The effect of inflammation on the generation of plasma DNA from dead and dying cells in the peritoneum

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Abstract: To assess the effects of inflammation on the generation of circulating DNA from dead and dying cells, plasma DNA levels were determined in BALB/c mice, administered apoptotic or necrotic Jurkat cells following induction of peritonitis by treatment with thioglycollate (TG), peptone (PT), or sodium periodate (NaIO₄). In mice receiving TG or NaIO₄, plasma DNA levels following intraperitoneal administration of Jurkat cells were significantly reduced compared with controls, whereas they were not affected in mice receiving PT. To determine the basis of these differences, the cellular composition of peritoneal fluids prior to the administration of the dead cells was analyzed. Among agents tested, TG administration led to the largest increase in cells—neutrophils and monocytes. As shown by flow cytometry, the exudates contained apoptotic neutrophils and macrophages, with the highest levels in the TG-induced exudates. Analysis of DNA and caspase 3 in the fluids also showed differences; TG exudates showed increases in DNA and caspase 3; and NaIO₄-induced exudates had an increase only in DNA. Fluid from PT-treated mice did not have increases in DNA or caspase 3. Together, these results indicate that prior inflammation can affect the generation of blood DNA from apoptotic or necrotic cells, although this effect may vary depending on the composition of the exudates with respect to cells as well as DNA. J. Leukoc. Biol. 77: 000–000; 2005.

Key Words: apoptosis · necrosis · neutrophils · peritonitis

INTRODUCTION

DNA is a nuclear macromolecule that can exist in an intracellular and extracellular form. In its extracellular form, DNA can appear in the blood as well as other biological fluids, with its level rising in a variety of clinical conditions [1–6]. These conditions include systemic lupus erythematosus (SLE), malignancy, pregnancy, and trauma among many others. Although often considered inert, extracellular DNA, depending on context and binding to other molecules, can display potent effects on the immune system [9, 10]. In SLE, for example, immune complexes containing DNA can stimulate interferon-α/β production by their action on plasmacytoid dendritic cells [11, 12]. Similarly, in mice expressing a rheumatoid factor transgene, such immune complexes can activate B cells by a mechanism involving Toll-like receptor 9 [13, 14]. Understanding the origin of blood DNA is therefore important for elucidating the mechanisms of autoimmune and other immune-mediated diseases.

In view of the conditions in which its levels are elevated, blood DNA has generally been considered the result of cell death, and apoptosis is implicated as the major mechanism for DNA release. Indeed, DNA in the blood shows low molecular weight and size-laddering, the hallmarks of the apoptotic process [3, 6, 15, 16]. Furthermore, in vitro, apoptotic cells can release DNA into the extracellular milieu. In contrast, necrotic cells do not release DNA under in vitro conditions [17]. The role of apoptosis in DNA release is strengthened by observations that treatment of mice with an anti-Fas antibody, which induces widespread hepatic apoptosis, causes a large increase in blood DNA [18].

The settings in which blood DNA levels are elevated are, in general, complex and involve cell activation and cell death, often occurring concomitantly [2, 7, 8, 19]. Thus, in tissue injury from trauma or toxin exposure, inflammation may follow the death-inducing insult. Similarly, in tumors, areas of necrosis and apoptosis may coexist adjacent to surrounding inflammation. These processes are dynamic, and the cellular composition of an inflammatory site as well as the activation state of cells present evolve over time. Furthermore, as these processes progress, the elaboration of cytokines and other inflammatory mediators may provoke subsequent waves of cell death and activation [20–22].

In a previous study, we used a murine model to elucidate the mechanisms of DNA release, testing whether cell death is sufficient to induce a blood DNA response and whether apoptotic and necrotic cells are similar in their behavior [23]. For this purpose, normal mice were administered apoptotic or necrotic Jurkat cells, and plasma DNA was determined using the fluorimetric dye PicoGreen. Results of these studies indicated that in vivo, apoptotic and necrotic cells can produce blood DNA and that the blood DNA arising from these cells shows laddering with both. Furthermore, we showed that in mice, in which macrophages were eliminated by clodronate...
treatment, a rise in blood DNA did not occur after administration of the dead cells. Together, these results indicate that the generation of blood DNA depends on macrophages and may not simply reflect the occurrence of cell death.

In view of the close relationship of inflammation and cell death and the role of macrophages in the clearance of dead and dying cells, we have therefore used an in vivo system to investigate whether prior inflammation can influence the generation of blood DNA from dead and dying cells. For this purpose, we induced peritoneal inflammation in normal mice prior to the administration of apoptotic or necrotic cells and then assessed plasma DNA by fluorimetry. In results presented herein, we show that in mice with peritonitis induced by thioglycollate (TG) or sodium periodate (NaIO₄), a plasma DNA response did not occur following administration of Jurkat cells treated with etoposide to induce apoptosis or ethanol to induce necrosis. In contrast, mice treated with peptone (PT) had blood DNA responses similar to those of untreated mice. Together, these results indicate that inflammation can affect the generation of DNA from dead and dying cells, although the nature of this effect may vary depending on the inflammatory stimulus and local cellular events.

MATERIALS AND METHODS

Preparation of mice and cells
Female BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and were used for experiments at ages 6–12 weeks. To study effect of inflammation on DNA release, mice were treated with 2 ml 4% TG (Sigma Chemical Co., St. Louis, MO) [24], 1 ml 10% PT (Becton Dickinson, Sparks, MD) [24], or 1 ml 5 mM NaIO₄ (Sigma Chemical Co.) [25, 26] at days 1 and 3 and then administered apoptotic or necrotic cells by the intraperitoneal (IP) route. For these experiments, cells were administered at day 4 after TG and NaIO₄ treatment and day 3 after PT treatment.

For the induction of apoptosis, Jurkat cells growing in RPMI 1640 with 10% fetal bovine serum were treated with etoposide (Sigma Chemical Co.) at 30 μg/ml for 24 h. For the induction of necrosis, cells were treated with 70% ethanol for 10 min. After treatment, cells were washed twice with phosphate-buffered saline (PBS; Gibco-BRL, Grand Island, NY) and suspended in PBS. The cells were administered IP at 10⁷ cells/mouse. Following these treatments, mice were bled at regular times thereafter as indicated in the figures. The blood was collected into tubes with 3–5 mice were bled at regular times thereafter as indicated in the figures. The blood was collected into tubes with 3–5

Measurement of DNA in plasma and peritoneum

Plasma DNA was assayed by a fluorimetric assay as described previously [23]. Briefly, plasma in various dilutions were mixed with the dye PicoGreen (Molecular Probes, Eugene, OR), diluted 1:200 in 10 mM Tris, 1 mM EDTA, pH 8 (TE buffer) in a black, 96-well microtiter plate (Costar, Corning Inc., Corning, NY). The DNA concentration was determined from fluorescence measurements using a TECAN GENios microplate fluorescence reader (Salzburg, Austria) with an excitation wavelength at 485 nm and an emission wavelength at 535 nm. Data were collected as relative fluorescence units. The concentration of DNA in plasma was calculated according to a standard curve using double-stranded calf thymus DNA (Sigma Chemical Co.). Peritoneal fluids were diluted 1:10 in TE buffer, and DNA levels were measured as described above.

Flow cytometry analysis

To assess the effects of various agents on immune cell populations, flow cytometry was performed on preparations of peritoneal washout cells. Briefly, mice treated with TG, PT, or NaIO₄ were killed by cervical dislocation, and peritoneal cells were harvested at various time-points by lavage with 1 ml cold PBS. The cells were pelleted at 300 g for 5 min and resuspended in hypotonic lysis buffer to remove red blood cells, followed by centrifugation and two washes with PBS/0.5% bovine serum albumin. The cell concentrations were adjusted to 1 × 10⁷/ml in PBS, and 1 × 10⁶ cells were used for staining. Cells were treated with anti-mouse CD16/CD32 antibody (BD Pharmingen, San Diego, CA) and then stained with phycoerythrin (PE)-anti-mouse F4/80 (Ro-rote, Raleigh, NC) or PE-anti-mouse Ly-6G or Ly-6C (Gr1; BD Pharmingen), along with Annexin V-fluorescein isothiocyanate (BD Pharmingen). Cells were analyzed using a FACScan flow cytometer (Becton Dickinson, Mansfield, MA). Data analysis was done using CellQuest software (Becton Dickinson Immuno-cytometry Systems, San Jose, CA).

RESULTS

To assess the effects of inflammation on the generation of plasma DNA from dead and dying cells, the release of DNA from apoptotic or necrotic Jurkat cells was assessed in mice receiving TG, NaIO₄, or PT to induce peritoneal exudates. Although these agents have all been used to peritonitis, they differ in the nature of the inflammatory stimulus, the cellular composition of the resulting exudates, and the time-course of cellular accumulation [24–26]. In these experiments, apoptosis or ethanol to induce necrosis, were administered by the IP route to treated mice.

As shown previously, plasma DNA rises following administration of either type of dead cell with DNA derived predominately, but not exclusively, from the Jurkat cells [23]. Figures 1–3 show the results of these experiments; for NaIO₄-treated mice, only apoptotic cells were studied. As these data indicate, consistent with previous observations for control mice, the magnitude and time-course of appearance of plasma DNA were similar with apoptotic and necrotic cells, returning to baseline by 24 h after administration. Among the three groups of mice treated to induce peritonitis, however, the appearance of plasma DNA differed. For mice treated with TG or NaIO₄, the plasma DNA levels were reduced markedly compared with controls, whereas for PT-treated mice, the levels of DNA were comparable with controls. These results suggest that local inflammation can affect the generation of plasma DNA from dead and dying cells, although the effect will vary depending on how the peritonitis is induced.

In these experiments, the dead cells were administered into an inflammatory site, where cell populations are undergoing changes in composition and properties. For example, although TG administration leads to an increase in the number of macrophages, the initial response consists of neutrophils, a population prone to die by apoptosis [27–29]. Therefore, the cellular composition of peritoneal exudates was characterized in the time-period prior to the administration of the apoptotic and necrotic cells to determine any features that could account
for the differences in the results obtained on plasma DNA levels.

Table 1 presents the results of experiments showing the total number of peritoneal exudate cells, the number of neutrophils (Gr1⁺), and number of macrophages (F4/80⁺). As these data indicate, the highest number of peritoneal cells observed occurred in the TG-treated mice at early time-points (6 and 16 h) as well as at later time-points (72 h). At 6 and 16 h, the predominant cells in all groups were neutrophils, and at 72 h, the predominant cells were macrophages. In the TG-treated mice, F4/80⁺ cells were especially abundant, far exceeding the numbers in the other two treatment groups.

The composition of these exudates was further analyzed to identify features that could be correlated with the levels of plasma DNA observed following administration of the apoptotic or necrotic Jurkat cells. This analysis focused on the occurrence of apoptosis among cells accumulating in the exudates, especially among neutrophils, as these are short-lived cells that undergo apoptosis as inflammation proceeds. As also shown in Table 1, peritonitis provoked by all three stimuli led to an increase in the number of apoptotic neutrophils, as assessed by Gr1⁺ cells, which were annexin⁺. These numbers were highest in exudates induced by TG. In particular, the exudates from TG had the highest number of apoptotic F4/80 cells at time-points from 6 h to 72 h, and exudates induced by PT or NaIO₄ did not differ in the number of annexin⁺, F4/80⁺ cells compared with baseline.

These findings suggest that prior to the administration of the apoptotic and necrotic Jurkat cells, the peritoneal cavity had been the site of cell death, and the extent and cell population involved depend on the inducing agent. To further assess this possibility, levels of caspase 3 and DNA were determined in the peritoneal fluid. Caspase 3 and DNA are released from apoptotic cells and provide a marker of apoptosis in biological fluids [17, 30, 31]. Figure 4 presents results of these determinations for peritoneal fluid. As these data indicate, each of the stimulating agents led to a distinct pattern of DNA and caspase 3 expression. Whereas exudates induced by TG showed DNA and caspase 3, exudates from mice treated with NaIO₄ showed only DNA. In contrast, exudates from mice treated with PT showed neither DNA nor caspase 3.

These data thus provide an opportunity to assess a correlation between events in the peritoneum and the generation of plasma DNA from apoptotic or necrotic Jurkat cells. Thus, plasma DNA from apoptotic or necrotic cells did not occur in mice receiving TG or NaIO₄, the two conditions in which exudates contained DNA. These results suggest that events that lead to the release of DNA into the peritoneal fluid affect
the subsequent generation of plasma DNA response from dead and dying cells.

RESULTS

Results presented herein provide new insights into the mechanisms for the generation of circulating DNA from dead and dying cells and indicate that prior inflammation can impact this process significantly. Thus, we have shown in a murine model that the presence of peritoneal inflammation can influence the generation of plasma DNA from apoptotic or necrotic cells administered by the IP route. This effect was not uniform, however, and depended on the inducing agent as well as local events in the peritoneum. These events were reflected in the cellular composition of exudates and the occurrence of local apoptosis, as indicated by DNA and caspase-3 in the peritoneal fluid. Taken together with previous experiments, these results indicate that the release of DNA into the blood is not the simple consequence of the presence of a large number of dead cells but rather results from a complex interplay with other cell types.

Fig. 3. Effect of NaIO4 administration on plasma DNA levels from apoptotic cells. 10⁸ Jurkat cells, treated with etoposide to induce apoptosis, were injected IP or intraperitoneally to induce apoptosis, were injected IP. Levels of DNA in the plasma were determined. Results are presented as means ± SD of three to six mice.

TABLE 1. Cellular Composition of Peritoneal Fluids*

<table>
<thead>
<tr>
<th></th>
<th>0 h</th>
<th>6 h</th>
<th>16 h</th>
<th>24 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TG</td>
<td>PT</td>
<td>NaIO4</td>
<td>TG</td>
<td>PT</td>
</tr>
<tr>
<td>Total</td>
<td>2.81 (1.62)</td>
<td>4.38 (2.86)</td>
<td>3.79 (1.50)</td>
<td>14.16 (6.12)</td>
<td>9.84 (2.52)</td>
</tr>
<tr>
<td>Gr1+</td>
<td>0.10 (0.11)</td>
<td>0.12 (0.09)</td>
<td>0.09 (0.03)</td>
<td>1.39 (0.06)</td>
<td>1.59 (0.06)</td>
</tr>
<tr>
<td>Gr1+/annexin+</td>
<td>0.07 (0.06)</td>
<td>0.07 (0.06)</td>
<td>0.09 (0.06)</td>
<td>1.39 (0.06)</td>
<td>1.59 (0.06)</td>
</tr>
<tr>
<td>F4/80+</td>
<td>0.99 (0.46)</td>
<td>1.46 (0.82)</td>
<td>1.44 (0.57)</td>
<td>2.00 (0.76)</td>
<td>2.05 (0.97)</td>
</tr>
<tr>
<td>F4/80+/annexin+</td>
<td>0.32 (0.18)</td>
<td>0.11 (0.06)</td>
<td>0.12 (0.05)</td>
<td>0.52 (0.28)</td>
<td>0.26 (0.25)</td>
</tr>
</tbody>
</table>

*Cell number = No. x 10⁶. Cells were analyzed by flow cytometry with staining Gr1, F4/80, and annexin V to assess cell populations at the time-points as indicated. Results represent the mean ± SD for three to six mice in each group at each time-point.
apoptosis or necrosis, the administration of dead and dying cells provides a more defined system in which the number of dead cells is known more precisely, and the response of the recipient animal can be manipulated. In previous studies exploring this approach, we showed that administration of $10^8$ apoptotic or necrotic Jurkat cells to normal mice leads to the appearance of DNA in the blood in a time- and dose-dependent manner [23]. This DNA was observed with two different cell lines made apoptotic or necrotic by a variety of means, suggesting a general property of dead and dying cells. As Jurkat cells are of human origin and were derived from a male, the contribution of this cell type to circulating DNA can be established unequivocally by the demonstration by PCR of Y-chromosomal sequences [23].

As shown by many experiments in vivo and in vitro, macrophages play a key role in the clearance of apoptotic and necrotic cells and can engulf these cells with high efficiency [23, 34–36]. To explore the role of macrophages in the generation of blood DNA, in previous experiments, we assessed the response to the administration of dead and dying cells of mice in which macrophage function was eliminated by clodronate liposomes. Clodronate is a bisphosphonate, which can induce macrophage apoptosis after uptake and render a mouse deficient in this cell population [37, 38]. Using Jurkat cells as a model, we showed that administration of apoptotic or necrotic cells to a mouse without macrophages fails to produce a blood DNA response. Similar results were obtained in mice treated with silica, which can also eliminate macrophage function [23].

Although these findings point to a central role of macrophages in the generation of circulating DNA, they do not define the role of these cells in the DNA release reaction. Most studies on macrophage clearance of dead cells have focused on the uptake of these cells and have not addressed the disposition of their contents. Thus, it is possible that the macrophages take up dead cells, degrade their contents, and expel the remnants into the extracellular milieu. In the absence of macrophages, the dead cells may gradually autolys or disintegrate in a manner that fails to cause a measurable elevation of circulating DNA. In this scenario, the released DNA is an end-product of the clearance process, with levels rising as the burden of material to be eliminated increases.

An alternative possibility posits that DNA release occurs when the phagocytic capacity of the macrophage is exceeded. In this situation, a phagocytic macrophage filled with dead cells and their breakdown products is induced to undergo apoptosis, releasing its own DNA and that of the engulfed DNA cells. Circumstantial evidence for this possibility is derived from data showing that the blood of mice receiving dead and dying cells contains murine DNA sequences as well as that of the administered human cell. Furthermore, the DNA of murine and human origin shows size-laddering, implying a role for nucleases activated during apoptosis [23]. In this scenario, DNA release thus reflects macrophage dysfunction and death rather than heightened physiological function.

The current results provide data relevant to distinguish between these mechanisms. Thus, findings presented herein indicate that inflammation can alter the subsequent clearance of apoptotic and necrotic cells, although the nature of the effect may vary depending on the inflammatory stimulus [39, 40]. Thus, we have tested the effects of three different agents that have been commonly used to induce peritonitis. TG and PT represent complex mixtures comprised of many components [24, 27–29, 41]. With TG, advanced glycation products, which develop as the mixture “ages” may be the key inducing agent. The proinflammatory components in PT have not been well defined. In contrast, NaIO$_4$ is a well-defined chemical that oxidizes surface molecules, including terminal sugars, to produce free aldehydes [25, 26, 42]. These modifications induce
macrophage activation and T cell mitogenesis, and these functional changes are associated with subsequent recruitment of cells into the peritoneum [25, 26, 42].

Because of properties of these irritants, the nature of the exudates following their administration may differ. Indeed, a variety of studies have compared macrophages arising in these settings with respect to their activation state, functional properties, and production of various cellular proteins. These studies have identified important differences in the features of the resulting exudates [27–29]. Although the mechanisms of cellular recruitment and activation in the peritoneum are not well understood, our findings suggest that patterns of peritonitis and likely other forms of local inflammation may differ significantly in their impact on the clearance and metabolism of dead and dying cells.

Among possible events that occur in inflammatory sites that may affect the subsequent clearance of dead and dying cells is the occurrence of apoptosis as the inflammatory reaction proceeds. Thus, each of the treatments produced an increased number of annexin+, Gr1+ cells, although these numbers were greatest with TG treatment, which also led to the highest levels of annexin+, F4/80+ cells. Furthermore, with TG-induced peritonitis, the occurrence of apoptosis could be inferred from the presence of DNA and caspase 3 in the peritoneal fluid. In mice with this treatment, a DNA response was inferred from the presence of DNA and caspase 3 in the peritoneal fluid. In mice with this treatment, a DNA response was not observed following administration with Jurkat cells. Similarly, in mice treated with NaIO4 administration of dead and dying cells did not lead to a blood DNA response. Although peritoneal fluid after NaIO4 administration did not contain caspase 3, it did contain DNA. In contrast, PT-treated mice showed an intact blood DNA response and lacked peritoneal fluid DNA as well as caspase 3.

These findings thus suggest that the effect of inflammation on the magnitude of the blood DNA response resulting from administration of dead and dying cells relates to the presence of DNA in the peritoneal fluid. At present, the mechanisms leading to this DNA and its effect on the functional properties of macrophages are speculative. It is possible, however, that DNA itself or other debris from apoptotic cells may affect the capacity of macrophages to clear dead cells that subsequently arise locally or in the case of these experiments, the administered Jurkat cells. In this regard, the exudates of mice treated with TG and NaIO4, despite their content of DNA, differed in the presence of caspase 3. Although apoptosis can account for simultaneous release of DNA and caspase 3, the mechanisms leading to DNA in the absence of caspase by NaIO4 are unknown. Possibilities include DNA release during cell activation, cellular leakiness secondary to membrane oxidation, or inhibition of caspase 3 by this agent. These possibilities are under investigation.

Studies from a number of investigators have suggested that in contrast to previous notions, apoptotic cells may have proinflammatory effects and modulate the function of macrophages [43–46]. Among these functions, clearance of apoptotic may be modulated, an issue to be considered in experiments. For example, studies about the role of macrophages in the clearance of dead and dying cells have involved mice that have been treated previously with TG to increase macrophage number and facilitate the analysis [47]. It is possible that such treatment may have affected important elements of this process, and the effects we have observed on DNA released also affected interactions such as binding and uptake of dead and dying cells.

As shown in studies in human and murine systems, DNA in the blood may have important immune effects that influence the pathogenesis of SLE [48–50]. These effects include the formation of immune complexes that promote cytokine production and can deposit in the kidney to induce glomerulonephritis [51, 52]. Other nuclear molecules released from dead and dying cells may also have pathogenic effects. For example, the high-mobility group protein has powerful, proinflammatory properties and can function as a cytokine once it has been released from necrotic cells [53–56]. Studies are therefore in progress to determine changes in macrophages that occur during inflammation, including the contact with apoptotic and necrotic cells and their impact on subsequent events in disease pathogenesis.

ACKNOWLEDGMENTS

This work was supported by a VA merit review grant, an Alliance for Lupus research grant, Lupus Research Institute grant, and National Institutes of Health Grant AI44308.

REFERENCES


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