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Emission of membrane vesicles: roles in complement resistance, immunity and cancer

Abstract Complement-mediated cell death is caused by C5b-9, the membrane attack complex (MAC) composed of the five complement proteins C5b, C6, C7, C8, and C9. Assembly of the C5b-9 complex initiates oligomerization of C9 and production of a transmembrane protein channel that inflicts damage to target cells. For protection, cells eliminate the MAC from their surface either by ectocytosis (direct emission of membrane vesicles) or by endocytosis (internalization). The process of ectosome release is rapid and involves cytosolic Ca^{2+} and activation of protein kinases, such as protein kinase C (PKC) and extracellular signal-regulated protein kinase (ERK). Recently, the involvement of mortalin (also known as GRP75 and mitochondrial hsp70) in MAC elimination has been suggested. Extracellular application of antibodies directed to mortalin increases cell sensitivity to MAC-mediated lysis. Release of membrane vesicles is ubiquitous and enhanced in apoptotic or tumor cells and upon cell activation. Composition of the ectosomes (also often referred to as microparticles) membrane proteins and lipids appears to be different from those of the original plasma membrane, indicating involvement of a selective sorting process during ectosome formation. Exosomes (unlike ectosomes) are membrane vesicles generated by endocytosis, endosome sorting into perinuclear multivesicular bodies (MVB) and exocytosis of MVBs. Exosomes appear to be different in size and composition from ectosomes. Exosome-associated MAC has also been described. Although research on ectosomes and exosomes is still limited, physiological roles in coagulation, vascular functions, angiogenesis, wound healing and development have been attributed to these shed membrane vesicles. On the other

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hand, there are indications that elevated levels of ectosomes and exosomes may predispose to morbidity. Membrane vesicles released by cells exposed to complement MAC may play roles in health and disease beyond protection from cell death.

Abbreviations   ERK: Extracellular signal-regulated protein kinase · fMLP: Formyl-methionyl-leucyl-phenylalanine · GPI: Glycosyl-phosphatidylinositol · GRP75: 75-kDa glucose-regulated protein · MAC: Membrane attack complex of complement · MVB: Endocytic multivesicular bodies · PKC: Protein kinase C

Introduction

The complement system plays important roles in activation, regulation, and execution of innate and acquired immune responses. Initiation of the complement activation cascade may occur through the classical, alternative, or lectin pathway. These three initiation pathways end up splitting complement C5 into C5a and C5b. C5b triggers a spontaneous, sequential assembly of the terminal complement complexes C5b-6, C5b-7, C5b-8, and C5b-9 followed by their membrane adhesion and insertion (reviewed in [62]). The C5b-9 complex, also known as the membrane attack complex (MAC), is composed of a C5b-8 complex attached to a transmembrane C9 oligomer (poly-C9, n=1–18) [62, 94]. The MAC is a potent membrane-damaging agent and a pore-former. The lytic capacity of the MAC was studied in depth with erythrocytes. The conclusion of these early studies was that a single functional MAC is sufficient to cause death of an erythrocyte [54]. Later studies have suggested that nucleated cells are more resistant to MAC-mediated lysis compared with erythrocytes and their lysis requires multiple MACs [44, 57]. The mechanism of cell death induced by the MAC is still not fully clear. MAC insertion is known to enhance dramatically $[Ca^{2+}{}_i]$ level [61], and this may be responsible for the ensuing mitochondrial damage, depletion of ATP, and osmotic cell lysis [71]. More recently, the MAC has also been implicated in induction of caspase-dependent apoptotic cell death in certain target cells in vitro and in vivo [21, 63, 83].

Unlike high doses of MAC that are lytic or apoptotic to nucleated cells, low, sublytic doses of MAC induce a variety of biological responses (reviewed in [6]). Depending on the cell studied, sublytic MAC has been shown to induce enhanced expression of adhesion molecules [92], release of proinflammatory mediators [40], entry into cell cycle [79], production of active oxygen metabolites [57], or increased DNA synthesis and cell proliferation [33, 65]. Sublytic MAC induces these activities by triggering several signaling pathways including Ca^{+2} influx, activation of phospholipases, generation of diacylglycerol (DAG) and ceramide, and activation of protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) pathways [46, 58, 66, 84].

Nucleated cells employ several defensive molecular mechanisms against complement (reviewed in [38]). Resistance is conferred by constitutive expression of membrane-associated complement regulatory proteins, such as CD46, CD55, and CD59 [43, 55, 64]. These complement regulators restrict binding of complement components to the cell surface. Defense is also conferred by ecto-proteases [24, 37] and ecto-protein kinases [5, 69]. Basal resistance of cells to complement can be augmented by treatment with various agents. Thus, treatment with sublytic MAC doses leads to desensitization to lytic MAC doses [76]. This phenotypic conversion depends on Ca^{+2} influx, activation of PKC and ERK and protein

synthesis [46, 76]. Both PKC and ERK [16, 23, 45, 46] as well as heat shock proteins [22, 88] are involved in cell protection from complement-mediated lysis. Sublytic MAC has also been shown to exert antiapoptotic effects [87].

Elimination of the MAC by membrane vesiculation

Cells also possess the ability to evade complement attack by elimination of the MAC. After a sublytic complement attack, cells emit the MAC rapidly and actively from their plasma membrane [12, 59, 74]. Bound MAC is also removed from the surface of erythrocytes, primarily by membrane vesiculation [36]. Following a complement attack, multiple membrane protrusions appear on the cell surface [68], and membrane vesicles are shed from the cells (Fig. 1). Membrane vesicles can be released from dying cells; however, release of the MAC-containing membrane vesicles appears to be clearly associated with cell recovery from complement attack. Such complement-induced membrane vesiculation was reported with neutrophils, oligodendrocytes, platelets, glomerular epithelial cells, and with the tumor cell lines Ehrlich, U937, and K562 [12, 57, 60, 82]. As described later in this review, many cells release plasma membrane vesicles either spontaneously or after a trigger unrelated to the insertion of the C5b-9 complex. Membrane vesiculation may occur by ectocytosis or exocytosis (Fig. 2). Ectocytosis leads to formation of ectosomes, often referred to as microparticles. These are vesicles generated upon budding out and pinching off from the cell surface. In contrast, exosomes are vesicles generated following an endocytic process. They are loaded within multivesicular bodies (MVB) and released by exocytosis. Ectosomes and exosomes differ in size and composition. It is conceivable that most cells produce both ectosomes and exosomes, yet erythrocytes can probably produce only ectosomes.

Rapid clearance of the C5b-9 complexes from the cells occurs under nonlytic conditions [10]. The membrane vesicles collected from complement-attacked neutrophils contain much of the cell-bound C9 but exclude normal cell surface constituents, suggesting selective disposal of the MAC [59]. High concentrations of the MAC cylinder-like structures, containing 12 C9 molecules per one C8 molecule, were visualized by electron microscopy on membrane vesicles shed from neutrophils following a sublytic complement attack [59]. Such ring lesions were also shown on vesicles recovered from the cerebrospinal fluid of patients with multiple sclerosis [82]. Analysis of the composition of the MAC-containing vesicles indicated a sorting process of membrane proteins and lipids in neutrophils attacked by sublytic complement [89]. The content of cholesterol and DAG was found higher in the shed vesicles. In contrast, the same quantity of sphingomyelin, phosphatidylcholine and phosphatidylinositol was found in shed vesicles and in the plasma membrane. Platelets attacked by sublytic MAC shed vesicles enriched in the plasma membrane glycoproteins GP Ib and GP IIb-IIIa, the membrane protein GMP-140, the receptor for coagulation factor Va, and the receptor for factor VIII [29, 85]. Membrane vesicles shed from erythrocytes after calcium ionophore treatment or complement attack are enriched in proteins anchored to the plasma membrane via glycosyl-phosphatidylinositol (GPI), such as acetylcholinesterase and decay-accelerating factor (DAF, CD55) [7, 100]. The presence of GPI-anchored proteins appears to be essential for ectocytosis from erythrocytes [100], but not from platelets [102].

Nucleated cells can also eliminate the terminal complement complexes from their surface by internalization or endocytosis. This was demonstrated with neutrophils, glomerular epi-

Fig. 1 Examples of vesicles re-
leased from activated cells. **a** and
b Membrane vesicles (see *arrows*)
released from K562 cells treated
with a sublytic dose of antibody
and complement. Light micros-
copy, vesicles size ~1 μm.
c Ectosomes (see *arrows*) released
from PMNs activated by fMLP.
TEM, negative staining, vesicles
size ~50 nm
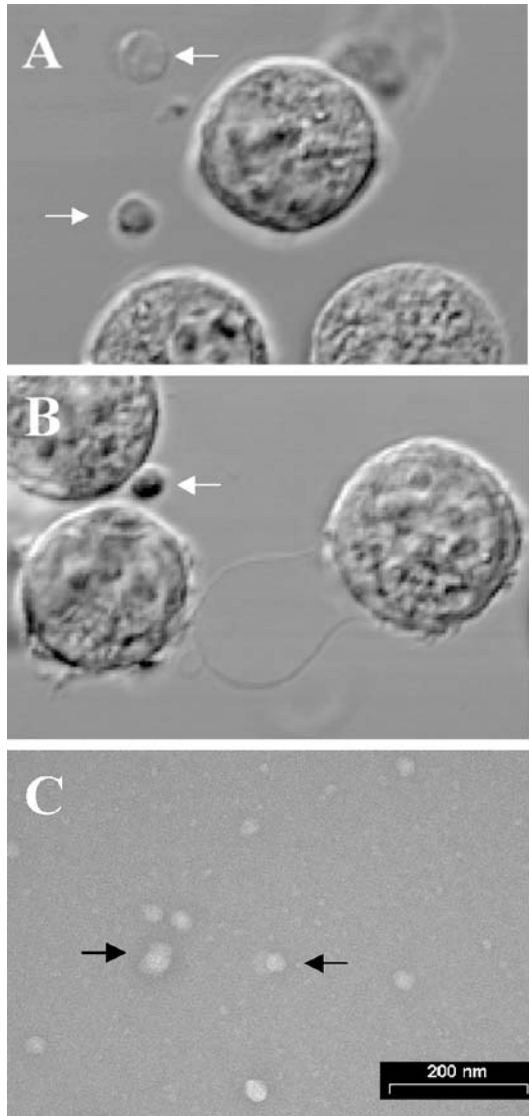
thelial cells, and Ehrlich tumor cells [13, 39, 59]. The internalization may be followed by
proteolytic degradation of the C5b-9 complex [61]. C5b-8 complexes deposited on the
surface of Ehrlich cells were seen in endocytic vesicles and in MVB [13]. C5b-9 complexes
deposited on glomerular epithelial cells in kidneys of rats with experimental membranous
nephropathy were found in clathrin-coated pits and also in MVB [39]. The latter report
suggested that C5b-9 complexes deposited in vivo on epithelial cells are endocytosed,
packed into MVB, and then released by exocytosis into the urine in a degraded form, attached
to membrane vesicles.
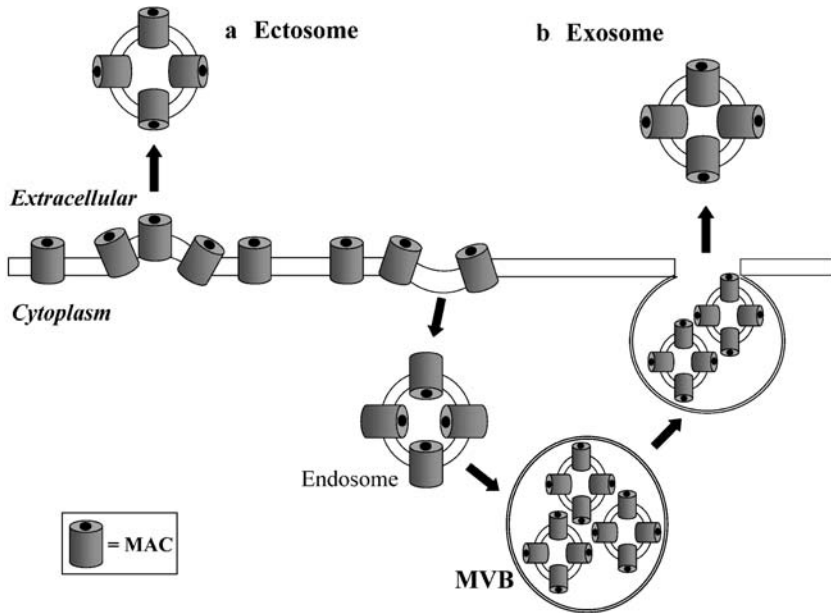
Fig. 2 Schematic presentation of modes of emission of ectosomes vs. exosomes. Under sublytic conditions, the complement membrane attack complexes (MAC) may either be released by ectocytosis (**a**), a budding-off process, or exocytosis (**b**) following endocytosis and packaging in multivesicular bodies (MVB). Currently, data support MAC emission by ectocytosis

Insights into the mechanism of MAC-induced membrane vesiculation

The mechanism of the MAC-induced vesiculation process is largely unknown. The earliest signal reported in cells undergoing a MAC attack is an increase in intracellular Ca^{+2} levels [8, 9]. It was suggested that this rapid rise in intracellular Ca^{+2} could signal MAC removal from the plasma membrane [12]. Chelation of intracellular Ca^{+2} inhibited MAC removal and increased cell death. Elevation of intracellular calcium concentration is known to activate certain protein kinases that regulate activity of key proteins by phosphorylation. Involvement of protein kinases in MAC elimination has indeed been suggested [14, 101, 103]. Activation of protein kinases, such as PKC and extracellular signal-regulated protein kinase (ERK), by sublytic MAC was demonstrated in several cell types [16, 45, 46]. MAC removal was suggested to be dependent on PKC activation [14]. Cell resistance to complement involves PKC-dependent activation of ERK [46]. The emission of proteins from K562 cells following a trigger by a sublytic complement attack can be reduced by treatment with an ERK inhibitor (Pilzer and Fishelson, unpublished). This suggests the involvement of ERK in the MAC shedding process.

Which proteins act downstream of PKC and ERK to eliminate the MAC from the cell surface? Recent data have suggested that mortalin is involved in MAC vesiculation [73]. Mortalin (also known as GRP75, PBP74, or mitochondrial hsp70) is a member of the hsp70 family (reviewed in [99]). It is found mainly in mitochondria but to some extent also in the cytoplasm, endoplasmic reticulum, and cytoplasmic vesicles [75]. Mortalin is constitutively

expressed in cells, participating in stress response [11] and mitochondrial import [98], and is frequently upregulated in tumors [18, 90]. We (Pilzer and Fishelson) found that mortalin is released from K562 cells within minutes after an attack by sublytic complement together with the MAC, probably on membrane vesicles. Mortalin release depends on C5b-8 and C5b-9 formation and on PKC and ERK activation. Interestingly, the presence of anti-mortalin antibodies outside the cells during complement attack reduces release of C9 and mortalin and augments cell death [73]. Therefore, it appears that mortalin is involved in the process of MAC elimination and in cell defense from complement. The mode of action of mortalin is still not known, yet its ability to bind C8 and C9 [73] may suggest a role for mortalin in MAC assembly prior to vesicle formation. The contribution of plasma membrane remodeling to MAC vesiculation still awaits further examination.

Complement-independent release of vesicles by normal and malignant cells

As described above, complement is a potent inducer of release of membrane vesicles. Ectocytosis and exocytosis are a general biological property of most eukaryotic cells. For instance, polymorphonuclear leukocytes (PMN) release ectosomes after stimulation with calcium ionophores, phorbol esters, and microbial peptides such as formyl-methionyl-leucyl-phenylalanine (fMLP) [26, 35] (Fig. 1c). Chondrocytes continuously release ectosomes loaded with annexin I, which induce calcium precipitation and bone formation [2]. The number of different cells which have been described to undergo ectocytosis in vitro as well as in vivo has been growing over recent years and now covers a large spectrum of cell types, in particular, many tumor cells and cell lines, endothelial cells, and almost all blood cell types. Ectocytosis has also been described in platelets, monocytes, and lymphocytes [52, 81, 85]. The physiological distinction between ectosomes and exosomes in platelets might be difficult since both are released concomitantly when platelets are activated [34]. Formation of exosomes and ectosomes can be well distinguished in developing erythrocytes. The loss of transferrin receptors occurs during erythrocyte maturation in part through the formation of exosomes, which are then continuously shed until complete loss of transferrin receptors [70]. In contrast, stressed erythrocytes shed ectosomes (e.g., upon ATP depletion activating in vitro aging or a complement attack) [72]. Whether or not ectosomes released in response to diverse stimuli have distinct structure and composition remains to be determined.

Recent studies, mainly of PMN and erythrocyte ectosomes, have characterized some of the biological properties of ectosomes. First, ectosomes express an array of proteins different from that present on the surface of the cell from which the ectosomes have been released. For example, ectosomes released by PMN express selectins, integrins, complement regulators, HLA-1, FcγRIII, and CD66b, but lack CD14 and FcγRII, which are found on the surface of resting PMNs [26]. Ectosomes released by a monocytic cell line THP-1 have been reported to be loaded with IL-1β [51]. Thus, the transfer of molecules into ectosomes is a selective process. Very little is known about the mechanism responsible for such a protein sorting. The selection of the proteins incorporated into ectosomes appears to be unrelated to the type of membrane anchor since both GPI-linked as well as transmembrane proteins have been found on the surface of ectosomes [26]. Both complement receptor type 1 (CD35, a transmembrane protein) and DAF (CD55, GPI-anchored) are similarly enriched in ectosomes released from erythrocytes [72]. However, the fact that major differences are observed in both lipid com-

position of ectosome vs. plasma membranes and the distribution of the lipids within the membranes suggests that certain membrane lipids play a major role in ectosome formation [49]. The process of ectosome formation, like apoptotic cell death, involves externalization of phosphatidylserine (PS). Interestingly, proteins that are degraded on activated PMNs, may remain intact on ectosomes. For example, L selectin is cleaved on activated PMNs to permit migration through the endothelial cell layer. In contrast, on PMN ectosomes, it remains intact, suggesting that the L selectin protease has been excluded from the ectosomes. Another surprising finding is that PMN ectosomes express on their surface enzymes, e.g., myeloperoxidase, which are normally stored in granules in resting PMNs. The expression of proteins in ectosomes may therefore depend on both packaging of proteins during ectosome formation and further on binding of soluble proteins after their release.

Ectosomes and exosomes express membrane complement regulatory proteins that have originated either from the cell surface or from intracellular stores, respectively. PMN-derived ectosomes express CR1 (CD35), MCP (CD46), DAF (CD55), and CD59. The expression of membrane regulators has been shown to confer protection from complement on the released membrane vesicles [15, 32]. Thus, membrane regulators might significantly modify the fate of microvesicles, allowing them to remain intact and functional. Synthetic liposomes containing PS activate complement and are consequently lysed. In contrast, PMN-derived ectosomes activate complement and fix C1q, C4, and C3 as well as a small amount of C5b-9, but are not lysed [28, 47]. Thus, ectosomes opsonised with C3 fragments may bind to cells expressing C3 receptors, in particular CR1. In blood, the bulk of CR1 is expressed by erythrocytes. Recent studies have indicated that PMN ectosomes attach rapidly to erythrocytes when formed in whole blood [28]. Thus, ectosomes, like immune complexes, are probably transported in the circulation and may reach the liver/spleen, where they are likely to be downloaded similarly to immune complexes. This observation may explain why only few ectosomes can be isolated from blood plasma, even in diseases such as sepsis, in which continuous and strong activation of PMN is a central feature.

Release of membrane microparticles, i.e., 0.05- to 1-μm membrane vesicles, from stressed and/or apoptotic cells has been shown to be accompanied by plasma membrane changes in the form of membrane blebbing and externalization of phosphatidylserine (PS) (reviewed in [25]). However, this is probably not a general phenomenon. Studies with endothelial cells suggest that a large fraction of microparticles released from activated and apoptotic cells do not bind measurable amounts of annexin V, implying that the expression of PS on them is absent or very low [1]. Release of microparticles from apoptotic neutrophils depends on different signals from those required for membrane blebbing and PS externalization and is therefore occurring independently of these two hallmarks of apoptosis [67]. It still remains to be determined whether apoptosis-derived microparticles are comparable to ectosomes [56] or exosomes recycling through MVB [67].

Biological consequences of vesicle shedding

Should the released membrane vesicles be regarded simply as waste products, which are disposed of by macrophages, or perhaps they play important roles in inflammation and other biological reactions? Exosomes are capable of eliciting specific immune responses [3, 86, 93]. Ectosomes of endothelial cells, monocytes, and platelets have been shown to induce and

enhance thrombosis [80, 81], whereas ectosomes of chondrocytes support ossification [2]. Many other essential functions might be related to vesicles in general. Monocytes may release IL-1β by ectocytosis. Recently, the cell-to-cell transfer of chemokine receptor has been suggested to be due to ectosomes shuttling from one cell to another [50]. PMN ectosomes express enzymes capable of attacking microorganisms, and they may well be doing so at sites of acute infection [26]. On the other hand, the same ectosomes, when ingested by monocytes/macrophages, downregulate the inflammatory potential of these cells [27]. It is conceivable that this latter property will protect the organism from excessive inflammation at a site of infection.

Release of membrane vesicles is also a common feature of tumor cells [91]. It is conceivable that these cells release both exosomes and ectosomes. Prostasomes released by prostate carcinoma cells and normal epithelial cells are found in semen [78]. Like exosomes, they are enriched in raft molecules found intracellularly in MVB, and their release is sensitive to wortmannin. Hence, prostasomes could actually represent specific exosomes of the prostate tissue [48]. The high cholesterol and sphingomyelin content of the extracellular membrane vesicles [4, 89, 95, 104] indicates increased membrane rigidity. Membrane vesicles released from tumor cells have been suggested to facilitate tumor growth, tissue invasion, and immune evasion by bearing matrix-degrading proteases [17, 31], angiogenic activity [41], and Fas ligand [91]. These membrane vesicles also contain protein kinase CK2 that has been shown to phosphorylate complement C9 and protect from complement-mediated lysis [5]. Membrane vesicles collected from malignant tumor fluids correlate positively with tumor malignancy. Therefore, measurement of membrane vesicle content and associated metalloproteinase level may serve as markers for tumor aggressiveness and progression [30].

Conclusions

Despite the fact that MAC removal by vesiculation is a widely accepted resistance mechanism against complement, still very little is known about the process of activation and regulation. Perhaps through mortalin [73], a deeper insight into the machinery of MAC vesiculation will be achieved. Mortalin is an intracellular hsp70-related protein, and it will be intriguing to realize whether and how such a protein supports membrane vesiculation. Hsc70, another intracellular hsp70-related protein that is externalized upon treatment with sublytic C5b-9 [22], is involved in clathrin-dependent endocytosis [19]. Mortalin may be similarly involved in the inverse activity, ectocytosis. To answer that, it is important to determine first whether the MAC vesicles are released from the plasma membrane by direct ectocytosis or following endocytosis, formation of MVB, and exocytosis pending fusion of the MVB with the plasma membrane (Fig. 2). At present, concomitant MAC vesiculation by ectocytosis and exocytosis cannot be excluded.

Exosomes derived from dendritic or cancer cells are also being considered as immunomodulators for cancer immunotherapy [3, 20]. Such exosomes express MHC-peptide complexes that can be targeted to antigen-presenting cells, thus amplifying specific immune responses [96]. Given that mortalin is capable of presenting antigens to T cells [42, 97] and that membrane vesicles released after sublytic complement attack may contain mortalin-peptide/protein complexes, it is intriguing to propose that, like MHC-loaded exosomes, mortalin-loaded membrane vesicles may play a role in immune regulation in normal and

pathological immune responses. It is also important to note that, under certain conditions, instead of augmenting immune responses, exosomes can suppress antitumor immune responses [77, 91] and ectosomes can have anti-inflammatory effects [27]. Therefore, MAC-induced vesicles may be added to the growing list of complement-derived immunomodulators produced at complement activation sites, and future studies will reveal their effects. Furthermore, MAC-induced vesicles are likely to have numerous effects beyond the immune system, as predicted from the reported effects of membrane microparticles (ectosomes) on coagulation, vascular functions, angiogenesis, wound healing, and development (reviewed in [53, 56]). Elevated levels of microparticles may be pathogenic [25] and have already been implicated in endothelial and vascular/heart dysfunction, inflammation, autoimmunity, and tumor escape.

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