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Microparticles as a source of extracellular DNA

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Abstract

Microparticles are small membrane-bound vesicles that display pro-inflammatory and pro-thrombotic activities important in the pathogenesis of a wide variety of diseases. These particles are released from activated and dying cells and incorporate nuclear and cytoplasmic molecules for extracellular export. Of these molecules, DNA is a central autoantigen in systemic lupus erythematosus (SLE). As studies in our laboratory show, DNA occurs prominently in microparticles, translocating into these structures during apoptotic cell death. This DNA is antigenically active and can bind to lupus anti-DNA autoantibodies. These findings suggest that microparticles are an important source of extracellular DNA to serve as an autoantigen and autoadjuvant in SLE.

Keywords

Microparticles; DNA; Apoptosis; Systemic lupus erythematosus; Innate immunity

Introduction

Microparticles are small membrane-bound vesicles that serve as mediators of inflammation as well as conduits of intercellular communication to modulate immune cell function in physiological and pathophysiologic settings. These particles range in size from approximately 0.1 to 1.0 microns and are released from cells during the processes of cell activation and cell death. In the context of cell death, microparticles may represent blebs that have detached from cells or, indeed, have been actively ejected. As extracellular organelles, microparticles contain cytoplasmic and nuclear constituents that are enclosed by the plasma membrane; the presence of cell surface markers allows identification of particle origin. The rich content of immunoactive cell molecules, including membrane receptors, endows

microparticles with pro-inflammatory and pro-thrombotic properties that can promote innate and adaptive immune responses [1–3].

As shown in studies of patients, levels of microparticles in the blood are elevated in a wide variety of diseases that encompass the full spectrum of medicine. These conditions include inflammatory diseases (e.g., rheumatoid arthritis, systemic lupus erythematosus), infections, malignancy and cardiovascular diseases including atherosclerotic heart disease and stroke. As shown by flow cytometry using antibodies to cell surface markers, microparticles can arise from a variety of cell types although those from hematopoietic cells, platelets in particular, are the most abundant [4]. Increased blood particle numbers may have direct effects on pathogenesis since particles can induce inflammation and thrombosis and can impact on many immune cell types as well as the endothelium[3].

In our laboratory, we are interested in the pathogenesis of systemic lupus erythematosus (SLE), a prototypic autoimmune disease characterized by multisystem organ involvement in association with the expression of antibodies to nuclear antigens (antinuclear antibodies or ANA). Among these ANA, antibodies to DNA (anti-DNA) represent markers of diagnostic and prognostic significance and serve as important biomarkers in assessing disease activity. Furthermore, anti-DNA antibodies play a direct role in pathogenesis and can form immune complexes which have two important functions. These complexes can deposit in the kidney to fix complement and incite renal injury [5]. In addition, immune complexes containing DNA can stimulate the production of cytokines, most prominently type 1 interferon, by plasmacytoid dendritic cells. This stimulation involves toll-like receptors (TLRs) as well as non-TLRs and depends on the nucleic acid content of the complexes. Other receptors such as Fc receptors and RAGE (receptor for advanced glycation endproducts) contribute to this immune activation, with RAGE likely responding to the nuclear protein HMGB1 (high mobility group protein B1) that is bound to DNA in the immune complex [6–8].

While the anti-DNA antibody response has been intensively investigated, relatively little is known about the origin of the DNA that either stimulates this response or forms immune complexes. In general, this DNA is thought to arise during cell death, with levels accentuated in lupus because of either accelerated apoptosis or impaired dead cell clearance [9]. Since defects in humoral and cellular systems involved in dead cell clearance can lead to lupus in both humans and murine models, the exposure of DNA in an immunologically relevant form has emerged as a central event in the immunopathogenesis of SLE. Coupled with data that even endogenous DNA has immunological activity, increasing evidence points to extracellular DNA as a prime suspect in initiating autoimmunity, serving as both an autoantigen and adjuvant [10].

Our studies have focused on microparticles as an important source of the extracellular DNA for several reasons and have explored the idea that a particulate form of DNA may have unique and potent activities. Thus, DNA and microparticles show similar patterns of expression in disease, with both displaying elevated levels in conditions marked by inflammation or cell turnover and death. Second, in experimental models in vitro, extracellular DNA and microparticles arise in similar situations, with apoptosis the most notable. Indeed, in apoptosis, there is extensive cleavage, rearrangement and translocation of DNA and other nuclear molecules as death proceeds, with blebs being a favored site for the movement of these molecules [11, 12]. Since blebs and microparticles have similar dimensions and dynamics of expression, it appears likely that at least some microparticles are released blebs and therefore contain bleb constituents (i.e., nuclear molecules).

Another reason for our interest in elucidating the nucleic acid content of microparticles relates to our previous observations on the immune activity of DNA in transfection agents.

Thus, we found that DNA encapsulated in lipoidal agents used for transfection (lipofectin) shows a dramatically enhanced ability to stimulate immune responses in comparison with free DNA [13, 14]. Furthermore, we found that DNA in lipofectin can stimulate macrophage responses irrespective of the content of the classical CpG (cytosine-guanine) motifs that serve as a PAMP (pathogen-associated molecular pattern) for recognition of bacterial and viral DNA. These motifs occur much more commonly in bacterial DNA than mammalian DNA because of CpG suppression in mammalian DNA and the lack of cytosine methylation in bacterial DNA. Thus, we showed that, whereas with free DNA, bacterial not mammalian DNA can stimulate murine macrophage production of nitric oxide (NO), with DNA in lipofectin, both DNA sources are active.

In view of these findings, we reasoned that with transfected DNA, DNA can engage other signaling pathways than the conventional TLR9 which responds to CpG motifs whether in bacterial DNA or synthetic oligonucleotides. At the time we performed these experiments, the non-TLR nucleic acid sensors had not yet been defined although it appears likely that such sensors are responsible for the responses we observed. In view of these findings, we reasoned that DNA in the context of lipids or vesicles may be the relevant immunostimulatory moiety in vivo, with DNA complexed with transfection agents mimicking the natural system. Thus, we became interested in the possibility that microparticles could be the endogenous source of encapsulated DNA and have immunostimulatory properties that differed from those of free DNA. We therefore turned our attention to microparticles as a source of extracellular DNA and investigated their structure and antigenic properties.

Generation of microparticles during apoptosis

In our experiments, we have extensively used in vitro culture systems to generate microparticles and characterize their nucleic acid content. An important issue in this type of study concerns the assay of microparticles. These structures are very small, with diameters approximately 10–100 times less than that of a conventional cell. As such, their surface area and volume are dramatically reduced in comparison with a lymphocyte, for example, with their measurement challenging the capabilities of the usual flow cytometer. To demonstrate particles, we have used analytic conditions that involve collection of flow data using side scatter detection at logarithmic gain. Even under these conditions, quantitation is difficult since the range of particle size can overlap with machine “noise.” As will be discussed below, nucleic acids provide an alternative approach to detection.

Using flow cytometry, we demonstrated that induction of apoptosis of Jurkat and HL-60 cell lines leads to the production of microparticles in a time-dependent process that is correlated with cell apoptosis as assessed by staining with annexin V and propidium iodide (PI). This production can be blocked by caspase inhibitors, indicating a close relationship to cell death. Among stimulants for microparticle release, staurosporine, a protein kinase C inhibitor, led to the most abundant particle production. Other agents capable of particle generation included etoposide, camptothecin, and UV irradiation [15]. Although blebbing is a feature of apoptosis, we have not directly determined whether the particles released originate from blebs; blebbing is an early process, however, whereas in our experiments, particle release occurs at a time when cells are annexin V positive/PI positive, a stage that can be considered late apoptosis.

Like apoptotic cells, microparticles are annexin V positive and PI positive [16]. While this phenotypic appearance is consistent with the release of particles from the membrane of apoptotic cells in which the phosphatidylserine (PS) has “flipped,” we fortuitously found evidence for another process. Thus, in time course experiments, we observed that in Jurkat

cells treated with staurosporine, the original population of particles released into the media was in fact annexin V low and PI low or negative. With subsequent culture, the particles changed their phenotype and became annexin V positive and PI positive [16]. These findings suggest that particles are dynamic structures and that, after release from cells, can undergo changes that lead to membrane permeabilization and PS “flipping.” This issue is important in particle assessment and could indicate the origin of annexin V negative particles.

In our experiments, the early release of particles from Jurkat cells occurred most readily with staurosporine and the related kinase inhibitor 7-hydroxystaurosporine but was not observed with other specific protein kinase C inhibitors or inhibitors of cyclin-dependent kinase (CDK). Thus, this particular aspect of particle release may result from the activity of another kinase that staurosporine inhibits or the simultaneous inhibition of multiple kinases [16]. Given the broad action of kinase inhibition by staurosporine, both mechanisms are possible. While many of the details of this release reaction remain to be determined, these studies nevertheless establish cell death as a setting for particle release.

Generation of microparticles during activation

In addition to cell death, cell activation is a setting in which microparticle release occurs. We were particularly interested in particle release during macrophage activation. In other studies in our laboratory, we have extensively investigated the mechanism for the release of HMGB1, a non-histone nuclear protein that is the prototypic alarmin. An alarmin is a cellular molecule that can promote immune activation when released from a cell during injury, death or activation. Alarmins can be large or small molecules although their common activity is cytokine and chemokine induction [17–19]. In many respects, microparticles are part of the continuum of alarmins although their large size and multicomponent composition may preclude their designation by a term that has been used for single molecular species. The biology of alarmins and microparticles is nevertheless very similar. Indeed, some alarmins may be components of microparticles.

As studies from other laboratories have shown, HMGB1 can exit cells following stimulation by LPS, translocating from the nucleus to the cytoplasm for entry into vesicles for secretion by a non-conventional mechanism [20, 21]. We extended these findings by showing the HMGB1 release also occurs with stimulation with poly (I:C), a ligand of TLR3 although, interestingly, stimulation by a CpG oligonucleotide, a ligand of TLR9, did not cause HMGB1 release by either RAW264.7 murine macrophage cells or bone marrow-derived macrophages. In the case of LPS, HMGB1 release depends on nitric oxide, while for poly (I:C) stimulation, interferon- α is the mediator of the translocation and release process [22, 23].

In view of these findings, we assessed similar processes with microparticle release. Using RAW264.7 cells as a model, we showed that both LPS and poly (I:C) can stimulate this release process in a time- and dose-dependent manner. As in the case of HMGB1 release, stimulation with a CpG oligonucleotide did not cause microparticle release, suggesting that this process occurs with stimulation of some but not all TLRs. Furthermore, for both LPS and poly (I:C) stimulation, microparticle release depends on NO production since inhibition of nitric oxide synthase by 1400W can block particle release. A direct role of NO in this response is demonstrated by the effects of NO donors which can induce microparticle production [24].

Because stimulation by both LPS and poly (I:C) can lead to apoptosis, it remains possible that particle release during TLR stimulation results from cell death rather than activation. It is difficult to make this distinction precisely because of the inevitable coexistence of cell

death and activation in macrophage cultures stimulated by certain but not all TLR ligands. Furthermore, attempts to distinguish the contribution of activation versus death by blocking caspase activation are confounded by the effects of caspase inhibitors. These inhibitors can affect cleavage of the NUR-77 orphan receptor, leading to an increase in cell death and therefore release of both HMGB1 and microparticles. Distinguishing the contributions of activation and death to the production of HMGB1 and microparticles during TLR stimulation may not be critical. One of the effects of TLR activation may in fact be the induction of cell death to restrain intracellular replication of viruses or bacteria and stimulate innate immunity. As such, cell death may be an intended effect of TLR activation, with release of microparticles and alarmins integral parts of an alarm system to promote host defense.

Nucleic acid content of MPs

Our studies using a variety of in vitro and in vivo models have established many similarities in the circumstances for the release of nuclear molecules, both DNA and HMGB1, and microparticles. We therefore directly tested whether DNA and RNA released from cells during apoptotic cell death are in the form of microparticles. For this purpose, we determined the DNA and RNA content of microparticles released from cells following induction of apoptotic cell death in vitro. For these experiments, we used the Jurkat and HL-60 lines and induced death by a variety of chemotherapeutic agents. Particles were isolated by differential centrifugation and nucleic acid content characterized by biochemical assays.

Results of these studies showed clearly that microparticles are an important source of extracellular DNA and RNA released during apoptotic cell death. In our experiments, the DNA in particles resembled that in the originating apoptotic cells since this DNA had evidence of nucleolytic cleavage, including laddering [15]. The microparticles from the apoptotic cells also contained RNA, both ribosomal and messenger. Interestingly, the ribosomal RNA in the particles showed evidence of cleavage which was much more pronounced than that in the cell itself. While selective degradation of ribosomal RNA during apoptosis occurs, these findings suggest that either the cleaved species are selectively packaged into particles or that cleavage occurs in the particles after release from the cell. We also showed that particles contained messenger RNA as demonstrated by RT-PCR.

The presence of DNA and RNA in particles suggested to us a new way to measure particles by flow cytometry that would reduce or bypass the difficulties using light scatter as well as the uncertainty in measuring particles on the basis of cell surface molecules from the cell of origin. Light scattering is limited by size considerations and the overlap of particles with machine "noise." Similarly, particle detection using surface markers can be limited by the small surface area of particles as well as the variable expression of different markers on the particle surface.

To exploit the nucleic acid content of microparticles for their detection, we investigated particle measurement by staining particles with the nucleic acid-binding dye SYTO 13. Unlike propidium iodide which does not penetrate intact membranes, SYTO 13 can enter into both live and dead cells and, by extension, particles of all kinds, whether or not the membrane barrier is retained. This approach had been used by others to measure bacteria by flow cytometry. Since SYTO dyes can interact with either DNA or RNA, this method is applicable to microparticle detection as long as the particle contains some nucleic acid.

Our studies demonstrated clearly that SYTO 13 staining can significantly increase the sensitivity of detection of particles generated in vitro during apoptosis [25]. Indeed, we could detect up to three times as many particles as observed with light scatter with particles

obtained from Jurkat cells undergoing apoptosis. This increased sensitivity results from better detection of small particles which would be excluded in the usual flow cytometric assays by light scattering. This approach likely will work the best with particles from nucleated cells because of the contribution of DNA that has translocated from the cell nucleus into the particle, thereby providing an important source of DNA for binding by SYTO 13. Studies are in progress to assess the utility of this approach using samples of blood and other biological fluids where platelet microparticles are the most abundant species. As platelet microparticles likely arise from platelets as opposed to megakaryocytes, they should be devoid of nuclear DNA which produces a strong signal with SYTO 13; we would therefore expect that the increased sensitivity for detection by a nucleic acid-binding dye may not pertain.

Antigenicity of microparticles

As our experiments have shown, microparticles obtained from cultures of apoptotic cells have a significant content of DNA. We next asked whether this DNA is antigenically active, thereby allowing binding by anti-DNA autoantibodies. To explore this possibility, we tested the interaction of microparticles derived from cultures of apoptotic Jurkat cells with a murine monoclonal anti-DNA antibody. As results of these experiments showed, the monoclonal antibody bound well to the particles generated in vitro, with treatment of DNase reducing or eliminating reactivity [15]. We have observed similar reactivity with other monoclonal anti-DNA antibodies as well as sera of patients with lupus (Fig. 1). Together, these observations indicate that microparticles display DNA in an accessible form. This DNA may be present on or near the particle surface such that an antibody has ready access. Alternatively, the microparticle may have a porous structure such that antibody may enter its interstices to interact with DNA even if the DNA is remote from the particle surface. While these issues may seem technical or semantic in nature, they nevertheless impact on the ability of microparticles to form immune complexes or for the ability of antibodies bound to particles to interact with Fc receptors to promote immune stimulation or mediate uptake for phagocytosis.

Conclusions

Together, our studies identify microparticles as an important source of extracellular DNA that can enter the blood during cell death or cell activation (Table 1). The presence of DNA outside the confines of the cell can have important immunological effects because of the potential to stimulate TLR and non-TLR nucleic acid sensors, induce antigen-specific antibody responses and form immune complexes that can drive cytokine production and deposit in the tissues. Future studies will define the details of DNA trafficking among compartments during cell death as well as the unique immunostimulatory properties of particle-bound DNA. The information content of this DNA is fascinating to consider, and hopefully, in the future the biology of DNA on the outside of the cell will become as important a topic of investigation as DNA on the inside of the cell, the usual but not invariable location of this most fundamental of all macromolecules.

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Fig. 1. Binding of a monoclonal anti-DNA antibody to microparticles. The figure illustrates the antigenicity of microparticles. For this experiment, microparticles were prepared from HL-60 cells induced to undergo apoptosis by treatment with staurosporine for 18 h. The particles were isolated and then incubated with a murine monoclonal antibody (QB-1) to DNA followed by a PE-labeled goat anti-mouse IgG reagent for FACS analysis. The *solid gray* peak shows fluorescence due to binding of the QB-1 antibody compared to conjugate control alone (*black line*).

Table 1**Properties of microparticles**

Production during apoptosis and activation
Contain DNA and RNA
DNA is cleaved and shows laddering
Nucleic acid dyes facilitate detection by flow cytometry
DNA is accessible to anti-DNA binding