neurodegenerative progression.

1. Introduction to central nervous system injury

Brain injury can be the consequence of physical, chemical or biological insults and can result in disabling or potentially fatal consequences. There can be multiple outcomes resulting from different forms of brain injuries and these are difficult to predict. Owing to the inability to biopsy components of the central nervous system (CNS), injury-linked biomarkers are needed to define pathophysiological mechanisms and to predict neurological outcomes. To date, some of the brain-associated biomarkers assessed have included cytokines (such as interleukin (IL)-1 β , IL-6, transforming growth factor- β (TGF- β)), S100B, glial proteins (such as glial fibrillary acidic protein and neurofilament light chain), and enzymes such as tissue transglutaminases), β -amyloid proteins and tau. The major clinical challenge to the accurate definition of prognosis is to identify the degree of CNS damage and the site of the lesion. A key goal is the development of assay markers that provide accurate prognosis in patients suffering from either acute or chronic CNS injuries, as well as definition of the extent and location of the primary injury, which is crucial for predicting mortality and morbidity. The review focuses on acute neurologic injuries.

Traumatic brain injury (TBI) is a significant public health issue with an estimated 1.6–3.8 million individuals experiencing TBI annually in the USA [1,2]. Of these, approximately 40% do not seek medical attention. For those seeking medical attention, the diagnosis of mild TBI is complicated by a lack of a consensus in defining mild TBI. Concussion and mild TBI are frequently used interchangeably. TBI commonly produces neurological morbidity and the neurologic consequences are seen even in mild TBI. Mild TBIs can lead to acute symptoms, including chronic traumatic encephalopathy, cognitive impairment, dementia, movement disorders and motor neuron dysfunction. Even within mild to moderate TBI, 51–54% exhibit long-term disability,

along with 78% of those with severe TBI [3]. While the incidence of mild TBI is higher in the military population, a similar percentage do not seek medical attention. Between 2000 and 2011, Veterans Affairs medical records report approximately 7% of veterans from Iraq and Afghanistan were diagnosed with TBI [4,5]. Among military personnel who serve in Iraq or Afghanistan, 11–23% may have sustained at least mild TBI [6]. While many cases of TBI go unreported or unrecognized, US military records indicate 233 425 TBIs; among those 178 961 were listed as mild [6]. In Iraq and Afghanistan, mild TBIs have become more prominent due to the use of improvised explosive devices (IEDs), accounting for approximately 75% of all combat-related injuries to military personnel [7]. Approximately 50% of IED injuries result in mild TBIs, with the lifetime prevalence for mild TBI in US military personnel estimated to be 19.5%.

2. Diagnostic gap

Imaging modalities such as computed tomography (CT) and magnetic resonance imaging exhibit suboptimal sensitivities for detecting concussions (3–10% for CT and 10–57% for magnetic resonance imaging), since these are designed to detect major structural damage that may not be present in concussions [8]. For clinicians who evaluate and treat individuals with concussions, it is critical to definitively identify those who have sustained a concussion and those who are at risk for adverse outcomes. Most concussions present with vague symptoms. While signs and symptoms are difficult to quantify, approximately 1–2% of the population exhibit lifelong TBI-related disabilities [9]. These disabilities include memory impairment, diminished reasoning and impaired motor functions. In many cases, TBI, including concussions, can result in adverse outcomes, such as chronic traumatic encephalopathy, cognitive impairment, dementia, movement disorders and motor neuron dysfunction [10]. The inability to quantitatively assess individuals with concussions can also lead to the risk of ‘second-impact syndrome’ (SIS). SIS is a frequently fatal condition that occurs when an individual suffers a second concussion prior to resolution of the symptoms from the first concussion. For many sports, ‘return-to-play’ guidelines have been established to prevent SIS; however, the grading systems for defining symptoms and recovery are subjective. The most common scale is the Glasgow Coma Scale (GCS), which is used to assess the level of consciousness following a head trauma. However, the GCS was not intended to be used alone to define the severity of brain damage or to predict outcome. There are significant concerns regarding the poor inter-observer reliability and the lack of prognostic utility. Additionally, the failure of clinical trials is due in part to the difficulty of defining a quantitative severity scale to accurately stratify the array of TBI disease, and to the absence of biomarkers that reflect specific pathophysiological mechanisms. Currently, there remains a significant need for new diagnostic approaches to identify brain injury and to predict the risk of neurological deterioration.

3. Immunologic sequelae

In CNS injury, primary damage occurs as a direct result of trauma, leading to immediate physical, biochemical and cellular alterations. These alterations occur within seconds of the

injury and are marked by systemic and local events. Diverse populations of cells and bioactive molecules from the nervous, immune and vascular systems are involved. The reactions of the immune system to acute injury are both cellular and molecular. At the injury site, the cumulative effect of the immune cells and regulatory proteins is inflammation [11]. Inflammation is a critical element in the secondary injury cascade that occurs immediately and can persist for weeks or months [12]. While the inflammatory response is essential for the clearance of cellular debris, the ‘over-activation’ of the inflammatory response can damage healthy tissue and exacerbate the primary injury. Microglia represent the innate immune cells of the CNS and these cells have a range of functions, which can vary dependent on time and the microenvironment [13]. Inflammation, resulting from the primary injury, attracts neutrophils, monocytes, microglia and T-lymphocytes [14]. Neutrophils release cytokines, proteases and free radicals, which activate other inflammatory and glial cells to perpetuate the inflammatory cascade, leading to neuron injury or death. Shortly after the primary injury, monocytes infiltrate and differentiate into macrophages, where activated microglia and macrophages secrete various pro-inflammatory cytokines, free radicals and growth factors [15,16].

The activated immune cells secrete pro-inflammatory cytokines, including IL-1 β , IL-6 and tumour necrosis factor- α (TNF- α), all of which increase the extent of inflammation. Following CNS injury, there is a marked increase in the blood–brain barrier permeability due to both direct mechanical disruption by the primary injury and the effects on endothelial cells by numerous inflammatory mediators and other compounds that are upregulated. While in normal rat brain, IL-1 β and its mRNA are present, both are increased in certain disease states, including ischaemic brain injury and TBI [17]. Two inflammatory cytokines (TNF- α and IL-1 β) are upregulated and are known to increase vascular permeability. Increased production of IL-1 β may contribute to injury processes by causing microvascular injury, which disrupts the blood–brain barrier and leads to oedema after brain injury [18]. Furthermore, IL-1 β stimulates the proliferation and hypertrophy of astrocytes and thus may be causally involved in the formation of the astroglial scar that develops in the injured CNS. Studies with an antagonist of the IL-1 β receptor suggest that neuronal damage produced by ischaemia is mediated in large part by IL-1 β [19].

Currently, there is no clear consensus on the role of endogenous TNF- α in CNS acute injury. TNF- α is a key inflammatory cytokine that is upregulated in neurons, glia and endothelial cells and its production at the injury site appears to be involved in secondary tissue [20]. Investigators have shown the presence of TNF- α protein and mRNA at the sites of traumatic lesions within minutes and the TNF- α concentrations peak in the cerebrospinal fluid (CSF) within 24 h [21,22]. Six hours after TBI, TNF- α expression is higher in the CSF than in serum; TNF- α expression did not correlate with outcome [23]. The differential patterns of localization of TNF- α receptors in neuronal and glial cells, their state of activation and the downstream effectors are all thought to play an important role in determining whether TNF- α will exert a beneficial or harmful effect on the CNS. Additionally, TNF- α contributes to the tissue injury induced by neutrophils by directly activating them, as well as by increasing the expression of such molecules as E-selectin, which cause the

activated neutrophils to adhere to the surface of the endothelial cells [24]. It has also been shown that the inhibition of neutrophil adhesion to the endothelial cell surface markedly reduces the severity of the CNS injury induced by compressive trauma.

4. Biomarkers

For clinicians who evaluate and treat individuals with mild TBI, it is critical to definitively identify those who have sustained a concussion and those who are at further risk for adverse outcomes. Most mild TBIs present as vague symptoms and imaging characteristics [25]. It is also difficult to monitor therapeutic responses; this failure is due in part to the difficulty of defining a quantitative severity scale to accurately stratify the array of TBI disease, and to the absence of biomarkers that reflect specific pathophysiological mechanisms.

Currently, functional neurologic measurements are used to stratify injury severity and predict neurologic outcome; however, in acutely injured patients these metrics are often difficult to determine. Biomarkers have potential utility as diagnostic, prognostic and therapeutic adjuncts in the setting of TBI. Circulating biomarkers have been investigated for the definitive diagnosis and monitoring of treatment for TBI. Such circulating biomarkers could also serve to monitor disease progression, by assessing tissue injury and predicting risk of neurological deterioration. They could be used to help determine which patients should receive which treatments. Two approaches are being used, namely, assessing markers of structural damage, and quantifying mediators of the cellular, biochemical or molecular cascades in secondary injury and repair. Circulating biomarkers have been proposed to be promising for the definitive diagnosis and monitoring of treatment in CNS injury. Defining TBI-linked biomarkers has numerous advantages, such as diagnosing the disease, identifying processes that are difficult to image (such as diffuse axonal injury), predicting outcome by identifying patients at risk for long-term neurocognitive consequences, defining injury-specific molecular and pathological alterations for developing therapeutic targets and monitoring responses to acute interventions [26,27]. These circulating biomarkers could also serve to monitor disease progression, by assessing tissue injury and predicting risk of neurological deterioration. However, circulating biomarkers are problematic and exhibit several critical issues. Free protein and nucleic acid biomarkers are extremely unstable in the circulation, thus a high steady-state must be reached for detection, which is generally not observed except in severe TBI. Minor changes over time (essential for monitoring) are difficult to quantify by current technologies. Furthermore, these current used biomarkers are sensitive to sample handling and require strict procedures for collecting, handling and processing. Thus, differentiating the degree of severity and predicting the extent of neurologic recovery remains enigmatic.

5. Exosomes

To circumvent these issues, we have initiated the use of exosome-associated biomarkers. Our work has demonstrated that these are extremely stable within the circulation, in the order of days (versus minutes for traditional soluble markers). In addition to serving as biomarkers of injury, our data demonstrate that exosomes may mediate *in vitro* events associated with TBI. Thus, their characterization

may provide insight into the ‘driver’ mechanisms of neurodegenerative disease.

In 1979, our group discovered and published the initial observation of circulating exosomes and proposed their diagnostic potential [28]. The release of 50–200 nm-sized membranous vesicles into biological fluids by viable cells has since been demonstrated in multiple cell types and systems over the past 30 years. *In vivo*, these nano-sized vesicles released by tumour cells accumulate in biologic fluids, including blood, urine, ascites and pleural fluids [29]. These released vesicles have been identified by various terms, including high molecular weight complexes, membrane fragments, exosomes, microvesicles, microparticles and extracellular vesicles. The terms, ‘microvesicles’ and ‘microparticles’ appear to be misnomers as these are nanometres in size and should have been termed ‘nanovesicles’. Additional terms have been created to differentiate vesicles from specific cell types, such as ‘oncosomes’, ‘texosomes’ and ‘tumoursomes’ for tumour-derived vesicles, ‘prostasomes’ for prostate cell-derived vesicles and ‘dexosomes’ for dendritic cell-derived vesicles. The use of these various terms has only served to confuse the field. While, more recently, restrictive definitions have been applied to these cell-derived vesicles, significant overlap (in terms of size, markers, cargoes and function) exists between structures identified as exosomes and microvesicles. Within the circulation, it may not be possible to differentiate 50–100 nm ‘exosomes’ from 50 to 200 nm ‘microvesicles’. With recent technological developments, we are beginning to define the extremely complex compositions of these vesicles, which are likely to provide insights into their classifications, cellular and molecular origins and biologic functions.

In many studies, uncharacterized cell-derived vesicles (in terms of markers or size) are termed ‘microvesicles’, while numerous studies define ‘exosomes’ solely based on density and the presence of the cell surface markers tetraspanins. These overlaps in vesicle properties and terms suggest that these distinctions may not be clear-cut. While many of the definitions are still used, their flaws are now recognized. The apparent size and shape of exosomes are artefacts of fixation and drying associated with electron microscopy. Principal markers of exosomes are tetraspanins, which as plasma membrane associated components are present on most vesicles, regardless of their origin. The importance of the endocytic pathway of vesicle formation has also been questioned as knock-out studies with Rab proteins only diminished vesicle release by approximately 30% (based on exosomal protein) [30]. The ‘exosome’ term was coined in 1981 for any ‘exfoliated membrane vesicles with 5′-nucleotidase activity’ [31]. This term, ‘exosome’, originated from the discovery of neoplastic cell line-derived exfoliated vesicles, which mirrored the 5′-nucleotidase activity of the parent cells [31]. For these reasons, this review uses the term ‘exosomes’ to include all 50–200 nm released vesicles.

Over the past three decades, shed vesicles have been characterized for multiple human cell types; they are not exact replicates of the plasma membrane or other membranous compartments of the originating tumour cells, but they represent ‘micromaps’ with enhanced expression of tissue-specific antigens as well as other macromolecules, including major histocompatibility antigens [29]. Exosomes can be viewed as cytoplasm enclosed in a lipid layer with the external domains of transmembrane proteins exposed to the extracellular environment in their normal cellular

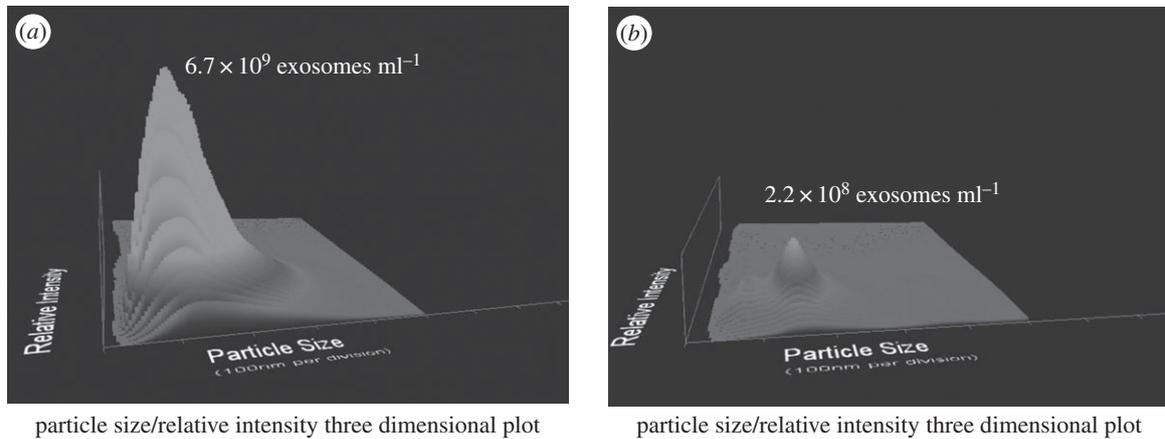


Figure 1. Quantification of brain-derived exosomes in the circulation of a patient with brain cancer. Following chromatographic isolation, circulating exosomes were quantified using a NanoSight NS300. The x -axis presents the size range of observed vesicles, the y -axis the concentration of vesicles and the z -axis the relative intensity. Panel (a) shows the circulating total exosome population, defined by the NS300 in light scatter mode. Panel (b) identifies those vesicles expressing a marker of brain-derived exosomes (β -amyloid), determined using the NS300 in fluorescent mode.

orientation. Electron microscopic studies have demonstrated the fusion of the limiting membrane of multivesicular bodies (MVB) with the plasma membrane as well as release of intraluminal vesicles in different cell types of haematopoietic origin, such as Epstein–Barr virus-transformed B cells [32], mastocytes [33], dendritic cells [34], platelets [35], macrophages [36] and cells of non-haematopoietic origin such as neurons and epithelial cells [37]. We have developed genomic, proteomic and metabolomic technologies to define stable, disease-specific exosomal markers for detection, disease characterization and predicting prognosis [38]. These temporal changes in exosomal profiles have been demonstrated to accurately predict disease recurrence and overall patient survival [39]. The proteomic and genomic profiles of circulating exosomes provide a real-time monitor of therapeutic response, serving as a companion diagnostic. By correlating these circulating markers with the molecular characteristics and real-time clinical parameters, we have established the use of circulating exosomes as a ‘liquid biopsy’. In 2008, our group published the initial demonstration of circulating exosomal RNA for diagnosis [40]. Many studies have examined the diagnostic utility of profiling total circulating microRNA in specific pathologies. While there is a significant exosome contribution from immune cells, platelets and vascular endothelial cells, little is known regarding these normal vesicular RNA contents or how they respond to exogenous ‘stressors’. Proliferating and injured cells, both *in vivo* and *in vitro*, have been demonstrated to release membranous structures, defined as microvesicles or exosomes, consisting of an array of macromolecules derived from the originating cells, including proteins, lipids and nucleic acids. In the peripheral circulation of brain cancer patients, we have demonstrated the presence brain-derived exosomes (figure 1), based on the combined presence of β -amyloid, tau and S100 β .

While described over three decades, only recently have the roles of these vesicular components in intercellular communication become elucidated. Significant evidence has demonstrated that exosomes can exert a broad array of detrimental effects on the immune system, ranging from apoptosis of activated cytotoxic T cells to impairment of monocyte differentiation into dendritic cells, to induction of myeloid-suppressive cells and T regulatory cells. Through the expression of molecules involved in angiogenesis promotion, stromal

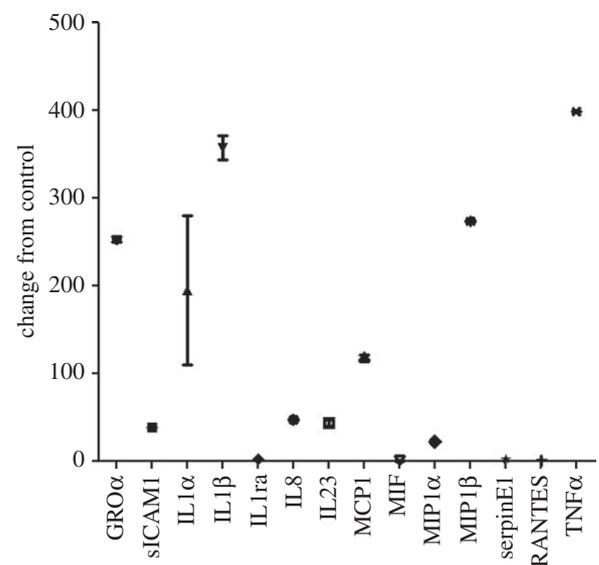


Figure 2. Induction of cytokines in human macrophages following exposure to $100 \mu\text{g ml}^{-1}$ exosomes. Human macrophages were incubated in the presence of $100 \mu\text{g ml}^{-1}$ exosomes for 20 h and the levels of specific cytokines were identified in the media by the Cytokine Profiler Array A. Exosomes were chromatographically isolated from pooled normal human serum (control) or from tumour cell conditioned media. Cytokine expression in tumour exosome-treated macrophages was expressed relative to the expression observed with addition of the ‘control’ exosomes.

remodelling, signalling pathway activation through growth factor/receptor transfer and genetic intercellular exchange, exosomes represent a central mediator of a tissue’s microenvironment. TBI is associated with induction of inflammation, oxidative stress, oedema and hypoxia, which can occur over hours to weeks, leading to brain tissue damage. Following initial injury, inflammatory mediators play a critical role in mediating leucocyte adhesion and cellular stress responses. There is a dramatic elevation of pro-inflammatory cytokines within minutes of injury, in particular TNF- α , IL-1 β , IL-6, IL-8, IL-10 and TGF- β . These cytokines are primarily produced by infiltrating lymphoid and mononuclear cells. Our work has demonstrated that these same pro-inflammatory cytokines are induced by specific exosomes (figure 2) from mononuclear cells. We have

demonstrated that injury-derived exosomes mediate the production of pro-inflammatory cytokines, including IL-1 β [41]. Following TBI, elevations in the IL-1 cytokine family are observed, including IL-1 β , IL-1 α , IL-1 receptor antagonist and IL-18. Of these, IL-1 β is the most prominently enhanced cytokine following TBI [42]. After TBI, IL-1 β is released primarily by microglia and acts as a pro-inflammatory pyrogen, increasing expression of other cytokines, proteases and matrix metalloproteinases (MMPs). IL-1 β is a potent mitogen for astroglia and has been shown to be elevated within 10 days after injury [43]. Studies have demonstrated that IL-1 β increases within the initial 24 h in the circulation and CSF [42]. These IL-1 β levels may exhibit prognostic value, as CSF IL-1 β levels have been shown to be greatest in severe CNS injuries with elevated intracranial pressures and unfavourable outcomes [44]. By contrast, IL-1ra, the receptor antagonist that diminishes IL-1 responses, has been shown to be greatest in patients with favourable outcomes [45].

6. Functions of exosomes

While some early studies implicated exosomes as ‘garbage bags’ of the cells [46,47], some vesicles released from tumour cells have gained increasing recognition as ‘vehicles’ for intercellular communication. Intercellular communication has been thought to be limited to cell-to-cell adhesion conduits (gap junctions) or secreted signals, such as hormones, neurotransmitters and cytokines, released from cells and acting in an autocrine or paracrine manner. These exosomes interact with the plasma membrane of a recipient cell by ligand/receptor binding, fusion or internalization (or a combination of these). If the exosomes fuse with the target cell, they can transfer their cargo to that recipient cell. Owing to the presence of cell-type-specific adhesion molecules, exosomes can interact with specific cells and deliver their ‘cargoes’, including bioactive lipids, cytokines, growth factors, receptors and genetic material. In this manner, exosomes represent a pathway for intercellular transfer of information, similar to that observed with direct cell–cell contact, but that can function at distance. Exosomes provide stable conformational conditions for their protein content, conserve bioactivity of their proteins, improve bio-distribution and support an efficient interaction with target cells.

The complexity of extracellular vesicle-associated bioactive macromolecules supports a critical role in generating the tumour microenvironment [48,49]. Exosomes can transfer specific proteins to target cells for the delivery of signalling pathways [50,51]. The presence of tumour-derived exosomes can increase MMP secretion and VEGF expression in target cells through the expression of pro-angiogenic molecules, such as members of the tetraspanin family, thereby promoting neo-angiogenesis even at secondary metastatic sites [52]. The released MMPs can digest the extracellular matrices where they arise. This degradation is enhanced when MMPs are co-released with exosome-associated extracellular matrix metalloproteinase inducer (EMMPRIN) [53].

Studies have shown that cancer ascites-derived exosomes carry extracellular matrix-remodelling enzymes, such as MMP-2 and MMP-9 [54,55], and urokinase plasminogen activator [56], leading to an increase in extracellular matrix degradation. The expression of matrix-remodelling enzymes increases the tumour’s invasive phenotype and promotes metastasis. The presence of pro-angiogenic factors supports neovascularization of the

developing tumour. A commonly identified cellular component of the tumour microenvironment is the monocyte/macrophage. Within the microenvironment, tumour-associated macrophages have been shown to assist in tumour progression by expressing cytokine/chemokine profiles that promote angiogenesis, stimulate tumour growth, and elicit immunosuppression by suppressing Th1 responses [57,58]. The tumour microenvironment is characterized by pro-inflammatory profiles, including IL-1 β . This profile is generally produced by infiltrating macrophages following interactions with tumours or their components. While we proposed that exosomes could ‘educate’ macrophages to produce pro-inflammatory cytokines following internalization, we recently demonstrated that the induction of IL-1 β was observed even when internalization of vesicles was blocked [59]. Arginine, glycine and aspartate containing peptides (RGD peptides), which are used to block fibronectin binding to macrophage $\alpha 5 \beta 1$ integrin, were observed to abrogate vesicle induction of IL-1 β production and downstream phosphorylation of Akt and c-Jun [60]. This approach reveals the importance of receptor/ligand interactions in vesicle communication.

When released exosomes fuse with their target cells, they can transfer specific membrane components, including receptors and ligands, which can express an activated phenotype. This transfer of receptors from exosomes to target cells was demonstrated by the observation that bystander B cells acquire antigen receptors from activated B cells by membrane transfer [61]. This transfer allows the amplified expansion of antigen-binding B cells with the ability to present specific antigens to CD4 T cells. Exosomes can transfer the adhesion molecule CD41 from platelets to endothelial cells or to tumour cells, conferring pro-adhesive properties to the target cell [62]. Exosome-mediated transfer of Fas ligand from tumour cells induces apoptosis of activated T cells enabling tumour immune escape [63]. Exosomes can also be protective for tumour cells by removing molecules such as Fas or the membrane attack complex from their membranes.

The horizontal transfer of macromolecules and their functional consequences has been demonstrated in human gliomas [64,65]. In this model, only a fraction of the cells exhibiting a transformed phenotype expressed the truncated epidermal growth factor receptor, EGFRvIII, associated with dysregulated tumour growth [64]. Al-Nedawi *et al.* [65] demonstrated transfer of the oncogenic EGFRvIII from human glioma cancer cells expressing the receptor to glioma cells without the EGFRvIII via the fusion of exosomes. After transfer, the glioma cells originally lacking the receptor were transformed to express EGFRvIII-regulated genes, including VEGF, Bcl-x_L and p27 [64]. Subsequent studies demonstrated that the oncogenic EGFRvIII from human squamous cell carcinoma cells was transferred via exosomes to tumour-associated endothelial cells to activate MAPK and Akt cell signalling pathways and promote endothelial VEGF expression [66].

Epigenetic changes have been frequently demonstrated in various tumours, resulting in regulation of gene transcription, altered proliferation, differentiation and therapeutic resistance [67]. Genetic information can be transferred through two proposed mechanisms: vertical gene transfer, which is gene exchange from parent to the next generation, and horizontal gene transfer, induced through, for example, bacteriophages or viruses. Since exosomes have been implicated as a potent source of macromolecule transfer to neighbouring and distant cells, viruses and other pathogens appear to exploit this system. Exosomes are postulated to contribute to the spread

of infective agents, such as human immunodeficiency virus type 1 [68]. In macrophages receiving chemokine receptors, this can induce an increased risk of HIV infection together with resistance to apoptosis. The transfer of the chemokine (CXC motif) receptor 4 and the chemokine (CC motif) receptor 5 chemokine co-receptors for human immunodeficiency virus type I by released exosomes can enhance the entry of the virus into cell types other than the lympho-haemopoietic lineage [69]. In addition to transferring receptors, exosomes can transfer viruses, contained within exosomes, by the 'Trojan exosome hypothesis' involving direct delivery [70].

Cell-derived exosomes represent another mechanism of horizontal gene transfer. Genomic instability may be mediated by horizontal transfer of tumour-derived materials via exosomes. Horizontal transfer of macromolecules, including RNA, proteins and lipids, via exosomes has been shown in multiple tumour systems, including gliomas, monocytes, mast cells and T cells [71]. Tumour-derived exosomes have been shown to be capable of transferring surface components (proteins and lipids) and RNAs to monocytes. Janowska-Wieczorek *et al.* [72] demonstrated that exosomes derived from murine embryonic stem cells (ESCs) could induce epigenetic reprogramming of target cells. ESC-derived exosomes were shown to improve survival of haematopoietic stem/progenitor cells, to induce upregulation of early pluripotent and early haematopoietic markers, and to induce phosphorylation of mitogen-activated protein kinase p42/44 and Akt. ESC-derived exosomes were shown to express mRNAs for several pluripotent transcription factors that can be delivered to target cells and translated to their corresponding proteins [73]. As RNase treatment inhibited their exosome-mediated biological effect, the involvement of mRNA in the observed biological effects was suggested. Yuan *et al.* [74] have shown that in addition to mRNA, exosomes can transfer microRNA to target cells. They demonstrated that exosomes derived from ESCs contain abundant microRNA and that they can transfer a subset of microRNAs to mouse embryonic fibroblasts *in vitro*. Since microRNAs are regulators of protein translation, this observation raised the possibility that stem cells can alter the expression of genes in neighbouring cells by transferring exosomal microRNAs. When shed vesicles fuse with their target cells, the portion of cytosol segregated within their lumen is discharged to and integrates with the cytosol of the target cell. Because this transfer can also include transmission of specific mRNAs, it can ultimately contribute to the epigenetic and proteomic properties of target cells.

One of the key mechanisms proposed for immune regulation by exosomes is the macromolecular transfer of small non-coding RNAs. These small non-coding RNAs, including microRNA, have been recognized in the regulation of cellular processes in maintenance of health and development of disease [75]. Specific microRNAs have been associated with the development of neurological disorders [76,77], contributing to the onset and progression of complications linked with TBI [78]. MicroRNAs associated with exosomes derived from injured brain tissue appear to represent surrogates and should have utility as stable, clinically accessible biomarkers to improve TBI detection and serve as sensitive measures for therapeutic outcomes.

7. Cargoes of exosomes

Exosomes contain proteins, non-coding RNAs and mRNAs, and the exosomal lipid bilayer appears to protect these

materials from degradation. While protein and RNA cargoes of exosomes vary depending on the originating cell, there are conserved proteins among exosomes from different cellular origins [79]. The protein composition of exosomes has been extensively analysed by various techniques including Western blotting, fluorescence-activated cell sorting, immuno-electron microscopy and mass spectrometry. All exosomes exhibit cytoskeleton proteins (such as ezrin and actins), proteins associated with the MVB biogenesis (such as alix and TSG101), membrane transport and fusion proteins (e.g. annexins and Rab proteins) and tetraspanins (e.g. CD9, CD63 and CD81). A catalogue of proteins, RNAs and lipids associated with exosomes can be found at www.microvesicles.org. Currently, Vesiclepedia (formerly ExoCarta) lists entries (many redundant) for 43 731 proteins, 20 196 mRNAs, 2400 microRNAs and 342 lipids associated with exosomes.

We have analysed the cancer patient-derived exosomal proteome using ion trap mass spectrometry and identified 232 unique proteins. These proteins were classified as percentage of the identified total proteins into molecular chaperones (8.5%), vesicle fusion (8.5%), cytoskeletal proteins and proteins involved in the assembly/disassembly of the cytoskeletal networks (17.6%), anionic and cationic ion transport channels (3.7%), proteins involved in lipid (6.9%), carbohydrate (3.2%) and amino acid (2.1%) metabolisms, and proteins involved in DNA replication (6.9%), mRNA splicing (5.3%), transcription/translation (5.3%), post-transcriptional protein modification (13.8%) and signal transduction (2.7%). Our studies demonstrated that cytosolic proteins were highly represented, and we observed a diverse array of cytoskeletal constituents (actin, α -actinin-1, cofilin, filamin-A, -B, -C, tubulins, gelsolin, profilin-1, spectrin, symplekin, talin, vinculin, myosins). We identified that transmembrane proteins were also abundant, including multiple integrins (β 1, α 3, α v), intercellular adhesion molecule 1 (ICAM-1) and mucin-4. A variety of channels were observed, such as the voltage-dependent anion-selective channel protein 2 and 3, chloride intracellular channel protein 1, sodium/potassium-transporting ATPase subunit β -3, long sodium/potassium-transporting ATPase subunit α -1 and transitional endoplasmic reticulum ATPase. In line with their endocytic origin, exosomal proteins belonging to the ESCRT complexes, which are important protein complexes involved in ubiquitin-dependent exosome biogenesis, have also been observed [80]. These ESCRT-associated proteins include vacuolar protein sorting-associated protein 35 (VPS-35), Alix, ubiquitin-like modifier-activating enzyme and ubiquitin carboxyl-terminal hydrolases. We demonstrated that proteins involved in membrane trafficking and fusion processes were enriched (annexin A2, A5, A6, clathrin heavy chain 1/2, coatmer subunit β , Rab1b, Rab2a and Rab7a). A group of markers of endosomes and lysosomes were also detected (cathepsin-C, -D, EH domain-containing protein 1 and β -hexoaminidase), and several chaperones were identified (HSP60, HSP70, HSP90, HSC70, GRP75, HSP110, GRP78, GRP94, HSP47, HSP27, HSP9 alpha members; T-complex protein 1, endoplasmin and protein disulfide-isomerase A3, A4, A6) [79,81,82].

8. Exosome-associated RNA

The current hypothesis for the stability of circulating RNAs is that they are released from cells in membranous vesicles.

Recent data confirm that extracellular RNA can exist in several forms: protein-associated RNA (including Argonaute 2-bound RNA), high-density lipoprotein-bound RNA and vesicle-associated RNA. This review focuses on RNA associated with exosomes. These exosomes are generated constitutively by most, but not all, cell types and contain both mRNAs and non-coding RNA. The ability of exosomes to transfer genetic information may facilitate cancer spread by delivering genetic material and oncogenic proteins. RNA profiles of exosomes differ from that of cellular RNA, since vesicles contain primarily small RNA, as mRNA (potentially debris and full length) and microRNA [40,71].

The presence of circulating RNAs has been extensively investigated, despite the presence of RNases, which should degrade any free RNA. The majority of the circulating RNAs have been defined as microRNAs based on the molecular weight [83]. Studies also demonstrated that microRNAs not only have high stability in body fluids, but also survive in the unfavourable physiological conditions such as freeze-thawing, extreme variations in pH and long time at room temperature [84–86]. Addition of detergents, such as Triton X100 or SDS, to serum or plasma allows the miRNAs to be easily degraded by RNases [86]. The results indicate there are at least two approaches responsible for the stability of extracellular microRNAs: packaging in membrane-encapsulated vesicles and protection by RNA-binding proteins. The stability of extracellular microRNAs has been hypothesized to be due to the formation of the RNA-vesicle. During RNA-induced silencing complex (RISC) disassembly in the cytoplasm, some microRNAs are found to be sorted into MVBs, which are commonly considered to form exosomes by fusion with the plasma membrane [87]. Both exosome and microvesicle can easily translocate across the cell membrane, which enables microRNAs to enter recipient cells easily and mediate cell-to-cell communication.

Our studies have indicated that many of the RNAs enriched in the exosomes may not be abundant, or even detectable, in the originating cell or the RNA might be highly expressed within the cell and low or absent within exosomes, indicating sorting of specific RNAs into exosomes. These released microRNAs can be classified into three categories based on the ratio between the amount of microRNA released from the cells and the amount retained in the cell [88]. The first group is selectively released microRNAs, which are characterized by being primarily released from tumour cells with relatively low concentrations remaining in the cell. By contrast, normal cells do not release appreciable quantities of these microRNAs [88]. An additional group of released microRNAs are those released in levels equal to those appearing within the cell, termed ‘neutrally’ released microRNAs. These neutrally released microRNAs include miR16 and miR21, where the abundance in exosomes reflects that in the tumour cells. The selective release of specific microRNAs differs depending on the cell type and appears to be influenced by malignant transformation. Breast and ovarian tumour cells have been demonstrated to release greater than 99% of the miR451 and miR1246 produced by the cells [88–90]. These selectively released microRNAs have been linked to the malignant phenotype. miR451 has been identified as a tumour suppressor, defining proliferation and cell polarity. miR451 has also been shown to induce chemosensitivity. miR1246 induces p53-dependent apoptosis triggered by DNA damage [91]. The changes in the release

of cancer-related microRNAs may suggest a role for selective microRNA export in malignant transformation, and it may provide a cancer signature within the exported, circulating microRNA population. While the mechanism of this selective sorting is unclear, some researchers have postulated that this selectivity relates to microRNA/RISC components. Exosomes contain components of the microRNA/RISC, such as Argonaute 2, together with several RNA-binding proteins known to regulate RNA traffic between the nucleus and the cytoplasm. It can therefore be hypothesized that, during vesicle biogenesis, these RNA binding proteins regulate the accumulation of selected RNAs within exosomes. Studies on the transfer of reporter mRNAs and their translation into proteins, demonstrated both *in vitro* and *in vivo*, suggest that the mRNA delivered by exosomes is functional [92,93].

Most investigations on small RNAs in exosomes have been limited to microRNAs; however, next-generation sequencing of small RNAs in exosomes is expanding the populations identified. While intracellular microRNAs have been defined in many biological processes, identification of extracellular vesicle-associated microRNAs represents a non-invasive approach to investigate disease-specific microRNA and may provide a method for disease diagnosis [85]. To detect, analyse and quantitate the RNA signatures of exosomes derived from biologic fluids, several approaches have been used, including microarrays, quantitative real-time PCR and next-generation sequencing. The development of high detection sensitivity in next-generation sequencing technologies has expanded the identification of the exosomal transcriptome, beyond microRNA. While most studies have focused on exosomal microRNAs, we now recognize the presence of numerous other small RNAs within these circulating exosomes, as well as fragments of larger RNAs. These exosomal small non-coding RNAs are less than 200 nucleotides in length (generally 20–30 nt). There are several primary populations of small non-coding RNAs, including microRNAs and Piwi-interacting RNAs (piRNAs) [94]. Small non-coding RNAs have been shown to be key regulators in development, apoptosis, stem cell self-renewal, differentiation and cell integrity maintenance. piRNAs are generated from intergenic elements, including transposable elements, through Dicer-independent pathways. These piRNAs function through the Piwi-Argonaute sub-family (AGO3, Aubergine and Piwi), leading to silencing of transposable elements. A link between piRNAs and cancer has been demonstrated in gastric cancers where two aberrantly expressed piRNAs, piRNA-651 and piRNA-823, were found in gastric tumour tissue versus paired normal tissue [95,96].

9. Exosomes as diagnostic markers

Circulating biomarkers have been proposed to be promising for the definitive diagnosis and monitoring of therapeutic responses. Defining circulating biomarkers has numerous advantages as diagnostic biomarkers. Their presence can serve to identify processes that are difficult to image. The RNA cargoes of these exosomes may predict outcome by identifying patients at risk for therapeutic failure, defining molecular and pathological alterations for developing therapeutic targets and monitoring responses to interventions [97]. Such biomarkers could also serve to monitor disease progression and predict risk of recurrence. Circulating

biomarkers are problematic and exhibit several critical issues, since free protein and nucleic acid biomarkers are extremely unstable in the circulation. Thus, to detect these a high steady state must be reached for detection, which is generally not observed except in late-stage disease, and minor changes over time (essential for monitoring) are difficult to quantify, in addition to these biomarkers being sensitive to sample handling. The use of exosome-associated biomarkers appears to be capable of circumventing these issues.

Exosomes provide stable, disease-specific markers for detection, disease characterization and predicting prognosis [98]. Temporal changes in exosomal RNA profiles have been demonstrated to accurately predict disease recurrence and overall patient survival [99]. The proteomic and genomic profiles of circulating exosomes provide a real-time monitor of therapeutic response, serving as a companion diagnostic. By correlating these circulating markers with the molecular characteristics and real-time clinical parameters, our group as well as others has established the use of circulating exosomes as a 'liquid biopsy'. Since our original description of exosomal microRNA, many studies have examined the diagnostic utility of profiling total circulating microRNA in specific pathologies. The release of exosomal RNAs provides features with utility for diagnostic biomarkers, as they can be detected at early stages, are present in routinely obtained biologic fluids (blood, CSF, urine and saliva), are derived from specific tissues and can be easily and accurately quantified.

10. Conclusion

While the use of exosomes as biomarkers in the clinical setting is in the development phase, the findings presented within this review demonstrate their significant diagnostic potential in TBI. In the 6 years since our initial demonstration of the diagnostic utility of RNA profiles from exosomes, new and sensitive techniques have been developed, including deep sequencing and technologies based on microarrays and real-time PCR, enabling the monitoring of pathology-derived exosomes. The potential of using circulating exosomal proteins and RNAs as biomarkers extends beyond the diagnostic arena and provides biomarkers linked with patient stratification, therapy selection, monitoring of therapeutic responses and pathologic disruptions. Early definitive identification of TBI will be critical for significant impact in improving patient outcomes, particularly in patients without symptoms as well as to stratify a heterogeneous patient population. Further, it appears that exosomes not only are diagnostic of TBI, but also may be an essential causative agent in cases of its progression to long-term neurodegenerative conditions. Biomarkers can be used to determine the potential to develop progressive disease, measure its progress or predict prognosis. Identifying informative biomarkers is an exceptionally valuable tool for evaluating clinical trial outcomes and for assisting physicians in choosing and personalizing treatment options.

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