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Exosomes: Secreted Membrane Micro Vesicles that Mediate Intracellular Communications

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Abstract

Exosome is an nano-particle with diameter of 40-100nm, generated in endosomes with bioactive molecules incorporated into lipid bilayer envelope. The micro vesicles migrate and are fused with membrane, then released to intercellular space. The compositions of lipids, protein and microRNAs in exosomes exert different effects on the cells which capture the microparticles. They impact on the host immune response against viruses and tumors, and interactions between host and invading viruses, as manifested by facilitating or suppressing viral infection. The oncogenic proteins and miRNAs encoded by Epstein Barr virus contained in exosomes contribute to the transformation of the surrounding recipient cells, and exosomes containing genomic products of HIV faciliate or suppress the viral infection. In view of the action of immune regulation and combating invading pathogens, exosomes show promising in antitumor and antiviral vaccine development.

Introduction

Previously known as particles containing disposals of waste from cells, exosomes, a type of extracellular vesicles (EV) has been identified as a ultra-structural particle that convey messages to deliver to recipient cells. EDVs differ in size, origin, and inside components. The exosome is an EV of 40-150nm, formed as endosomal particle [1]. EVs play a role in the intercellular communication, through the transfer of biologically active molecules of lipids, proteins and microRNAs (miRNAs) [2]. As other type of EV, exosomes comprise lipid bilayer envelope, and enclosed compositions of lipids, proteins, and micro RNAs (miRNAs). They are exocytose on fused with the cytoplasmic membrane, and released to intercellular space, to exert different types of biological activities, like regulation of neuronal function and immune response, transmission of viral infection [3-5], and modulation of growth and proliferation of the surrounding cells which capture them. The viral proteins and nucleic acids are delivered by exosomes from infected cells to uninfected; this process is implicated in the spread of the infection [6] host antiviral immune response [7], and also contributes to such events like viral tumorigenicity [8,9]. A crucial role for exosomes in the viral life cycle as well as antiviral and antitumor immunity has been suggested.

Bioactive materials contained in exosomes are delivered from one cell to another [2,10-12]. The capture of exosomes by recipient cells is determined by fusion of vesicles to with cellular membranes mediated by a group of protein, tetraspanins, expressed on the EV membrane [13,14]. These molecules, as well as molecules enclosed in EVs (e.g., transcription factors and cytokines), constitute signals that can affect the function of recipient cells. Proteins on the surface of exosomes also determine adhesion to the plasma membrane of specific target cells. The intercellular adhesion molecule 1 (ICAM 1), present on dendritic cell (DC)-derived EVs, mediates EV recruitment by activated T cells and other DCs[15,16]. The combination of integrin proteins on tumor cell EVs was implicated in the delivery of exosomes to specific target organs, such interactions facilitate metastasis[17].

The Biogenesis and Incoporated Contents of Exosome

Generation and capture of exosomes

Exosomes are micro vesicles at nanometer scale, formed by the fusion of the endosome vesicles

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Table 1: Programs of EBV infection in latency and associated diseases.

Type of latency/expression profile/ disease	Gene expression pattern	miRNAs	Associated diseases
Latency I	EBNA 1	EBER 1 & 2, BARTS (miR-BART6)[a]	Burkitt´s Lymphoma, NPC (35%)
Latency II	EBNA 1, LMP 1, LMP 2b (& a), BARF 0	EBER 1 & 2, BARTs(miR-BART7 and miR-BART13) [b]	NPC (65%), Hodgkin´s Disease, T-cell lymphoma
Latency IIb	EBNA 1 & 2, BARF 0	EBER 1 & 2; BARTs; ebv-miR-BART4-5p[c,d]	Gastric carcinoma
Latency III	EBNA 1-6 LMP 1, LMP 2a & b	EBER 1 & 2, BHRF1 (miR-BHRF1-1, -2, and -3)[e]	Immunoblastic, Post-transplant & AIDS-lymphomas, Primary infection (IM); *In vitro* transformed LCL

Note: a: ref.4; 1b: ref.42; c: ref. 43; d: ref 44; e: ref 45.

with membrane; the particles are surrounded by a phospholipid bilayer (approximately 50–100nm in diameter), with size range roughly overlaps that of the viruses [18,19] they are secreted by a diverse range of cell types, released to the intercellular environment, and some migrate to distal site with circulation. Molecules of lipid, proteins, and microRNAs with biological activities are incorporated into these particles during the biogenesis of exosomes. Exosomes have been predominantly characterized in the immune competent cells including antigen presentation cells (dendritic cells, macrophages), T cells, B cells, and tumors, and exert functions of exosomes include antigen presentation [20] and immunostimulatory and inhibitory activities [21].

An early endosomes contain only few vesicles in their lumen, and the vesicles accumulate during the maturation of endosomes. The late endosomes are increased in size and accumulate internal vesicles, and are hence called Multiple Vesicle Bodies (MVB). During the maturation of late endosomes, biologically active molecules including the protein and lipid compositions in the small bodies are incorporated in exosomes, and exert their functions when released to extracellular microenvironment. Two steps of event occur during the protein sorting: The first step is the lateral segregation and selection of the membrane, and the second one is the inward budding of the vesicle with the concomitant incorporation of the selected cargo [20]. The surface molecules including receptors could also be engulfed by cytoplasmic membrane, to form endosome.

Activities of exosome released by immunoregulatory cells

Exosomes are regarded as the source of self-antigen for modulating the immune response against various self-tissues, notably tumors. The binding of exosomes to target cells in which the regulatory effects to be mounted involve the interactions of them with a class of surface molecules acting as receptor, and the target cells include antigen presentation cells (APC) in the marginal zones in the spleen, and follicular dendritic cells in the B cells region in the lymph nodes. [15,21-25]. Macrophages that capture exosomes are present in the marginal zone of the spleen and the lymph node sinus. And sialoadhesin (CD169; Siglec-1) captures B cell derived exosomes through α2,3-linked sialic acids expressed on their surface. Research suggested that Cd169 plays an essential role in the capturing exosomes, and immune response against exosomal antigens mediated by CD169 [26].

Immune Regulation by Exosomes Derived from Tumor Cells and Dendritic Cells

Immune regulation by Exosomes derived from tumor cells and dendritic cells: suppression of antitumor immunity

Exosomes, are recognized as a means of intercellular communication. The most important signals transduced by exosomes include immunoregulation. A major group of antigen presentation cells, dendritic cells emit signal to activate T cells, to mount host defense against tumors and viral infection, and tumor derived exosomes, play a role in counteraction of antitumor immune surveillance by the host, the activities are exerted by blunting specific T cell-mediated cytotoxicity and skewing innate immune cells towards a phenotype to support tumorigenicity [27]. In addition, the signals delivered by exosomes also modulate angiogenesis, and remodeling stroma, hence favors tumor the onset of metastasis and contributes to tumor progression.

Human *in vitro* generated dendritic cells and the exosomes they release are potential tools for the modulation of immune responses. Monocyte-Derived Dendritic Cells (MDDCs) and their exosomes have been characterized [28]. When peripheral CD14+ cells were co-incubated with interleukin (IL)-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (conventional MDDCs) or with IL-4 and IL-3 to generate immature MDDCs, The IL-4/IL-3-generated MDDCs had significantly lower percentages of CD1a+, CD40+ and CD80+ cells and a higher percentage of CD86+ cells as compared with conventional MDDCs. And they had significantly higher densities of Major Histocompatibility Complex (MHC) class I [human leucocyte antigen (HLA)-ABC], MHC class II (HLA-DR), CD11c and the tetraspanin CD81, more efficient than the conventional MDDCs at inducing interferon (IFN)-gamma release in response to viral peptide. Thus, phenotypic ally the exosomes largely reflected their MDDCs of origin. The findings suggested development of DC- and exosome-based therapies.

Compositions frequently found in exosomes are MHC class I and MHC class II molecules. It has been reported that these molecules cause immunosuppression [29- 31]. In mice, exosomes, especially from DC, have been shown to reduce inflammation due to IL-10, B7, and MHC class II. Exosomes from plasma in mice can contain other protein markers such as CD71, FasL (also aids in suppressing inflammation), and CD86 [32].

Anti-tumor immunity

Exosomes from tumor cells can contain transforming growth factor-b (TGF-b) on the lipid surfacetosuppressTcells. [33]. Manycytokinesin macrophage-derivedexosomes, suchasCCL3, CCL4, CCL5, TNFa, G-CSF, CSCL2, and IL-1RA, causeimmunestimulationin target cells. Pathogen-associated molecular patterns (PAMPs) are also packaged into the exosomes from bacterially-infected cells to cause a faster immune response [34]. Exosomes from B cells can contain LAMP-1, CD20, BCR, various tetraspanins, as well as heat shock proteins[35]. Overall, immunoregulatory molecules incorporated into the exosomes may cause both immuno-suppressive and immuno-activation effects depending on their composition and cellular source of the exosomes.

Exosomes released from tumor cells and other types of cells contributes to the immune suppression through interactions with target cells. When co-cultured with antigen specific dendritic cells,

CD+8 T cells are stimulated to produced exosome targeting to the CLT specific for the same antigen, including what is expressed on melanoma cells [36]. The inhibition, however, is reduced by LFA-1 expression. T cell derived exosomes expressing a death ligand, Fas L has been shown to promote the invasion of cancer cells in vitro through Fas/Fas ligand interaction. The exosomes increased the amount of the inhibitors of FLICE/caspase-8 and subsequently activated intracellular pathways of NFkappaB and ERK, to elevate matrix metallothionase 9 (MMP9). A Fas-MMP9 axis triggered contributing to tumor invasion, triggered by T cell derived exosomes is suggested [32]. The finding suggested a T cell exosome in suppression of antitumor immunity, Table 1 as well as a possible use of the exosomes in treatment of autoimmune diseases.

Their release by tumor cells may represent the future for targeting therapeutic interventions and for development of multiplexed diagnostic biomarkers.

The Effect on T cells, NK cells

Exosomes are released by a great variety of cells, including normal cells of either hematopoietic or epithelial origin, but also tumor- and virus-infected cells [37,38]. The exosomes derived from Natural Killer (NK) cells are thought to play pivotal roles in both innate and adaptive immunity. Data suggested that activated and resting NK cells freshly isolated from the blod of healthy donors, express protein markers of NK cells, including CD56 and also proteins participating the cell death process, for example, Fas ligand and perforin molecules[39]. Molecules involving the functions of NK cells, including the rceptor NKG2D, its multiple ligands, MHC class I-related chain A and B (MIC A/B) form a powerful stress-induicble danger detector, targeting various micro environmental stress including infections, inflammation and malignant transformation. It plays a vital role in the host immune surveillance against tumorigenesis. These molecules are enriched in the endosomal compartment of the tumor cells, and released in the form of exosome to extracellular environment, including intercellular space and body fluids [40]. The nanovesicles display cytotoxicity against tumor cells, as well as to activated immune cells. The cell type specificity may be based on the surface molecule profile. And the molecules expressed on the target cell surface may guide the uptake of the exosome: by tumor cells but not by resting peripheral hemopoietic cells. The work proposes an important role of NK cell derived exosomes in immune surveillance and homeostasis, and the use of exosome in the future therapeutic approaches in various diseases, including cancers and viral infection.

The Impact of Secreted Viral Genomic Products on Cellular Gene Expression

Exosomes support replication and infection of EBV

During the viral latency of human viruses, viruses, notably herpes viruses exploit cellular biosynthesis machinery to support a long-lasting, well-balanced infection with their host. Human herpes virus 4 (HHV4) or Epstein-Barr virus (EBV) is a human herpes virus which establishes life-long latent infection in the host, playing pathogenic role in a variety of malignancies, ranging from lymphomas arising in immune suppressive individuals infected by HIV or receiving immune inhibitors on organ transplant to Burkitt Lymphoma (BL), undifferentiated nasopharyngeal carcinoma (NPC) and other types of malignancies. EBV genomic products are detectable not only in tumor cells, but also in extracellular milieu, in free soluble form or loaded in exosomes.

Cell type dependent latent infection of Epstein barr virus (EBV)

EBV genome codes for a number of products of miRNAs and proteins with transforming potential. The viral genetic products are expressed in a pattern dependent on latency types. Different host ranges of the two human herpes viruses, as EBV infects B lymphocytes and through immortalization process mediated by latent transforming genomic products, confers the infected B cells lymphoblastoid phenotypes. EBV also infects less differentiated or undifferentiated epithelial cells; and it is tightly associated with occurrence of NPC, which is arisen in individuals with normal immune competence. The viral genome is integrated in virtually all tumor cells, and antigenic viral proteins are expressed. It remains therefore, to elucidate the mechanisms of immune evasion in NPC. The viral genomic products may in turn impact on the host cells, released to microenvironment either in free, soluble form or incorporated in exosomes.

The effect of EBV encoded transforming proteins on tumorigenicity and antitumor immunity

EBV encoded LMP1 is recognized as an oncoprotein because of its potential to transform rodent fibroblasts and human lymphocytes. It is also one of then transforming EBV protein that is expressed in EBV associated malignancies. From one third to two thirds EBV positive undifferentiated NPC and a considerable amount of Hodgkin disease, adopting latency II are positive for LMP1. It is a member of Tumor Necrosis Factor (TNF) super family, and has been shown to activate intracellular signal pathways through its interactions with TRAFs, TRADD to induce NFkappaB, MAPK and Akt. LMP1engages intracellular pathways to regulate cell growth and proliferation, including NF-kB and JNK pathways, to induce anti-apoptotic factor Bcl-2[46], Mcl-1,[47] A20 [48] and A1/Blf1, [49] has been found to be secreted and localized in exosomal component in culture medium of LMP1 recombinant Baculovirus [50, 51], and the components are likely to inhibition T cell activation [52].

As an EBV associated B lymphoid tumor, considerable cases of Hodgin's Disease (HD) are positive for LMP1. Exosomes harboring LMP1 are released to enable B cells to gain the capacity to proliferate [53]. LMP1 is actively secreted by Hodgkin-Reed Sternberg (H-RS) cells in EBV positive HD cells and direct immunosuppressive properties of LMP1 fragments have been demonstrated [52].

LMP1, together with another transforming EBV protein BARF1 were expressed in NPC; they have been described to be secreted in the serum and saliva of NPC patients and BARF1 protein and LMP1 complexed with exosome showed powerful mitogenic activity *in vitro* [54]. Exosomes containing LMP1, when released from EBV infected cells, exert differential effects on the neighboring uninfected cells. A downstream target, ICAM1, an adhesion molecule, ICAM1 is induced by LMP1 in an NFkappaB dependent manner, and contributes to the clumping features of the EBV transformed lymphoblastoid cells. It has been recently reported that LMP1 expressed from cells latently infected with EBV elevates the level of ICAM1 when LMP1 is incorporated in exosomes, and the exosomes are endocytozed by recipient cells [55].

HLA- class II positive exosomes collected from LMP1 positive and negative NPC xenografts were analyzed. It was found that LMP1 and galectin 9 existed in LMP1 positive cell supernatants, and only galectin 9 in LMP1 negative supernatants. Intrinsic T inhibitory activity by LMP1 was confirmed by the study [56]. Galectin-9 is a

ligand of the membrane receptor Tim-3. Its ligation leads to apoptosis in mature Th1 lymphocytes. The incorporation of galectin 9 into exosomes prevents the proteolytic cleavage on it, and maintains its Tim-3 binding activity. The effect inhibits the counteraction of EBV-specific CD4+ cells by the tumor through induction of apoptosis. Such exosomes might play a role in the immune evasion of NPC cells [58].

The cell type or latency dependent expression of miRNAare transferredby exosome on EBV infection

MicroRNAs (MiRNAs) are also among composition of exosomes. They are short, non-coding RNAs that are negative post-transcriptional regulator of host gene expression. miRNAs are 19 to 24 nucleotides in length and regulate posttranscriptional gene expression by blocking translation or causing the degradation of target mRNAs [59,60]. These potent gene regulators control up to one third of all genes, affecting such biological functions, including differentiation, cell growth, and disease, especially cancer [61].

EBV-transformed cells express at least 44 mature viral miRNAs generated from 25 precursors targeting to different viral and cellular genes [62]. Their existence in the serum of EBV infected individuals and the correlation of the serum loads with the intracellular copy number suggested that they exert certain biological effects on recipient cells when released from cells where they are synthesized [63]. The EBV encoded small non-coding miRNAs capable of regulating the expression of the host target genes. The miRNAs can be transferred from an infected cell to uninfected neighboring cells by delivery by exosomes, to down regulate specific target genes. EBV exploits intracellular trafficking and function of miRNA-containing protein complexes to promote or restrict miRNAs sorting into exosomes to perform such regulations [64].

EBV is the human virus in which the earliest miRNAs were discovered, and it is also the virus with the largest number of miRNA. The 25 pre-miRNAs are matured to 44 miRNAs. The earliest study identified EBV miRNAs clustered in the regions near the mRNA of BHRF1 and the intronic region of the BART [65]. EBERs are the microRNAs encoded by EBV and had been identified before the term of microRNA was proposed. EBERs are abundant RNA in all EBV infected cells, and in the recent years it has been noted that it contributes to the surviving of EBV infected cells by reducing the cellular sensitivity to interferon-induced apoptosis [55]. This activity may explain the genesis of EBV associated tumors in which latency I viral infection is adopted.

The microRNAs, BARTs, BHRF, etc shows a latency dependent expression pattern. BARTs are particularly abundant in the EBV-associated carcinomas and encode a large number of microRNAs (miRNAs). [66, 67] It has been shown Cluster 1 BART miRNAs have been reported to down regulate the expression of the viral LMP1. miR [68]. miRNAs from the BHRF1 region have been associated with replication of the virus and regulation of the chemokine CXCL-11 [69].

Similar with the host cell dependent protein expression spectrum during EBV latent infection, the expression of EBV encoded miRNAs is also host cell type dependent. During latency III pattern of EBV infection, seen in lymphoblastoid cells *in vivo* immortalized by EBV, and lymphomas arising in the immune suppressive individuals in vivo, the BHRF1 miRNAs are expressed at high level. The profile, together with the expression of all proteins coded by EBV genome, is due to the EBV transcripts expressed under the control viral promoters Wp or Cp [70]. The cluster of miRNAs are not detectable in cells infected with EBV adopting latency I, e.g. Burkitt lymphoma or latency II, nasopharyngeal carcinoma (NPC) in which an EBV encoded miRNA, BART7 is detected but not in EBV associated lymphomas [71,72,73].

As seen with numerous examples in viral infections, that the invading viruses code for genomic products to counteract the host defense. It has been shown that miRNAs expressed during different types of latent infection modulate the level of host genetic products to support viral parasitism. miR-BHRF1-3, which is highly expressed in type III latency down-regulates interferon-inducible T cell attracting chemokine, CXCL-11/I-TAC [69]. Through blocking the chemokine to activate the chemokine receptor CXCR3 miR-BHRF1-3 inhibit the host interferon response on viral infection.

Exosomes Regulate HIV pathogenesis

Products encoded by viral genome have been detected in exosomes released virally infected cells. It is postulated that the exosomal compositions are implicating in the regulation of viral replication as well as the the interactions between the host and the viruses. Among HIV proteins and RNAs in EVs released from HIV-infected cells HIV Transactivation response element (TAR) RNA was detected [74]. TAR is an RNA stem-loop structure located at the 5′ ends of HIV-1 transcripts; it binds Tat, thereby facilitating recruitment of elongation factors and increased production of viral RNA [75]. When transferred via EVs, the population of susceptible target cells was increased by TAR RNA. Exosomes also suppresses HIV infection. When HIV-Nef is incorporated into these exosomes, it competes for the incorporation of CD4 molecules into the vesicles, and decreases the number of CD4, as HIV receptor CD4 to attach to viral particles, thereby decreases the numbers of virions that infect CD4+ T cells [76].

Exosomes derived from infected cells were found to contain Tax protein and proinflammatory mediators as well as viral mRNA transcripts, including Tax, HBZ, and Env. An exosomes released from HTLV-1-infected Tax-expressing cells contributed to enhanced survival of exosome-recipient cells when treated with Fas antibody. This survival was cFLIP-dependent, with Tax showing induction of NF-κB in exosome-recipient cells. The results suggest that exosomes may play an important role in extracellular delivery of functional HTLV-1 proteins and mRNA to recipient cells [74].

The Potential Application of Exosomes in Vaccine Development: A Gene Transfer Vehicle

It has long been observed that lesions of tumor are infiltrated by considerable amount of lymphocytes, mostly T cells. The crucial point for mounting efficient antitumor immunity is stimulating the cytotoxicity T response of these infiltrating lymphocytes, to eradicate the malignancies. Mature DC is the most potent APCs for the priming of naive CD8+ T cells. As discussed above, secretion of exosomes incorporated with active immunregulatory factors plays a role in priming T cells response. It has been discovered that DC can secrete MHC class I-bearing exosomes, a possibility exists that exosomes pulsed with synthetic peptides could subserve the DC function consisting in MHC class I-restricted, peptide-specific CTL priming *in vitro* and *in vivo* [77]. MHC-I molecules in purified exosomes can be directly loaded with peptide at much greater levels than indirect loading. The direct loading method performed in mildly

acidic conditions was effective, and the increase in peptide binding greatly enhanced exosome potency, so the further study the biologic activity of exosomes *in vitro* was facilitated. Exosomes in Antigen-Presenting Cells (APC), directly loaded with the HLA-A2 restricted tumor peptide stimulated an HLA-A2 specific T-cell line in the tumors. MHC-II molecules, which are abundantly expressed on DC exosomes, were also functionally loaded under the same conditions as MHC-I. This feature allows for delivery of multiple peptide antigens that can stimulate both CD8+ cytotoxic T cells as well CD4+ T helper cells critical for an effective antitumor response. Exosomes loaded with major histocompatibility complex class II/peptide are released by Intestinal epithelial cells have been shown to interact with DCs, strongly inducing activation of T cells [78]. Both peptide- and whole protein-loaded dexosomes induced potent antigen-specific CD8+ T cell activation in vitro, but only protein-loaded dendritic cell derived exosomes (dexosomes) containing both T- and B- cell epitopes induced specific cytotoxic T-lymphocyte (CTL) responses *in vivo*. In this context, the activation of CD8+ T cells was totally dependent on CD4+ T cells as well as on Marginal Zone B cells (MZBs) [79].

Tumor associated antigens, including over-expressed oncogene products elicit specific cytotoxic T cell response and can serve as the target of antitumor vaccination. MAGE antigens initially described in melanoma, and the immune responses have been correlated with clinical outcome of melanoma [80]. The antigens are found to express on malignant cells of other tumors like Non-Small Lung Cancer (NSCL) [81,82]. Exosomes pulsed with MHC-I molecule and melanoma specific peptides have been observed to prime T cell response, suggesting that exosome transfers mechanism of functional MHC class I/peptide complexes to DC so as to activate CTL efficiently in vivo [84]. The anticancer therapy using immature autologous DC-derived exosome loaded with peptides derived from such tumor associated antigens with MHC class I and II restriction has entered clinical trial [8384]. HLA A2 + NSCLC patients positive for MAGE 3 and 4 were enrolled to the study; their DCs were isolated and DEX was produced, loaded with MAGE 3and 4 derived peptides. The results suggested that T cell response as well as NK lytic activity were augmented [85]. The work is expanding to strategies using many tumor antigens, including immunodominant peptides alone, or peptide pulsed exosomes.

Conclusion

As a nanoscale particle formed by the MVB of the late endosomes, exosomes incorporate biologically active molecules of proteins and miRNA during their biogenesis. They play a role in intercellular communication, exerting influence on such biological events like cell adhesion, motility, as well as immunoregulation. Exosomes also impact on the host virus interactions like entry to permissive cells by viruses, and antiviral immune defense. As rapid and efficient means to transfer macromolecules, exosomes are promising in utilizing in vaccine development and biotherapy against different diseases.

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