Extracellular Vesicles in Malaria Infection

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ABSTRACT

Malaria is one of the tropical diseases which cause high rate of morbidity and mortality. The disease is caused by the infection of protozoan parasites in the genus *Plasmodium*. The severe syndromes of malaria infection arise from the complex sequences of parasite-host interactions. It starts with parasite invasion and followed by the rupture of infected red blood cells causing the release of parasite products that activate the host immune response. During the past decade, research on the functions of extracellular vesicles (EVs) in many diseases including malaria has increased dramatically. This article reviews the role of EVs in malaria immunopathogenesis. Investigations into modulators in immune response, ubiquitous mechanism for intercellular communication between parasite-parasite and parasite-host, as well as its usefulness as the diagnostic biomarkers are highlighted.

Keywords: Extracellular vesicles; exosomes; Malaria; *Plasmodium* spp. (Siriraj Med J 2019;71: 89-94)

INTRODUCTION

Extracellular vesicles (EVs) are heterogeneous types of small membrane-enclosed particles which originated from many cell types and can be found in body fluids such as serum, plasma and cerebrospinal fluids (CSF). EVs released from the cells in physiological conditions have significant role in homeostasis. Its level tends to increase under the pathological conditions. EVs transport cellular components such as proteins, lipids and genetic materials from the originating cells to the recipient cells. These EVs can be directly fused at plasma membrane or internalized by endocytosis resulting in the EVs cargo transfer and function in the recipient cells. Nowadays, the roles of EVs have been widely demonstrated in many diseases such as cardiovascular diseases, cancer and autoimmune diseases.[1,2] The contribution of EVs is also well-established in infectious diseases. However, study of EVs in the context of a parasitic infection is complicated because both the parasites and the host cells release the EVs into the extracellular space which play a role in disease pathogenesis. The EVs in niche environment provide the intercellular communication between parasites and other parasites or host cells. It leads to either activation or modulation in the host immune response to parasites. This review provides an overview of the research studies on EVs in malaria infection.

Characteristics of EVs in malaria infection

Although the definite terminology for different types of EVs has not yet finalized. Three types of commonly known EVs, apoptotic bodies, ectosomes and exosomes, are classified based on their biogenesis and characteristics. Because of their different biogenesis and originating cells, each EV type contains distinct active biological components and has specific biological functions.[3,4]

The apoptosis bodies are the largest particles with 1-5 micron in size. They result from outward blebbing of plasma membrane of cells at the end stage of apoptosis.

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They carry various molecules including the nuclear fractions, cell organelles and genomic DNA which involve in facilitating apoptotic process. The term of ectosomes, also referred as microvesicles (MVs) or microparticles (MPs), are used for defining the membrane-enclosed particles ranging in size from 0.1 to 1 micron. MPs are released by outward budding from plasma membrane of activated cells or early apoptotic cells. The plasma membrane is composed of a bilayer of lipids, including phospholipid and oily substances. Phospholipids normally enriched at the outer leaflet of plasma membrane are cationic and neutral phospholipids such as sphingomyelin and phosphatidylcholine (PC) while anionic phospholipids located at the inner leaflet are phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidylethanolamine (PE). Once cells are in the activated stage or undergo apoptosis, there is the alteration of plasma membrane asymmetry, externalization of anionic phospholipids and vesiculation from the plasma membrane. Therefore, the types of ectosomes can be identified by specific surface markers such as phosphatidylserine, integrins, selectins and other compositions expressed on the membrane of originating cells. The exosomes are the smallest particles with the diameter 40-100 nm. They are generated from the endocytosis of plasma membrane into endosomes and later are accumulated as vesicles in multivesicular bodies (MVB). The fusion of MVB with the plasma membrane allows the release of exosomes from the originating cells to the extracellular space. The common proteins used for identifying exosomes include tetraspanins (CD81, CD63, CD9), flotillin, constitutive heat shock protein 70 (Hsc70) and vesicle trafficking-related proteins (TSG101, ALIX, and RAB proteins, syntenin-1).[5] Both ectosomes and exosomes deliver numerous proteins (cytoplasmic proteins, transmembrane proteins and membrane associated proteins), lipid and nucleic acids (mRNA, miRNA and other non-coding RNAs), similar to those expressed in the parental cells, to the membrane or cytosol of the target cells and function in the same way as parental cells do.

The characteristics of EVs derived from plasma of malaria-infected patients and malaria culture supernatant have been determined. The biophysical analysis of EVs derived from *P. falciparum*-infected red blood cells (iRBCs) showed that the majority of EVs were 50-300 nm diameter with single bilayer membrane.[6] The proteomic analysis of EVs revealed that they contained both host and parasite proteins. The MPs isolated from plasma of malaria-infected patients contained host proteins including complement-associated proteins, coagulation-associated proteins and cytoskeletal proteins. Meanwhile, major parasitic protein components were involved in parasite invasion and parasite growth.[7] The EVs from *P. falciparum* culture supernatant are enriched with red blood cell lipid rafts proteins and membrane-associated parasite antigens, especially proteins associated with red blood cell membranes and proteins involved in parasite invasion.[8,9] These abundant parasitic proteins included the parasite proteins found in cytosol of iRBCs and exported to red cell membrane such as Maurer's clefts; the merozoite secretory proteins such as RhopH protein complex (RhopH1, RhopH2 and RhopH3), the RAP complex (RAP2 and RAP3), RALP1 and RON3; the microneme resident proteins such as EBA-175 and EBA-181; and the dense granule proteins such as HSP101, PTEX150, EXP2, SBP1, RESA, and MAHRP1.[9] Not only proteins, but other genetic materials including genomic DNA(gDNA), functional mRNA, miRNA and other small non-coding RNA have also been detected in EVs.[6,8,10] The parasitic gDNA, human and parasitic small RNA between 4-150 nucleotides were present in EVs derived from *P. falciparum*-iRBCs, especially those from ring-stage iRBCs.[6] The miRNA profile of plasma EVs and peripheral blood cells under normal healthy conditions showed that red blood cell contributed the highest cellular miRNA to the blood and different cell lineage expressing different patterns of miRNA. The miR-451 and miR-150 established crucial function in erythroid- and lymphoid differentiation, respectively. The level of miR-223 is abundant in granulocytes and platelets.[11] The highest miRNAs expressed in plasma EVs were miR-223, -484, -191, -146a, -16, -26a, -222, -24, -126, and-32.[12] Since *Plasmodium* spp., do not have a mechanism to produce miRNA, all miRNAs isolated from malaria-infected red blood cells or from the plasma of malaria-infected patients have been confirmed to be of human origin.[13-15] The majority of miRNA found in iRBCs-derived EVs were miR-451, let-7b and miR-106b.[10] However, the expression levels of miRNAs, miR-19b, -4732, let-7a, -16, -183, -18a and 148b in iRBCs-derived EVs were lower than those in uninfected RBCs-derived EVs.[6]

EVs as an amplifier in the pathology of malaria infection

Human malaria is caused by the infection of intracellular protozoan genus *Plasmodium* transmitted by Anopheles mosquitoes. There are five species of *Plasmodium* which are *P. falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlesi.* Among these, the *P. falciparum* and *P. vivax* are highly prevalent in Thailand and the Southeast Asian region.[16]

Malaria infection begins with the injection of

sporozoites from infected mosquitoes during a blood meal. Sporozoites invade hepatocytes and proliferate into merozoites. It leads to rupture of infected hepatocytes. The hepatic merozoites enter the blood circulation, invade erythrocytes and begin the erythrocytic schizogony. The clinical symptoms are associated with the invasion of asexual erythrocytic-stage parasite to erythrocyte, and the inappropriate immune response to iRBCs and diverse parasite-derived products. The severe *P. falciparum* infection results from the sequestration of iRBCs, leukocytes and platelets to endothelial cells (ECs) within microvessels, the excessive proinflammatory cytokine production and the severe red blood cell hemolysis. The parasite sequestration leads to accumulate host cells within vasculature, injure ECs and disrupt blood flow, causing tissue hypoxia and lactic acidosis. The sequestration mechanisms contribute to organ-specific syndromes such as cerebral malaria and placental malaria.[17] Recent studies in both animal models and malaria-infected patients suggest the significant contributions of EVs to severe malaria.

In rodent malaria model, the association of EVs and malaria pathogenesis was initially studied in ATP-binding cassette transporter (ABC) knockout mice. The ABC is a cholesterol transporter involved in controlling the outward translocation of PS at the plasma membrane.[18] The deletion of this gene results in reducing the externalized expression of PS on the cell surface and inhibit the MPs production. The *Plasmodium berghei* strain ANKA (PbA) infection in the ABCA1 knockout mice ($ABCA^{-/-}$) showed the decreasing of plasma tumor necrosis factor (TNF) level and resistance to cerebral malaria. The MPs from these mice had lower procoagulant activity than those from wide type mice.[19] Treatment with pantethine, a provitamin regulated lipid metabolism, to PbA-infected mice also reduced the MPs production, decreased platelet reactivity and impaired endothelial cell activation by MPs resulting in prevention of cerebral malaria development.[20] By contrast, the adoptive transfer of MPs from PbA-infected mice with the neurological symptoms led to localize MPs in cerebral microvessels of PbA-infected recipient mice. The transfer of endothelial cell-derived MPs (EMPs) also induces the signs of pathologies in the brain and lung of the recipient mice.[21] These findings indicate that host cell-derived EVs have pathogenic roles such as procoagulant activity and proinflammatory potentials in the pathogenesis of cerebral syndrome.

Accumulating evidence of host cell-derived MPs in plasma of malaria-infected patients has also supported the contention that the elevated levels and origin of MPs are associated with the disease severity. The levels of red blood cell-derived MPs (RMPs), platelet-derived MPs (PMPs) and EMPs were markedly increased in severe falciparum malaria patients. The level of RMPs were significantly elevated in patients with severe anemia while the levels of EMPs and PMPs correlated to coma depth and thrombocytopenia.[22-24] Similarly, the levels of MPs derived from leukocytes, platelets, and erythrocytes were significantly increased and the level of PMPs correlated with the presence of fever in acute *P. vivax*-infected patients.[25] These elevated levels of MPs were reduced to normal level at the convalescent phase and the clearance of parasitemia.[24,26]

Although the precise mechanisms underlying the induction of MPs production during the course of infection are not completely understood, many factors have been described. The CD40L-induced platelet apoptosis and thrombocytopenia were associated with increasing plasma PMPs in PbA-infected mice with severe syndrome.[27] The exposure to febrile temperature led to the significant increases of PS expression on the surface of iRBCs, particularly at the late schizont stage before the red blood cell egress which was corresponding to releasing EVs from iRBCs.[28] Moreover, the level of proinflammatory cytokine TNF was positively correlated with the level of circulating plasma MPs in malaria-infected patients, thus TNF might be another factor that induces the releasing of MPs in malaria infection.[26]

The major cell sources of EVs in malaria infected patients were from platelets and red blood cells. The *in vitro* study showed that infected red blood cells produced more EVs per cell than uninfected cells.[8,24] The components that were carried by EVs were associated in driven malaria pathogenesis. The EVs derived from plasma of malaria-infected patients and *P. falciparum* culture supernatant contain the proteins which are involved in parasite invasion and parasite growth. Therefore, these EVs might play a role in facilitating red blood cell invasion by merozoite during intraerythrocytic life cycle. The plasma MPs from cerebral malaria infected mice, but not from non-infected mice, carried proteins that were implicated in molecular mechanisms relevant to cerebral malaria pathogenesis, including endothelial activation.[29] In addition, the reticulocyte-derived exosomes of non-lethal *P. yoelii* 17X-infected mice contained the parasite antigens. The immunization of these purified exosomes induced the specific immune response to *P. yoelii* infected red blood cells.[30] The EVs also transferred the functional miRNAs, especially miR-451, from red blood cells to endothelial cells and targeted on the genes of proteins required for barrier function leading to vascular alteration.[10]

In addition to these biomolecules, hexanal compound was also encompassed with EVs of iRBC, because the

volatile organic compound presented in EV of iRBC is diacetin which is the insect attractant like hexanal. However, both compounds have been proven to play a role in malaria transmission.[31]

EVs as a messenger in parasite communication

Within the past decade, EVs have emerged as important mediators of communication between EVs-secreting cells and recipient cells. Two crucial experimental studies demonstrated the crosstalk between intraerythrocytic stage -*P. falciparum* by EVs. Regev-Rudzki *et al* exhibited the role of EVs cargo in parasite crosstalk by using the transgenic parasites. The EVs derived from drug resistance *P. falciparum*-iRBCs can transfer DNA encoding for a drug resistance marker between individual parasites leading to spreading of drug resistance in the parasite population. Moreover, this study showed that EVs transferring between *P. falciparum* parasites under drug pressure-induced stress conditions allows increased differentiation of gametocytes.[32]

In parallel, Mantel et al also revealed that iRBCs are able to internalize EVs isolated from malarial culture supernatant and transfer them into the parasite cytosol leading to increased gametogenesis.[8] However, the exact underlying mechanism of this phenomenon was not obviously proven in this work. However, another study showed that the endogenous translocation of human miRNA-451 from red blood cells into the parasites leads to chimeric fusion RNAs with regulatory subunit of cAMP-dependent protein kinase (PKA-R) transcripts of *P. falciparum* resulting in reduction of the translation of the regulatory PKA subunit. The suppression of PKA-R is associated with reduced parasite growth and increased numbers of gametocytes.[33] All these findings suggested that the EVs transfer genetic materials of parasite and host cells to parasites of other infected red blood cells resulting in alteration of the parasite cycle.

EVs as a modulator on the host immune response

Internalization of EVs by immune cells leads to either activation or suppression of the immune cell function. Because monocytes are the key immune cells that play a role in phagocytic eradication of iRBCs and free merozoites in blood circulation during intraerythrocytic life cycle, there are several interesting studies in monocyte/macrophage immunomodulation by EVs during malaria infection. The iRBCs-derived MPs from the plasma of PbA-infected mice induce macrophage activation by up-regulation of CD40 expression and proinflammatory cytokine TNF production. The activation is via TLR-4 and MyD88 dependent pathways resulting in systemic inflammation which impacts on both progression of disease complications and generation of adaptive immune responses.[34] In addition, the internalization of iRBCs-derived MVs isolated from malarial culture supernatant by macrophages triggers the strong pro- and anti-inflammatory cytokine responses of TNF and IL-10 production, respectively. The pre-incubation of these MVs with neutrophils also reduces neutrophil function.[8] Not only in malaria infection, the malaria EVs also attenuate neutrophil function in response to bacterial infection by inhibiting ability to produce reactive oxygen species and suppression of cytokine secretion.[35] These data demonstrate that EVs from iRBCs, but not from uninfected red blood cells, strongly modulate the cells of the innate immune system.

Immunization with reticulocyte-derived EVs from *P. yoelii* 17X-infected mice can induce the *P. yoelii* infected red blood cells-specific IgG antibody production.[30] Study showed that the immunization with the combination of these reticulocyte-derived EVs and CpG-ODN to BALB/c mice leads to significant increase in the percentage of effector T cells of both CD4 and CD8 T cells, especially effector memory CD4 subset when compared to mice immunized with reticulocyte-derived EVs isolated from uninfected mice. In addition, *in vitro* experiment showed that the exosome isolated from *P. vivax*-infected patients are captured by splenocytes leading to significant increase of the number of CD3 T cells and CD8 T cells, but there is no change on B or NK cell population. These data suggested that EVs also activate the immune cells of the adaptive immunity.[36] In addition to the immunization of iRBCs-derived EVs in malaria infection, the subcutaneous immunization with the exosomes from excretory/secretory products of *Echinostoma caproni*, an experimental intestinal helminth, in mice can reduce symptom severity during infection.[37] The intraperitoneal immunization of mice with EVs isolated from *Heligmosomoides polygyrus*, a gastrointestinal nematode, in alum adjuvant Is resulted in induction of specific antibody response against larval challenge and reduction of intestinal worm burdens.[38] Collectively, the parasitic EVs can also modulate diverse aspects of the immune system, suggesting that these EVs might be the candidate strategy used for vaccine development.

EVs as a source of diagnostic biomarkers

Today, the circulating miRNAs have been the research subject of interest as they can be used as biomarkers in many diseases. Alteration of miRNA expression reflects the pathological status. Upon malaria infection, infected

hepatocytes or other tissues at pathological sites, such as brain, placenta and bone marrow, may produce and release the EVs containing tissue-specific miRNAs to blood circulation. Detection of such miRNAs may allow discrimination between infected individuals with uncomplicated symptoms and those with specific organ complications or to be used as the surrogate markers for detecting the hypnozoites that remain dormant in the liver.

The differential miRNA expression study in the brain tissues from PbA-infected mice showed that the levels of miR-27a, miR-150, and let-7i, miRNAs in regulation of cellular proliferation and the innate immune response, are upregulated in infected mice with cerebral malaria when compared to those without cerebral malaria symptoms.[39] The development of protective immunity against malarial blood stages of *P. chabaudi* or the lethal outcome of *P. chabaudi* infection in mice are also associated with alteration of miRNA expressions in the liver during the infections.[40,41] As human miRNAs, mir-451 and miR-16, are highly expressed in red blood cells, it is not surprising that these miRNAs are also detected in plasma of both normal healthy and malaria-infected patients.[14,15] However, their levels were significantly downregulated in *P. vivax* infection and negatively correlated with the severity of parasitemia.[42] This might be due to the consumption of miRNAs by parasites during parasite growth inside the red blood cells and the clearance of circulating miRNAs by spleen during infection. A recent study of the EVs-bound miRNAs from plasma of mothers with placental malaria showed overexpression of placenta miRNA, a miRNA in regulatory process during gestation, compared with non-infected group.[43]

CONCLUSION

The involvement of EVs in the pathophysiology of malaria infection have been extensively shown in both malaria-infected patients and in experimental animal studies. EVs are considered as a vehicle necessary for transferring of proteins and nucleic acids which trigger intercellular communication between parasites and host immune cells which lead to change in the parasite biology and regulation of host immune responses. Due to their association with the disease severity, they are now being researched as potential biomarkers and for their use in future vaccine development.

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