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REVIEW

Exosomes/microvesicles: mediators of cancer-associated immunosuppressive microenvironments

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Abstract Cancer 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. While only recently have the roles of these vesicular components in intercellular communication become elucidated, significant evidence has demonstrated that tumor 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. Immunosuppressive exosomes of tumor origin can be found within neoplastic lesions and in biologic fluids from cancer patients, implying a potential role of these pathways in in vivo tumor progression and systemic paraneoplastic syndromes. Through the expression of molecules involved in angiogenesis promotion, stromal remodeling, signaling pathway activation through growth factor/receptor transfer, chemoresistance, and genetic intercellular exchange, tumor exosomes could represent a central mediator of the tumor microenvironment. By understanding the nature of these tumor-derived exosomes/microvesicles and their roles in mediating cancer progression and modulating the host immune response will significantly impact therapeutic approaches targeting exosomes.

Keywords Exosomes · Cancer · Immunosuppression · Microenvironment

Introduction

The release of nano-sized membranous vesicles by viable tumors was initially described by our group over three decades ago [1] and has since been verified in multiple tumor and cell types. These membranous vesicles have been identified by various terms, from "high molecular weight complexes," "membrane fragments," "microvesicles," "microparticles," and "exosomes." While restrictive definitions have been applied to these vesicular structures [2], considerable overlap exists between various circulating cell-derived vesicles isolated from cancer patients, suggesting the distinctions may not be clear-cut, and these different terms may include the same components.

The term "exosome" was coined by Trams et al. in 1981 [3] for "exfoliated membrane vesicles with 5′-nucleotidase activity." The "exosome" term originated from a discovery of the secretion of neoplastic cell line-derived exfoliated vesicles, which mirrored the 5′-nucleotidase activity of the parent cells [3]. Subsequently, the canonical pathway of "vesicle" release following multivesicular endosome fusion with the cell surface was demonstrated in cultured sheep [4] and rat [5] reticulocytes. After purification by ultracentrifugation, the sedimented microvesicles were found to contain transferrin receptors, which were also found in

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native reticulocytes [6]. These microvesicles were redefined as "exosomes" [7].

The release of tumor-derived microvesicles was initially demonstrated in ovarian cancer patients [1, 8, 9]. Within this patient population, intact membrane fragments or vesicles from the peripheral circulation and malignant effusions (ascites and cyst fluids) were found to express molecular markers that were inherent to the tumor plasma membrane, 5′-nucleotidase, and placental-type alkaline phosphatase [10, 11]. In addition to tumor cells and embryonic cells, microvesicles/exosomes are released by a variety of cells, particularly activated cells of the immune system, including dendritic cells, macrophages, B cells, T cells, and NK cells [12–14]. We now recognize that these shed microvesicles are key intercellular communication vehicles, serving to regulate normal immune responses. Exosomes from activated dendritic cells can present antigens in the context of MHC II to T cells [15]. Exosomes from activated T cells can mediate "activation-induced cell death" in a cell-autonomous manner, defined by the nature of the initial T cell activation events and can play central roles in both central and peripheral deletion events involved in tolerance and homeostasis [16]. Exosomes released by tumors may elicit a tolerogenic response and participate in other immune mechanisms, such as platelet activation, mast cell degranulation, germinal center reaction, and potential engulfment of apoptotic cells. Since, under normal condition, microvesicles/exosomes are enriched in MHC class I and II antigens and costimulatory molecules, they are thought to be an alternative antigen delivery pathway mediated by cell surface molecules. The aberrant release of exosomes by tumors may allow them to circumvent these immunoregulatory antigen delivery pathways and evade immunosurveillance [10].

Microvesicle/exosome formation

There are multiple mechanisms leading to the release of cellular components into the extracellular space. Three mechanisms have been proposed for the release of membranous vesicles. These are exocytic fusion of multivesicular bodies (MVBs) resulting in exosomes, budding of vesicles directly from the plasma membranes resulting in shed "microvesicles," and cell death leading to apoptotic bodies [17]. The first two mechanisms are properties of viable cells and are energy-requiring events. While most isolation protocols readily exclude apoptotic bodies, they do not differentiate exosomes from shed "microvesicles." Thus, these extracellular vesicle populations may include a mixture of exosomes and microvesicles, which may confuse interpretation of biochemical data. In terms of their characteristics, exosomes/microvesicles derived from the extracellular environment of tumors, either in vitro or in vivo, exhibit overlapping similarities in size (defined by dynamic light scattering), morphology (defined by electron microscopy), density (defined by of sucrose gradient centrifugation), and protein markers of both the endosomes and plasma membranes (defined by western immunoblotting and mass spectrometry) [18, 19]. We have compared the vesicle populations obtained from biologic fluids of ovarian cancer patients by both the technique described to isolate exosomes and our original chromatographic method isolating "microvesicles" [18]. This comparative study demonstrated that these in vivo-derived vesicles from both techniques isolated cup-shaped vesicles, with a density between 1.13 and 1.17 g/ml, a diameter between 50 and 100 nm, and expressing CD63, Alix, VPS35, galectin 3, HSP90, fibronectin, and placental alkaline phosphatase (Fig. 1). Thus, these patient-derived circulating vesicles fall within the definition of exosomes. However, it is unclear whether this distinction between exosomes and shed microvesicles is critical to understand the biologic activities of these vesicles as they can interact with target cells of the host as a mixture. This review focuses on the vesicle populations actively released by viable cells.

The increased release of exosomes/microvesicles and their accumulation appear to be important in the malignant transformation process. Although extracellular shedding of exosomes occurs in other types of cells under specific physiological conditions, the accumulation of membranous vesicles from non-neoplastic cells is rarely observed, in vivo [20]. In contrast, exosomes released by tumor cells accumulate in biologic fluids, including sera, urine, ascites, and pleural fluids. Exosome release and its accumulation appear to be important features of the malignant transformation. Shed tumor-derived exosomes do not mirror the general composition of the plasma membrane of the originating tumor cells, but represent 'micromaps,' with enhanced expression of tumor antigens [21, 22].

While the exact mechanisms of exosome/microvesicle release remain unclear, this release is an energy-requiring phenomenon, modulated by extracellular signals. The most common process is the release of large biomolecules through the plasma membrane by a process termed exocytosis, which has regulatory and signaling functions. Exocytosis can be either constitutive (non-calcium-triggered) or regulated (calcium-triggered) [23]. Constitutive exocytosis occurs in all cells and serves to secrete extracellular matrix components or to incorporate newly synthesized proteins into the plasma membrane following fusion with transport vesicles. Regulated exocytosis is critical to events, such as neurologic signaling, as synaptic vesicles fuse with the membrane at the synaptic cleft. The formation of these endosomes is initiated cell surface-mediated invagination to generate endocytic vesicles that migrate and fuse with the

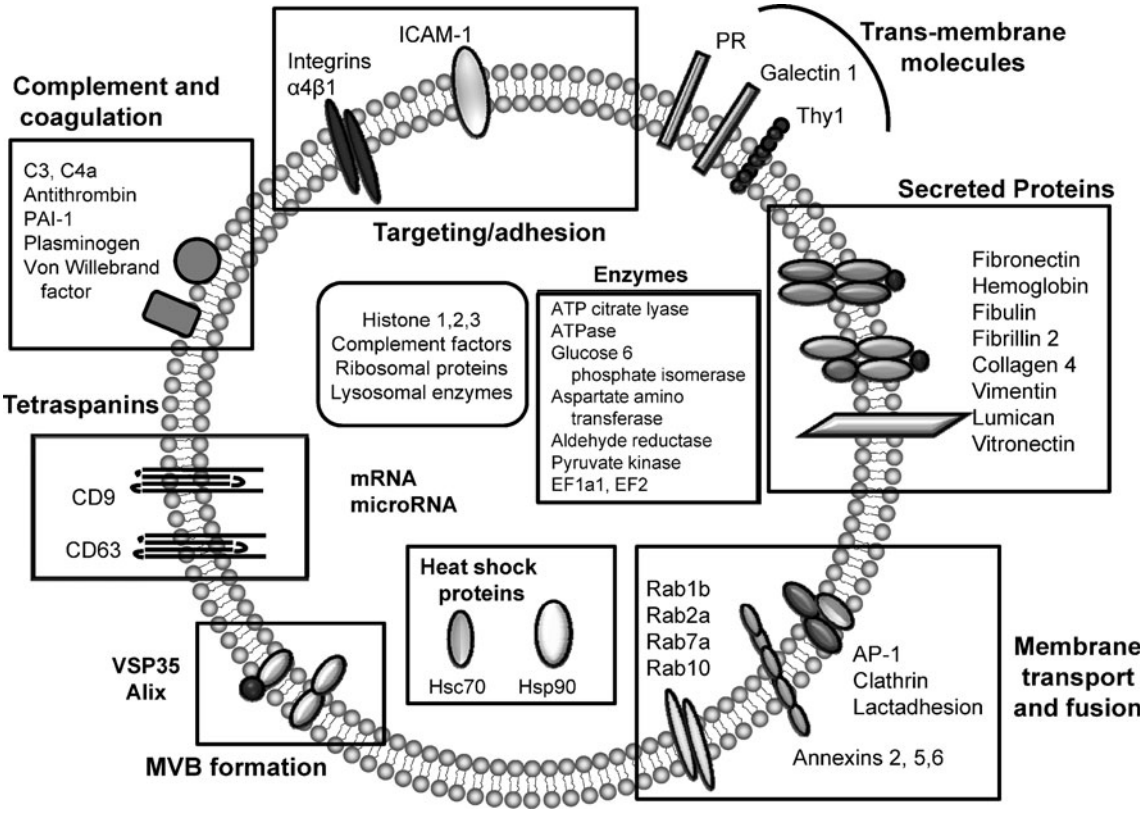

Fig. 1 Schematic diagram of a tumor-derived exosome, presenting protein components defined by ion trap mass spectrometry

early endosome [24]. Exosomes appear to form by invagination and budding from the limiting membrane of late endosomes, resulting in vesicles that contain cytosol and that expose the extracellular domain of transferring receptors at their surface. Using electron microscopy, studies have shown fusion profiles of multivesicular endosomes with the plasma membrane, leading to the secretion of the internal vesicles into the extracellular environment [24].

Since the formation of these membrane vesicles has an endocytic origin, this mechanism is a secretion process of the endosomal system, including endocytic vesicles, early endosomes, late endosomes, and lysosomes. These endocytic vesicles form through clathrin- or non-clathrin-mediated endocytosis at the plasma membrane and are transported to early endosomes [25]. The late endosomes develop from early endosomes via acidification, changes in their protein content, and their ability to fuse with vesicles or other cellular membranes. Early endosomes are localized near the outer margin of the cells and exhibit a tubular appearance, in contrast, late endosomes are localized proximal to the nucleus and are spherical in shape. A critical step in the formation of MVBs from late endosomes is reversed budding. In this step, limiting membranes of late endosomes "bud" into their lumen, resulting in a continuous enrichment of internal luminal vesicles [26] (http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B6T75-4M4KJ04-1&_user=134779&_coverDate=11%2F15%2F2006&_rdoc=1&_fmt=high&_orig=search&_origin=-search&_sort=d&_docanchor=&view=-c&_acct=C000011238&_version=1&_urlVersion=0&_userid=134779&md5=347321f447ba003d55a8380cc328666d&-searchtype=a-bib6). MVBs have been demonstrated to be involved in the exocytic fusion of their external membrane with the plasma membrane of the cell, resulting in release of their segregated vesicles to the extracellular space.

The molecular composition of the cell surface can be modulated by endosomes, facilitating segregation of proteins destined for degradation from proteins destined to be recycled [27]. As a consequence of multiprotein complex, the Endosomal Sorting Complexes Required for Transport (ESCRTs), specific proteins and lipids of the MVB external membrane are enriched in the exosomes, while other components are excluded. The formation of MVBs relies on ubiquitin-binding proteins [28]. The protein-sorting mechanisms include ubiquitination of the target protein and preferential sorting. The ligation of one ubiquitin molecule (mono-ubiquitinylation) can serve as a signal for endocytosis and segregation into MVBs; however, the attachment of multiple ubiquitin molecules (poly-ubiquitinylation) can earmark proteins for degradation in the proteasome [29]. Some studies have also suggested that oligoubiquitination may also be a sorting signal for

trafficking to MVBs, which may increase MVB sorting efficiency. ESCRT-I, -II, and -III recognize monoubiquitinated cargoes and promote their inclusion in MVBs [30]. The ESCRT complex recognizes the ubiquitinylated proteins via vacuolar protein sorting (VPS)-27 [31]. Subsequently, VPS27 recruits an additional ESCRT complex and TSG101, activating AIP/Alix. This complex initiates sorting specific proteins into the budding vesicles. Although mono-ubiquitinylation prompts their uptake into MVBs, not all proteins in exosomes are ubiquitinylated, potentially resulting from a passive mechanism involved in protein sorting to MVBs. It has been proposed that the signals responsible for the "passive" processes are, in some cases, the presence of tetraspanin-enriched [32] or cholesterol-enriched (i.e., lipid rafts) membrane microdomains [33]. A similar but unclear mechanism is potentially associated with the accumulation of specific cytosolic proteins in the exosome lumen.

Exosome/microvesicle characterization/composition

Exosomes have been described as microvesicles containing 5′-nucleotidase activity that are released from neoplastic cells [3]. These small nanovesicles, present within MVBs (endosomes), contain transferrin receptors, a marker used to identify endocytosis and recycling of internalized plasma membrane proteins [34]. Biophysically, exosomes are equivalent to cytoplasm enclosed in a lipid bilayer with the external domains of transmembrane proteins exposed to the extracellular environment. Exosome composition varies depending on the cell type of origin.

The lipid and protein content of exosomes has been extensively analyzed by various techniques including western immunoblotting, fluorescence-activated cell sorting, immuno-electron microscopy, and mass spectrometry.

Ultrastructural studies, western blot, and mass spectrometry analysis of exosomes from different sources have allowed the distinction between commonly expressed proteins, as well as cell type-specific proteins [22, 35]. Exosomes contain a number of common protein components. Based on their endosomal origin, exosomes, independent of the parental cell of origin, exhibit multiple proteins involved in MVB formation (annexins, Rab family GTPases, and ESCRT complex proteins (TSG101, Alix)) [36]. Additional protein markers linked with exosomes include tetraspanins (CD81, CD63, and CD9) and heat shock proteins (HSP60, HSP70, and HSP90) [22]. Exosome-associated HSP70 and HSP90 can facilitate peptide loading onto MHC class I and class II proteins. Exosome express cell type-specific markers, such as class I and II MHC, co-stimulatory proteins (CD80 and CD86) on antigen presenting cell-derived exosomes [37], integrin CD41a and Von Willebrand factor on platelet-derived exosomes [38], and perforin and granzyme on cytotoxic T lymphocyte-derived exosomes [39]. The cytosolic proteins present on exosomes include Rabs, which promote exosome docking and the membrane fusion events [40]. The annexins, including annexin I, II, V, and VI, may regulate membrane cytoskeleton dynamics and membrane fusion events [41]. Adhesion molecules, including intercellular adhesion molecule-1, CD146, CD9, EGFRvIII, CD18, CD11a, CD11b, CD11c, CD166, and LFA-3/CD58, have also been identified in exosomal preparations [42, 43]. One of the characteristic features of exosomes is the presence of tetraspanins, including CD9, CD63, CD81, and CD82 [44]. Exosomes are also enriched in proteins that participate in vesicle formation and trafficking, such as the lysobisphosphatidic acid (LBPA)-binding protein, Alix [45]. Other proteins demonstrated to be present exosomes include metabolic enzymes, such as peroxidases, pyruvate and lipid kinases, and enolase-1 [46].

Recently, we have analyzed the patient-derived exosomal proteome using ion trap mass spectrometry (Table 1). This study identified 232 unique proteins. These proteins were classified as percent 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 acids (2.1%) metabolisms, proteins involved in DNA replication (6.9%), messenger RNA (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, and myosins). We identified that transmembrane proteins were also abundant, including multiple integrins (β1, α3, and αv), 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 of sodium/potassium-transporting ATPase subunit α-1, and transitional endoplasmic reticulum ATPase. In line with their endocytic origin, exosomal proteins belonging to the ESCRT complex that are important protein complexes involved in ubiquitin-dependent exosome biogenesis have also been observed. 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

Table 1 Proteomic analysis of tumor-derived exosomes, isolated from the peripheral circulation and defined by ion trap mass spectrometry

Name	Name
Abhydrolase domain containing protein 12	Guanine nucleotide binding subunit beta 4
Alpha-1 acid glycoprotein-1	Haptoglobin
Alpha-1-antitrypsin	Heparin cofactor 2
Alpha-2 macroglobulin	Immunoglobulins
Annexin A2	Insulin-like binding protein 3
Ankrd26-like family C member	Keratin type I and II
Antithrombin-III	Kininogen 1
Apolipoprotein (A, B-100, D, H)	Lipopolysaccharide binding protein
Armadillo repeat containing protein	Moesin
Calmodulin	Profililin-1
Cellular retinoic acid binding protein 2	Serum amyloid
Choline transporter-like protein 4	Serum paraoxonase/arylesterase
Complement and complement fragments	Talin
Disheveled associated activator of morphogenesis 2	Triose phosphate isomerase
Ezrin	Trypsin 3
Fibrinogen	Ubiquitin
Fibronectin	Vitronectin
Galectin 3 binding protein	

(annexin A2, A5, A6, clathrin heavy chain 1/2, coatomer 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 chaperonnes were identified (HSP70, HSP90, HSC70, HSPA4, -8, -9, HSPA1A/B, HSPB1, HSP47, HSPA5, HSPβ1, HSPD1, HSP90AB1, B1, AA1; T-complex protein 1, endoplasmin, and protein disulfide-isomerase A3, A4, and A6).

The initial identification of exosome release by tumor cells was envisioned as the discovery of a new cell-free source of tumor antigens for in vivo immune priming or tumor vaccine design [47]. Exosomes are close replicas of the originating cells in terms of selected protein content and express a large array of tumor antigens when secreted by neoplastic cells, including highly immunogenic antigens MelanA/Mart-1 and gp100; colon carcinoma cells express CEA and HER2. This antigenic content is not only a feature of in vitro-released exosomes but also can be found in microvesicles isolated from plasma of cancer patients as well, evidence that demonstrates the tumor origin of these organelles [48].

Exosomes as vehicles for intercellular communication

Tumor-secreted exosomes have recently gained increased attention as a "vehicle" for intercellular communication with extensive autocrine/paracrine functions. One of the most important functions of cell-derived microvesicles/exosomes appears to be intercellular communication. By exposing cell type-specific adhesion receptors or ligands, exosomes can interact with specific cells and deliver their "signals," including bioactive lipids, cytokines, growth factors, receptors, and genetic materials. Thus, the microvesicle/exosomal pathway may constitute a mechanism for local and systemic intercellular transfer of information, with a complexity superior to that of secreted soluble factors, but similar to that observed with direct cell–cell contact.

Exosomes provide stable conformational conditions for their protein content (due to maintenance of their three-dimensional transmembrane structure), conserve bioactivity of their proteins (based on the protective membrane structure), improve bio-distribution (based on their capacity to circulate in biologic fluids and migrate to secondary sites), and support an efficient interaction with target cells (due to the fusogenic properties of exosomes) [49, 50]. Due to these features, tumor-derived exosomes are efficient platforms for the in vivo transfer of cross-talk signals. The multiplicity of bioactive molecules associated with exosomes suggests that they exhibit a central role in generating the tumor microenvironment [51, 52]. Exosomes have the ability to transfer specific proteins to homologous and heterologous target cells for the delivery of signaling pathways [53, 54]. The presence of tumor-derived exosomes can increase matrix metalloproteinase (MMP) secretion and VEGF expression in target cells through the expression of proangiogenic molecules, such as members of the tetraspanin family, thereby promoting neoangiogenesis even at secondary metastatic sites [55]. 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) [56].

Studies have shown that cancer ascites-derived exosomes carry extracellular matrix-remodeling enzymes, such as metalloproteinases 2 and 9 (MMP-2 and MMP-9) [57, 58], and urokinase plasminogen activator [59], leading to an increase in extracellular matrix degradation. The expression of matrix-remodeling enzymes increases the tumor's invasive phenotype and promotes metastasis. The presence of proangiogenic factors supports neovascularization of the developing tumor. A common cellular component of the tumor microenvironment is the monocyte/macrophage. Within the microenvironment, these tumor-associated macrophages have been shown to assist in tumor progression by angiogenesis, growth, metastasis, and immunosuppression [60].

When shed vesicles fuse with their target cells, they can transfer important membrane components, such as receptors and ligands. The transferring of receptors between exosomes and 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 a specific antigen to CD4 T cells. Exosomes can transfer the adhesion molecule CD41 from platelets to endothelial cells or to tumor cells, conferring pro-adhesive properties to the target cell [62]. Exosome-mediated transfer of Fas ligand from tumor cells induces apoptosis of activated T cells favoring tumor immune escape [63]. Exosomes can also be protective for cells that remove from their membranes to the extracellular compartment the potentially harmful molecules, such as Fas or the membrane attack complex.

Exosomes have also been postulated to contribute to the spread of infective agents, such as human immunodeficiency virus (HIV) type 1 [64]. 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 HIV type I by released exosomes, can enhance the entry of the virus into cell types other than the lympho-hemopoietic lineage [65]. In addition to transferring receptors, exosomes can transfer viruses, contained within exosomes, by the "Trojan exosome hypothesis" involving direct delivery [66].

In human gliomas, only a fraction of the cells, exhibiting a transformed phenotype, expressed the truncated epidermal growth factor receptor, EGFRvIII, associated with dysregulated tumor growth [67]. Al-Nedawi et al. [68] 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, lacking the receptor, were transformed to express EGFRvIII-regulated genes, including VEGF, Bcl-x_L, and p27 [69]. Subsequent studies demonstrated that the oncogenic EGFRvIII from human squamous cell carcinoma cells was transferred via exosomes to tumor-associated endothelial cells to activate MAPK and Akt cell signaling pathways and promote endothelial VEGF expression [69].

The occurrence of epigenetic changes has been frequently reported in cancer. Epigenetic regulation of gene transcription, mediating cell proliferation, differentiation, and survival are additional targets in tumor progression, resulting in genomic instability [70]. One explanation for this genomic instability lies in the mediation by microvesicular horizontal transfer [71]. Horizontal transfer via microvesicles has been validated in a number of tumor-associated cells including gliomas, monocytes, mast cells, and T cells [72]. One explanation of this phenomenon is the transfer of genetic information between cells. It has been shown that tumor-derived exosomes may transfer not only surface determinants but also mRNA of tumor cells to monocytes. Janowska-Wieczorek et al. [73] 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 hematopoietic stem/progenitor cells, to induce upregulation of early pluripotent and early hematopoietic 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 [74]. As RNase treatment inhibited their exosome-mediated biological effect, the involvement of mRNA in the observed biological effects was suggested. Yuan et al. [75] 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 opens the possibility that stem cells can alter the expression of genes in neighboring 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.

It has been suggested that tumor cell progression could use multiple forms of exosome/microvesicle-mediated communication to simultaneously affect multiple effector populations, based on release of tissue factors, immunoregulators, and oncogenic molecules. Thus, the signals transferred to neighboring cells via exosomes may mirror

the transcriptional status of the parent cell, but due to the exosomal mRNA and microRNA being transferred, their consequences on the translational machinery of the target cells are extensive.

Exosomes/microvesicles as mediators immune regulation

Cancer cells are postulated to modulate components of the microenvironment and affect immune system function, primarily through pathways involving cell-to-cell contact and the release of suppressive soluble factors. However, a unique alternative mechanism has emerged that involves the active release of immunosuppressive microvesicles/exosomes by tumor cells [76, 77]. As tumor-derived microvesicles/exosomes are abundant in the blood and malignant effusions derived from cancer patients [78, 79], their release appears to be important features of intercellular communication. Evidence supports the concept that tumors constitutively shed exosomes with pleiotropic immunosuppressive effects [80, 81] that are protective and supportive of the tumor to facilitate escape from lymphocyte immunosurveillance [82]. Since released exosomes express molecules with biologic activity (such as Fas ligand, PD-1, MICA/B, mdr1, MMPs, CD44, and autoreactive antigens), the ability of exosomes to modulate lymphocyte and monocyte functions has been analyzed [83–85]. The immunological significance of exosomes, while far from clear, has been linked with their potential to modulate the host's immune system, which may be their major function. Supporting this view is that the topology of the macromolecules displayed on exosomes is analogous to that observed on the tumor's plasma membrane, making them well positioned for interactions with target cell surface receptors. This positioning can mediate signal transduction without the need for direct cell–cell contact. Further, these microvesicles can also fuse with the recipient cell, leading to the acquisition of novel molecules by the cells and the delivery of mRNA and miRNA through this route.

Tumor exosome release has been described as capable of modulating the evasion from anti-tumor immune responses [86]. It has been suggested that the anti-tumor immune response can be divided into three different phases, and tumor-derived microvesicles/exosomes can exhibit roles in each phase [87]. The first step includes the recognition of tumor cells by innate immune cells. The progression and development of tumors is coordinated by biochemical and biophysical signals from the tumor microenvironment [88]. After growing to a certain threshold size, solid tumors exceed their capacity to acquire oxygen and nutrients in this hypoxic environment [89, 90]. For tumor progression to occur, the "angiogenic switch" has been reported to be required [91]. The "angiogenic switch" promotes the transition of the tumor to a highly vascularized and progressive outgrowth [51]. This process further induces extracellular matrix remodeling and the production of a pro-inflammatory environment leading to the recruitment of innate immune cells, including NK, macrophages, and dendritic cells and the mediation of T cells into the tumor microenvironment [57–59].

Recent studies have demonstrated that the MHC class I-related chain (MIC) A and MICB ligands for the NK cell activating receptor NKG2D is released by tumor cells as a component of exosomes [92]. This shedding of MICA/B by tumors not only prevents recognition of MICA/B-expressing tumor cells but also results in the downregulation of NKG2D expression on circulating CD8 T cells, NK cells, and γδT cells, leading to impairment of the antitumor immune response [93]. Ashiru et al. [92] demonstrated that treatment of NK cells with MICA-expressing exosomes suppressed NK expression of NKG2D on the cell surface and also suppressed NK cytotoxicity, which is independent of NKG2D ligand expression. Thus, their findings demonstrated exosomal MICA/B expression as a mechanism of NK suppression, facilitating immune escape and progression.

The second phase of an anti-tumor immune response involves the maturation and migration of tissue dendritic cells and priming of naïve T cells. Blood-derived exosomes from melanoma patients have been shown to promote the generation of myeloid-derived suppressor cells (MDSCs) from peripheral blood monocytes [94], which acts as one of the major mechanisms used by tumors to escape immune recognition [95]. MDSCs have potent immunosuppressive functions that can suppress T cell immune responses by a variety of mechanisms [96–99]. Further, tumor exosomes have been shown to be involved in the regulation of the adaptive immune responses to cancer cells in animal models and cancer patients [12, 100] by impairing peripheral blood monocyte differentiation into dendritic cells [101]. As a result, the generation of tumor-specific T cells has been reported to be a very inefficient process. Most importantly, tumor exosomes have the ability to induce a series of functional defects in tumor reactive-effector T cells [102], through the expression of apoptosis-inducing ligands, such as FasL and TRAIL [103] or PDL-2, which directly stimulate their T cell targets to negatively regulate T cell activation [104]. For example, ovarian cancer patient-derived exosomes inhibit T cell functions by increasing expression of FasL (on the exosome surface) and suppressing CD3-zeta (on the T cells) to collectively induce T cell apoptosis [105]. An investigation of human prostate cancer exosomes added to activated T cells exposed a dose-dependent inhibition of $CD8^+$ T cell proliferation stimulated by Fas–FasL interaction [106]. Additionally,

tumor-derived exosomes block innate immune effector cell function as seen in NK cells via production of exosome-associated MICA/B to downregulate NKG2D expression, thereby decreasing NKG2D-mediated killing [107, 108]. Production of tumor exosome-associated MICA/B and FasL has been shown to decrease the effectiveness of not only the innate immune system but also the adaptive immune system to reject the tumor [109, 110]. It has been theorized that these released exosomes modulate lymphocyte functions by mimicking "activation induced cell death" (AICD) [111, 112]. Lymphoid cells appear to release exosomes following activation, and these appear to play an essential role in immunoregulation, by preventing excessive immune responses and the development of autoimmunity [113]. It was postulated that exosome release by tumor cells is a re-expression of the fetal cell exosomes and that both constituted pathways to circumvent immunosurveillance.

The third and final phase involved in the anti-tumor response is the generation and homing of tumor-specific T cells. Tumor-reactive $CD4^+$ and $CD8^+$ T cells homing to the primary tumor site is an important step in eradicating the tumor [114, 115]. Tumor exosomes express membrane bound ICAM-1 that efficiently blocks the interaction between lymphocytes and endothelial cells [42] and therefore decreases the recruitment of adaptive immune cells. Several studies have shown that tumor-infiltrating T cells are impaired by the tumor and display altered expression in intracellular signal transduction molecules such as CD3-zeta [108, 116]. Tumor exosomes co-incubation with T cells leads to a decrease in CD3-zeta, which suggests that tumor exosomes may be an additional mechanism used by tumors to evade immune recognition [82, 108]. The alteration of TCR-CD3-zeta has been observed in several types of tumors namely malignant melanoma [117], ovarian [118], and pancreatic [119]. Furthermore, studies have reported that cancer patients display a high frequency of suppressive peripheral blood regulatory T cells when compared to normal controls [120–122]. These cells have been shown to infiltrate the tumor and are involved in the induction of $CD8^+$ tumor-reactive cytotoxic T lymphocyte (CTL) apoptosis [42, 123, 124]. A study from Szajnik et al. shows that tumor-derived microvesicles expand and promote the suppressive activities of human regulatory T cells (Treg) by upregulating the expression of FasL, interleukin (IL)-10, TGF-β1, CTLA-4, granzyme B, and perforin [125]. Therefore, the immunoregulatory properties attributed to tumor-derived exosomes might be essential in regulating peripheral tolerance and promoting immune evasion of tumors [53]. Collectively, these studies support a role for exosomes in adapting the host microenvironment to allow escape from immune surveillance via stimulation of angiogenesis and metastasis of tumors [126, 127], which suggests that tumors may use exosomes to keep the host immune system under control without a direct interaction with host immune cells.

Critical components of the immune response, such as antigen presenting cells, are significantly affected by interactions with tumor exosomes. These microvesicles not only impair the capacity of circulating monocytes to differentiate into functional DCs but they also skew the differentiation of these cells towards altered $CD14^+$ monocytes expressing low or absent levels of HLA-DR [128]. These cells, which are present in relatively high numbers in PBMCs of melanoma patients, exert suppressive activity on lymphocyte proliferation and impair the expression of effector molecules (such as perforin and IFN-γ) in a TGF-β-mediated fashion. $CD14^+HLA\text{-}DR^-$/low cells behave as MSC, undergoing in vivo expansion upon administration of GM-CSF. These hallmark alterations induced by tumor microvesicles on target immune cells in vitro can also be detected on immune cells isolated from cancer patients, which supports the hypothesis that these suppressive pathways are present in vivo.

These exosomes activated a stronger pro-inflammatory response in the form of NF-κB activation and TNF-α release from untreated macrophages as compared to macrophages exposed to control exosomes. Parallel evaluations of the structural components of tumor-derived microvesicles demonstrated enhanced expression of tumor antigens on the vesicular surface [129, 130]. In murine B16 melanoma cultures, the expression of surface glycoproteins on the isolated microvesicles represented a profile similar to that found in the melanoma cell membrane [130]. Continued characterization of tumor-derived microvesicles was achieved through biochemical analyses, which identified molecules with immunologic and biologic activity. Microvesicles released by late stage tumor cells were found to exert a dose-dependent suppression of MHC II in monocytes/macrophages in comparison to early stage tumor cells [131, 132]. In addition, microvesicles suppressed lymphocyte activation induced by phytohemoglutinin, anti-CD3, concanavalin A [10, 11, 130], and IL-2 [133].

As evidence of the pleiotropic effect of tumor-secreted exosomes, tumor exosomes can interfere directly with T cell effector functions. Exosomal expression of bioactive FasL and TRAIL has been shown on exosomes derived from human tumors to induce apoptosis in activated tumor-specific T cells. This phenomenon highly resembles the one utilized under physiological conditions not only by T cells to downsize immune responses [134] but also by placenta cells that recently have been shown to promote a state of immune privilege by inducing FasL-mediated apoptosis and defects in the expression of crucial TCR signaling components (such as CD3-zeta and JAK3) in local T cells, which have been reported with microvesicles isolated from

plasma of cancer patients and may help to explain the high frequency of apoptotic or CD3-zeta$^-$ lymphocytes that are often found in the peripheral circulation of these patients [135, 136]. Natural killer cells lose their cytolytic potential, through the suppression perforin expression, upon encounter with tumor-secreted microvesicles.

A pro-inflammatory microenvironment is associated with the development and progression of cancer. Macrophages are prominent in the development of the pro-inflammatory environment, with IL-1β serving as a "master" cytokine regulator. Macrophages are critical for the resolution of inflammation by producing anti-inflammatory cytokines and chemokines and by increasing phagocytic activity. Based on Th1/Th2 polarization, phenotypically polarized macrophages are termed pro-inflammatory M1 (classically activated) and anti-inflammatory M2 (alternatively activated) [137]. In vitro, macrophages can be polarized to the M1 state by treatment with IFN-γ and inducers of TNF-α, such as lipopolysaccharide (LPS) [138]. These M1 macrophages induce synthesis of pro-inflammatory cytokines and chemokines, including TNF-α, IL-12, IL-6, CCL2, and IL-1β, as well as increased production of reactive oxygen species [139, 140]. Elevated levels of IL-1β are present in M1 polarized macrophages due to activation of the NF-κB and MAPK pathways [141], while no IL-1β protein is found in M2 polarized macrophages [142]. Using cytochalasin D, a known inhibitor of actin polymerization, while a dramatic suppression of exosome internalization was observed, this internalization was not essential for the induction of IL-1β mRNA and protein, following the exposure to exosomes.

IL-1β exhibits profound effects on immune cell function during inflammation. Components of the extracellular matrices have been demonstrated to be capable of stimulating the expression of IL-1β [143]. Fibronectin is highly expressed in injured tissues [144] and appears to be positioned to modulate the expression of IL-1β in diseased tissues [143]. In vitro, fibronectin stimulates the expression of IL-1β mRNA and its translation into the 31-kDa intracellular precursor protein, along with secretion of the 17-kDa active form in human mononuclear cells [145]. This effect of fibronectin is mediated by specific cell surface α5β1 integrin receptor, which activates poorly understood intracellular signals to induce IL-1β expression [146]. Fibronectin contains a sequence, termed Arg-Gly-Asp (RGD), which promotes its attachment to integrin receptors [147]. Monocytes and macrophages have been shown to possess fibronectin receptors that recognize the RGD motif and mediate pro-inflammatory cytokine production. The effect of fibronectin has been shown to be dependent on binding of the RGD sequence of fibronectin to integrin receptors, as this effect could be inhibited by integrin receptor blocking peptides (anti-RGD sequence

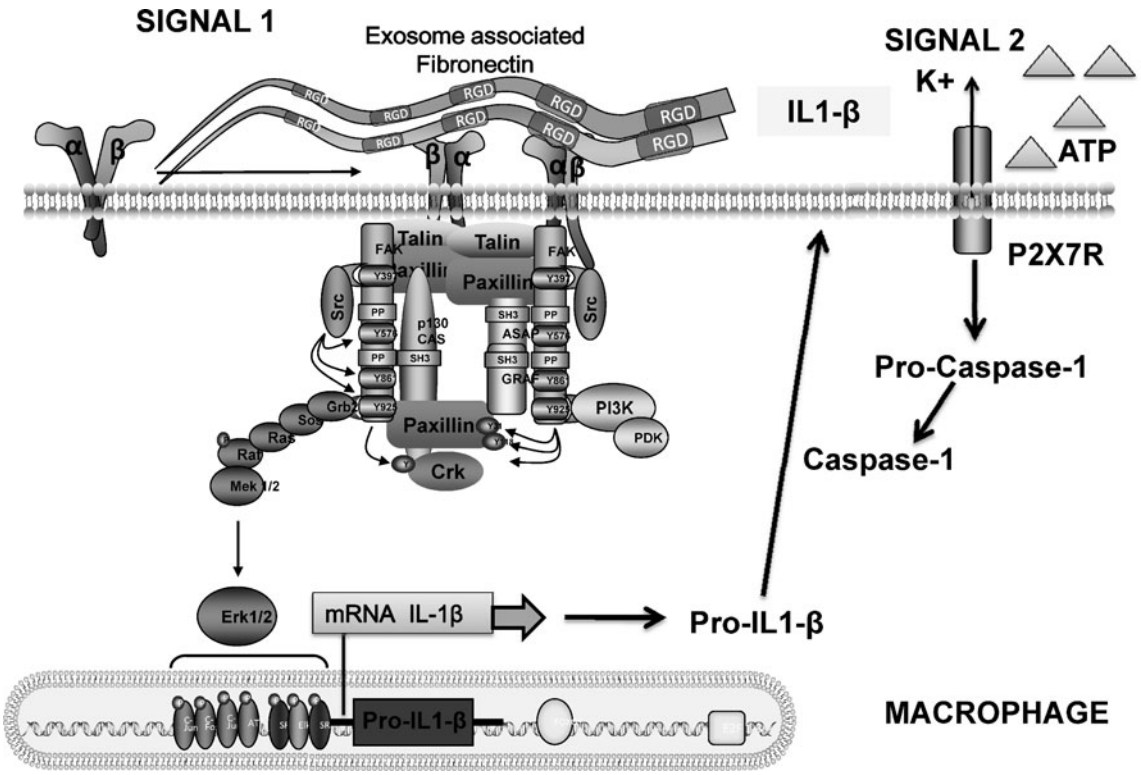

Fig. 2 Schematic presenting exosome-associated fibronectin induction and release of IL1-β by macrophages

mimics) [148]. The use of RGD antagonists demonstrates that exosome-induced IL-1β production by macrophages is mediated by exosome-associated fibronectin (Fig. 2).

Shedding vesicles released by various cell types are now known to participate as well [149, 150]. Their role can vary depending on the stage of the process. At an early stage, the vesicles shed by neutrophils stimulate the release of anti-inflammatory factors such as TGFβ1 and IL-10 from macrophages with reduction of TNFα and IL-8 [151, 152]. At later time points, however, shed vesicles can become pro-inflammatory, mediating the transfer of chemokine receptors, such as CCR4 and CCR5, and stimulating release of other mediators, such as IL-6 and the monocyte chemotactic protein 1 (MCP1), which induce and strengthen inflammatory responses [153].

Conclusions

It has become increasingly clear, as new exosome studies are published, that these small bioactive vesicles are important in a number of biological functions. Exosomes exhibit important roles in intercellular communication, and under normal conditions, this communication mediates the activation of the immune response. However, in cancer, tumor exosomes can induce apoptosis of activated cytotoxic T cells, impairment of monocyte differentiation, induction of myeloid-suppressive cells and T regulatory cells, and suppression of lymphoid activation signaling molecules. Tumor-derived exosomes express molecules involved in angiogenesis promotion, stromal remodeling, signaling pathway activation through growth factor/receptor transfer, chemoresistance, and genetic intercellular exchange. Tumor exosomes induce a pro-inflammatory environment from macrophages due to expression of exosomal fibronectin. As a result of these exosomal effects, they can represent a central mediator of the tumor-supportive microenvironment. From the removal of unwanted proteins from maturing reticulocytes to their role in immune surveillance, the inventory of functions continues to grow.

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