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The Relationship between Apoptosis and High-Mobility Group Protein 1 Release from Murine Macrophages Stimulated with Lipopolysaccharide or Polyinosinic-Polyctydylid Acid

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High-mobility group protein 1 (HMGB1) is a nonhistone nuclear protein that has both intracellular and extracellular functions. Inside the cell, HMGB1 binds to DNA and promotes transcription as well as other functions (1, 2). Outside the cell, HMGB1 can serve as a cytokine to promote inflammation and induce an array of proinflammatory responses including the expression of TNF-α, IL-1, and NO, as well as induce the maturation of dendritic cells (3). This translocation event can be induced by stimulation of certain TLRs, with HMGB1 serving as a late mediator of the effects of the TLR4 ligand, LPS, or endotoxin (4, 5). An important role of HMGB1 in disease pathogenesis is suggested by the finding of increased levels of HMGB1 in the blood and tissue in disease settings and the beneficial effects of anti-HMGB1 Abs in models of sepsis and arthritis (4, 6).

In the expression of the cytokine activity of HMGB1, translocation from inside to the outside of the cell represents a key event. As indicated by in vitro studies, HMGB1 can exit cells in at least two distinct settings: activation and death. During activation of macrophages by LPS, TNF-α, and other stimulating agents, HMGB1 translocates from the nucleus to the cytoplasm following acetylation, which alters its intracellular trafficking and location; phosphorylation may also affect this event (7, 8). In the cytoplasm, HMGB1 moves into vesicles from which it can be secreted. Following macrophage stimulation, the release of HMGB1 is delayed and more sustained than that of cytokines, perhaps reflecting the series of events required for exteriorization.

In contrast to the secretion by activated cells, the release of HMGB1 from dead cells appears to be a more rapid and passive process that occurs readily with necrosis rather than apoptosis (9). In the nucleus, HMGB1 is weakly adherent to chromatin and thus can exit cells readily when membrane permeability breaks down during necrotic cell death. In contrast, during apoptosis for at least certain cell types, posttranslational modification of HMGB1 appears to increase its adherence to chromatin as shown by fluorescence loss in photobleaching analysis of HeLa cells treated with etoposide to induce apoptosis (9). In these experiments, even as cells transitioned to late apoptosis, a stage that can be called secondary necrosis, HMGB1 remained inside the nucleus. Thus, these experiments suggested that, because of events early in apoptosis, HMGB1 becomes fixed to the nucleus so that even with permeability changes in late apoptosis, exit from cells does not occur. A number of observations suggest, however, that the release of HMGB1 from cells may not be entirely dichotomized but may vary by cell type as well as inducing stimulus. Thus, we have shown that, in Jurkat cells induced to undergo apoptosis by staurosporine, etoposide, and camptothecin, HMGB1 is released in a time-dependent process (10). This release can be blocked by Z-VAD-FMK (zVAD), indicating the role of apoptosis. Furthermore, activation of macrophages by certain TLR ligands and cytokines is ordinarily accompanied by apoptosis, with some of the agents that can induce macrophage activation also capable of inducing apoptosis (11–14). Thus, culture systems that have been studied to characterize

Abbreviations used in this paper: HMGB1, high-mobility group protein 1; poly(I:C), polyinosinic-polycytidylic acid; ODN, oligodeoxynucleotide; iNOS, inducible NO synthase; LDH, lactate dehydrogenase; PI, propidium iodide; TSA, trichostatin A; TRIF, Toll/IL-1R domain-containing adaptor-inducing IFN-β.

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2 These abbreviations were used in this paper: HMGB1, high-mobility group protein 1; poly(I:C), polyinosinic-polycytidylic acid; ODN, oligodeoxynucleotide; iNOS, inducible NO synthase; LDH, lactate dehydrogenase; PI, propidium iodide; TSA, trichostatin A; TRIF, Toll/IL-1R domain-containing adaptor-inducing IFN-β.
HMGB1 release during activation by TLR ligands most likely include a mixture with apoptotic cells. In this regard, CpG DNA that is a potent activator of macrophages is unable to induce HMGB1 release, suggesting that activation may not be sufficient to cause this process (5).

Together, these studies raise the possibility that HMGB1 release may be related to the process of apoptosis, with apoptotic as well as activated cells contributing to HMGB1 release following stimulation of cell macrophages with agents such as TLR ligands that can also induce apoptosis. Defining this relationship is important in terms of understanding the release of a key inflammatory mediator, interpreting the results of pathological studies, and defining immunological properties of dead and dying cells. Therefore, to define further the relationship between apoptosis and HMGB1 translocation during the process of cell activation, we investigated the ability of various TLR ligands to induce apoptosis and HMGB1 release by RAW 264.7 cells as well as other cell types. Results of these studies indicate a strong relationship between the induction of HMGB1 release and the occurrence of apoptosis during macrophage activation, suggesting that the operation of common mechanisms in these processes and a contribution of apoptotic cells to the generation of extracellular HMGB1.

Materials and Methods

Cells and reagents

The murine macrophage-like RAW 264.7 and human T cell leukemia Jurkat cell lines were obtained from American Type Culture Collection. Peripheral macrophages from female BALB/c mice (6–8 wk of age, purchased from The Jackson Laboratory) were collected 3 days after i.p. injection of thioglycollate (Sigma-Aldrich). RAW 264.7 cells were maintained in RPMI 1640 supplemented with 10% FBS and 20 μg/ml gentamicin (Invitrogen Life Technologies). LPS (Escherichia coli 0111:B4) was purchased from Sigma-Aldrich and poly(I:C) was purchased from InvivoGen. CpG oligodeoxynucleotide (ODN) 1826 and control GpC ODN was purchased from Midland Certified Reagents. JNK inhibitor was purchased from Calbiochem. PAPANO (PAPANOate (PAPANO), DETA NONOate (NOC-18), and indacube NO synthase (nNOS) inhibitor) 1400W were purchased from Alexis. rIFN-α and rat anti-IFN-α Ab were purchased from PBL Biomedical Laboratories. Anti-caspase 9 and anti-β-actin Abs were purchased from Chemicon. Etoposide and zVAD were purchased from Sigma-Aldrich. All protocols for animal use were approved by the Institutional Animal Care and Use Committee of the Durham Veterans Affairs Medical Center.

Cell treatments

For stimulation of the RAW 264.7 cell, cells were plated in either 6-well culture plates (3 × 10⁶/well) or 8-well chamber slides (1.5 × 10³/well) for 3 h or overnight (1.5 × 10³/well). The overnight growth was performed to allow resting before stimulation. Then cells were washed twice with Opti-MEM (Invitrogen Life Technologies) and stimulated with 0.5 μg/ml LPS or 0.25 μg/ml poly(I:C) or 1.5 μg/ml CpG ODN 1826. Thioglycollate-elicited peritoneal macrophages were plated in 6-well plates for 3 h and vigorously washed twice to remove nonadherent cells before the addition of LPS (1 μg/ml) or poly(I:C) (5 μg/ml) or ODN 1826 (1.5 μM). For Jurkat cells, cells were plated in 6-well plates and treated with poly(I:C) (50 μg/ml) or PAPANO (500 μM) for 4 or 24 h. For inhibition assays, all cell types were incubated with various inhibitors (1400W, anti-IFN-α, JNK inhibitor, and 10 μM zVAD) for 30 min before the addition of LPS, poly(I:C), or PAPANO. Etoposide (30 μg/ml) was used to serve as positive control for apoptosis.

Immunochemical and biochemical assay

Lactate dehydrogenase (LDH) levels, caspase activities, and TUNEL assay were used to determine cell death. Culture supernatant was collected and assayed for LDH level using a commercial kit (CytoTox96 Non-Radioactive Cytotoxicity Assay; Promega). Cellular caspase-3 activity was measured by a commercial kit (EnzCheck Caspase 3 Assay kit; Invitrogen Life Technologies) according to the manufacturer’s suggestions. For TUNEL assays, cells plated in 8-well chamber slides were washed twice with PBS, fixed, and permeabilized using the Cytofix/Permeable kit (BD Biosciences). A Fluorescein In Situ Cell Death Detection Kit (Roche) was used, and TUNEL-positive cells were visualized by an indirect fluorescent microscope (Olympus America). TUNEL results were expressed as percentage of fluorescent-positive cells per viewing area. Three viewing areas were counted and all experiments were repeated at least twice.

Western blotting was used to detect HMGB1 release and caspase 9 cleavage. HMGB1 assays were performed as described previously (5). Briefly, culture supernatants were concentrated by Centricom YM-10 (Millipore). The concentrated samples were then volume adjusted and resolved on 4–12% NuPAGE Tris-Bis/SDS polyacrylamide gel (Invitrogen Life Technologies). Protein was transferred to polyvinylidene difluoride membranes (Invitrogen Life Technologies), blocked with 5% dry milk in TBS-Tween 20, and blotted with rabbit anti-HMGB1 polyclonal Ab (a gift from Dr. K. J. Tracey North Shore Long Island Jewish Research Institute, Manhasset, NY). The membrane was then incubated with HRP-conjugated anti-rabbit IgG followed by SuperSignal West Femto substrate (Pierce). Images were captured by exposing the membrane to a CCD camera (FluoChem8900; Alpha InnoTech).

For caspase 9 cleavage detection, cells were lysed by M-PER (Pierce). Protein concentration was determined by the Bradford method. Western blotting was done as described above. Membrane was blotted with rabbit anti-caspase 9 Ab followed by HRP-conjugated anti-rabbit IgG. Caspase 9 cleavage was visualized by adding the SuperSignal West Femto substrate followed by exposing the membrane to a CCD camera.

FACS analysis

Jurkat T cells were treated with or without zVAD for 30 min followed by poly(I:C), PAPANO, or etoposide for 20 h. Jurkat cells were washed twice with PBS and stained with annexin V-FITC and propidium iodide (PI) in 1 × annexin binding buffer for 30 min. Cells were then washed twice with staining buffer and subjected to FACSscan (BD Biosciences). Data were analyzed by CellQuest software (BD Biosciences).

Confocal imaging

Cells were cultured in 8-well chamber slides for 2–3 h and stimulated with LPS (0.5 μg/ml) or poly(I:C) (0.25 μg/ml) for 20–24 h. Cells were then washed with ice cold PBS, fixed, and permeabilized using the Cytofix/Permeable kit (BD Biosciences). The rabbit anti-HMGB1 Ab was added followed by Alexa-anti-rabbit IgG 488 (Molecular Probes). Cell-permeable DRAQ5 (Alexis) was used for nuclear staining. Images were captured by a confocal laser scanning microscope (Zeiss LSM510; Carl Zeiss).

Statistical analysis

TUNEL assay results were analyzed by a two-tailed, unequal variance Student’s t test. A value of p < 0.05 was considered significant.

Results

The effects of LPS, poly(I:C), and CpG ODN on HMGB1 release and apoptosis

In this study, we have investigated the relationship between activation, apoptosis, and HMGB1 release by macrophages stimulated by TLR ligands, specifically testing the hypothesis that apoptosis and the release process are closely correlated in cultures of activated cells. As shown in a previous study, lipoteichoic acid, poly(I:C), and LPS but not CpG DNA can induce the release of HMGB1 from RAW 264.7 cells, although these stimuli induce similar patterns of inflammatory mediator production (5). Because these TLR ligands differ in their ability to induce HMGB1 release, we therefore performed experiments to determine whether differences in the induction of HMGB1 translocation are related to signaling properties of the TLR ligands, including their ability to induce apoptosis.

In these studies, we therefore first assessed the ability of poly(I:C), LPS, and CpG DNA to induce apoptosis in the RAW 264.7 macrophage cell line. As shown in Fig. 1A, caspase activity increased as early as 4 h in RAW 264.7 cells stimulated by poly(I:C), whereas caspase activity in LPS-stimulated cells increased at a later time point. At the time points tested, however, an immunomodulatory CpG ODN 1826 did not cause elevated caspase activity in RAW 264.7 cells. In these experiments, the medium control groups displayed detectable readings. These readings varied among experiments and were likely related to spontaneous apoptosis or background fluorescence.
LDH is a stable enzyme that is normally released upon cell membrane disruption or cell death. In this study, LDH level was evaluated as another indicator for late apoptosis or secondary necrosis. Thus, as these studies showed, cells stimulated by poly(I:C) showed elevated LDH levels in the culture medium as early as 6 h, with much higher levels at 20 h; under these conditions, cells stimulated with LPS showed elevated LDH level at 20 h. In contrast, the CpG ODN did not lead to elevated LDH levels at the time points assayed.

As shown in Fig. 1, LDH levels (Fig. 1A) increased in the culture medium in a pattern similar to that of HMGB1 (Fig. 1B). As these data indicate, HMGB1, as detected by Western blotting, appeared in a time-dependent way in the medium of RAW 264.7 cells treated with either LPS or poly(I:C) but not CpG ODN. As shown previously, HMGB1 release induced by LPS is a late event, whereas release induced by poly(I:C) occurs as early as 4 h (4). These results suggest a relationship between the induction of apoptosis and the process of HMGB1 release. Similar results were also obtained from RAW 264.7 cell cultures that were rested overnight before stimulation (data not shown).

To further evaluate the extent of apoptosis induced by poly(I:C) or LPS, we used the TUNEL assay in RAW 264.7 cells stimulated by poly(I:C), LPS, or CpG ODN. As shown in Fig. 1C, LPS-treated RAW 264.7 cells showed a marked increase in TUNEL-positive cells (7.76 ± 0.81%) at 20 h compared with the medium control (2.52 ± 0.21%). RAW264.7 cells stimulated with poly(I:C) showed increased TUNEL positivity as early as 4 h (7.57 ± 1.28%) and a dramatic increase at 20 h (39.04 ± 6.67%). In agreement with results of caspase determinations, the CpG ODN did not lead to a detectable increase of TUNEL positivity (2.51 ± 0.78%) at 20 h.

For comparison to stimulation by LPS and poly(I:C), RAW 264.7 cells treated with etoposide were studied. As indicated in Fig. 1C, under conditions of these experiments, poly(I:C) caused more apoptosis than etoposide as assessed by TUNEL assays. We also measured the extracellular release of HMGB1 induced by etoposide by Western blotting in the culture medium. As shown in Fig. 1D, the treatment with etoposide increased HMGB1 release in RAW 264.7 cell culture supernatants as well as LDH levels and cellular caspase activities. These studies are consistent with HMGB1 release during apoptosis and indicate the similarity in the responses of RAW 264.7 cells treated with LPS or poly(I:C) and that of a chemical inducer of apoptosis.

Confocal imaging was performed next to evaluate HMGB1 translocation and to analyze the morphology of cells undergoing this process. As the data in Fig. 2A show, HMGB1 translocation was observed in RAW 264.7 cells stimulated with LPS or poly(I:C). In these experiments, cells lacking HMGB1 nuclear staining also demonstrated condensation or fragmentation of DNA as shown with staining with DRAQ5. The appearance of these nuclei was similar to that of cells treated with etoposide. As previously observed, cytoplasmic staining of HMGB1 occurs with RAW 264.7 cells, reflecting the distribution of this protein in this particular cell type (5, 15). To evaluate further
the occurrence of apoptosis in these cultures, we assayed for caspase 9 cleavage by Western blotting. As shown in Fig. 2B, RAW 264.7 cells stimulated with LPS or poly(I:C) showed procaspase 9 cleavage. This finding further indicates increased apoptotic activity. These results suggest a correlation between HMGB1 release induced by poly(I:C) or LPS with late apoptotic events.

The effects of NO, IFN-α, and JNK on apoptosis and HMGB1 release

In a previous study, we showed that NO and IFN-α differentially mediated the release of HMGB1 by LPS and poly(I:C) in a process that may depend on JNK activation (16). In these experiments, we showed that inhibiting iNOS or neutralizing IFN-α by an Ab reduced HMGB1 release. Because both NO and IFN-α can cause apoptosis (4, 17, 18), we investigated whether the decrease in HMGB1 release resulting from inhibition of iNOS or neutralizing IFN-α is related to effects on apoptosis.

As shown in Fig. 3A, the inhibition of NO production by a specific iNOS inhibitor, 1400W, reduced the release of HMGB1 stimulated by LPS but did not affect that release stimulated by poly(I:C). In these experiments, inhibition of NO caused a reduction in caspase 3 activity stimulated by LPS. This inhibition did not affect induction of apoptosis induced by poly(I:C) as reflected by LDH levels. As noted for experiments presented in Fig. 1B, the levels of caspase 3 and LDH at the time points measured differed...
depending on stimulus, with caspase 3 levels more reflective of apoptosis with LPS and LDH with poly(I:C).

The effects of iNOS inhibition (i.e., decreased apoptosis with LPS but not poly(I:C)) were also evident in the levels of HMGB1 in the supernatants (Fig. 3A). These results suggest that HMGB1 release results from the effects of NO on apoptosis. To determine whether NO can cause apoptosis as well as HMGB1 release, the effect of NO donors was tested. As shown in Fig. 3B, addition of NO donors, PAPANO (fast NO releaser; t_{1/2} = 76 min) or NOC-18 (slow NO releaser; t_{1/2} = 21 h), led to apoptosis and HMGB1 release (Fig. 3B) to varying extents. Thus, PAPANO induced much more HMGB1 release than did NOC-18, although both induced this response. These results indicate that NO can induce both HMGB1 release as well as apoptosis.

The role of IFN-α was next tested. As shown in Fig. 4A, in cultures of RAW 264.7 cells stimulated by poly(I:C), neutralizing IFN-α by Ab reduced LDH activity and the level of extracellular HMGB1 in culture supernatant. Furthermore, addition of rIFN-αA in RAW 264.7 cell culture increased caspase activity that was also correlated with the extracellular release of HMGB1 (Fig. 4B). Neutralizing IFN-α did not affect HMGB1 release induced by LPS or LPS-induced apoptosis (data not shown).

MAPKs are key signaling molecules that act downstream of TLR activation and are important for the production of NO and cytokines (19–22). In previous experiments, we showed that inhibiting JNK activation reduced extracellular release of HMGB1 induced by poly(I:C) or LPS (16). To evaluate the role of JNK in apoptosis induced by LPS or poly(I:C), we analyzed the effects of JNK inhibitor on caspase activities and LDH levels in RAW 264.7 cells stimulated with LPS or poly(I:C). As shown in Fig. 5A, the addition of JNK inhibitor reduced caspase activity in LPS- or poly(I:C)-stimulated cells. The JNK inhibitor also reduced LDH release from cells treated with poly(I:C). Under these conditions, JNK inhibition reduced HMGB1 release induced by LPS or poly(I:C).
poly(I:C) (Fig. 5B), providing further evidence for the relationship of HMGB1 release and apoptosis.

The effects of LPS and poly(I:C) on primary macrophages

In these experiments, the relationship between the induction of apoptosis and HMGB1 release was evaluated in RAW 264.7 cells, although another murine macrophage cell line J774A.1 showed similar results (data not shown). To evaluate further this phenomenon in primary cells, we used thioglycollate-elicited peritoneal macrophages. Similar to the findings with cell lines, a relationship between apoptosis and HMGB1 released was observed in the primary macrophages. As shown in Fig. 6, the level of extracellular HMGB1 in culture supernatants was correlated with the levels of LDH and TUNEL positivity in peritoneal macrophages. A similar correlation was also observed in bone marrow-derived macrophages (data not shown). For peritoneal macrophages, the CpG ODN also did not cause either detectable HMGB1 release or apoptosis.

The effects of caspase inhibitor on HMGB1 release by RAW264.7 cells

The correlation between apoptosis and HMGB1 translocation induced by LPS or poly(I:C) suggests that the extracellular release of HMGB1 during activation could result, at least in part, from leakage from cells undergoing late apoptosis or secondary necrosis. We, therefore, evaluated the effect of a pancaspase inhibitor, zVAD, on HMGB1 release induced by LPS or poly(I:C). As shown in Fig. 7A, however, the addition of zVAD to RAW 264.7 cells stimulated with LPS or poly(I:C) resulted in even greater LDH release than cultures without this inhibitor. Similarly, more extracellular HMGB1 was detected in culture medium by Western blotting from cells treated with zVAD. Inhibition of caspase activity with zVAD in RAW 264.7 cells treated with etoposide, however, did reduce HMGB1 release and LDH levels in the cultures (Fig. 7B). Because caspase-3 activities in these experiments are diminished by treatment with zVAD, LDH levels were used to measure cell death.

The effects of poly(I:C) and NO on HMGB1 release by Jurkat cells

Because cell types differ in events during apoptosis and the response to caspase inhibition, we therefore investigated the responses of a human leukemia T cell line Jurkat cells, treated with poly(I:C) or PAPANO. As shown in Fig. 8A, Jurkat cells treated with poly(I:C) or PAPANO had an increased number of annexin V-positive cells (5.6 and 8.9%, respectively, compared with 3.6% in medium control) 4 h after treatment. After 20 h of treatment, the PI and annexin V double-positive cell population increased to ~20% in Jurkat cells treated with poly(I:C) or PAPANO, almost three times the amount of the double-positive cell population (late apoptotic cells) in control Jurkat cells (7.3%). The addition of zVAD to Jurkat cell cultures treated with poly(I:C) or PAPANO, however, reduced the percentage of apoptotic cells to the level of zVAD-alone cultures (~1–2%) at 4 h. The addition of zVAD to Jurkat cells treated with poly(I:C) or PAPANO also reduced the percentage of PI and annexin V double-positive cells at 20 h to zVAD or medium-alone levels (~5–7%).

As shown in Fig. 8A, the presence of zVAD in cultures of cells treated with poly(I:C) or PAPANO caused a reduced level of extracellular HMGB1 that was related to reduction in the number of apoptotic cells as assessed by FACS. As a control for this effect, Jurkat cells treated with etoposide were studied. As shown in Fig. 8B, 20 h after etoposide treatment ~70% Jurkat cells became either annexin or annexin and PI positive compared with only 4% in control Jurkat cells. Supernatants of these cells showed readily detectable HMGB1 as previously shown. For these cultures, the addition of zVAD reduced cell death from 70 to ~10%. This reduction was also reflected in the amount of HMGB1 detected in the supernatant. Together, these studies indicate that, for both RAW 264.7 and Jurkat cells, the process of HMGB1 release and
apoptosis are related, with poly(I:C) capable of inducing both responses.

**Discussion**

Results presented herein provide new insights into the release of HMGB1 by macrophages in cells stimulated by TLR ligands and suggest that the translocation of this protein and apoptosis are closely related processes that may reflect common mechanisms. Thus, as our findings show, stimulation of RAW 264.7 cells by either LPS or poly(I:C) leads to the translocation of HMGB1 from the nucleus into the extracellular milieu under conditions in which the frequency of apoptotic cells increases dramatically; similar results were obtained with primary macrophages cultured from mice. Although the presence of apoptotic cells in cultures of activated cells does not prove that they are the source of HMGB1, the failure of cells stimulated by CpG DNA to release HMGB1 suggests that activation by itself is not sufficient for this process. Thus, for macrophages, it appears that apoptosis is closely associated with events leading to HMGB1 release and, indeed, may be the origin of at least some of the HMGB1 released following macrophage stimulation by TLR ligands.

Previous studies using cell lines as well as primary cell populations have indicated that the release of HMGB1 occurs in two distinct settings. The first is immune cell activation where stimuli such as LPS, poly(I:C), or cytokines can initiate a cascade of events that lead to the posttranslational modification of HMGB1 (5, 15, 16, 23). These modifications, which may include protein acetylation as well as phosphorylation, can alter the charge of HMGB1 and intracellular trafficking (7, 8). As a result, HMGB1 translocates from the nucleus into the cytoplasm to enter vesicles for eventual secretion. Once secreted into the extracellular milieu, HMGB1 can function as a cytokine to induce inflammation and serve as a mediator of events such as sepsis. This process has been most clearly defined for macrophages, including cell lines such as RAW 264.7 cells.

The other setting in which HMGB1 release occurs is cell death, most notably, necrosis. As shown for a number of cell lines, the induction of necrotic cell death by physical or metabolic injury leads to the extracellular release of HMGB1 (9). Because HMGB1 is loosely bound to chromatin, it may readily leave the nucleus when the membrane permeability barriers are broken. Outside the necrotic cell, HMGB1 can induce inflammation and, indeed, may promote the proinflammatory activity of cells dying by this pathway.

In contrast to findings with necrotic cells, a previous report indicated that HMGB1 release does not occur with apoptotic cell death, with the absence of this release possibly contributing to the anti-inflammatory properties of this type of dying cell (9). Furthermore, these studies have suggested that biochemical changes during apoptosis anchor HMGB1 to the nucleus so that it remains intracellular even into late apoptosis or secondary necrosis at time when cell permeability barriers break down. Evidence for this idea comes from studies using HeLa cells treated with etoposide (9). These studies, which involved fluorescence loss in photobleaching...
analysis, demonstrated that HMGB1 showed drastically reduced mobility in apoptosis, with diffusion values far lower than those of histone proteins.

The distinction between HMGB1 release during apoptosis and necrosis, however, may not be as rigid as originally proposed. Thus, recent studies using Jurkat cells have shown that, with apoptosis induced by a number of chemical agents, HMGB1 appears in the medium in a time-dependent process (10). This release was coincident with the appearance of cells in which the nuclear content of HMGB1 was diminished, suggesting translocation events. In contrast to results of Scaffidi et al. (9), in these studies, induction of apoptosis in HeLa cells by TNF-α and cycloheximide did lead to HMGB1 release with trichostatin A (TSA) potentiating the process. It is important to note that TSA alone is a strong inducer of apoptosis in many cell types including HeLa cells (24–26). In our preliminary studies, TSA, even at a dose 10 times less than what was used in the previous study (7), induced apoptosis in Jurkat, HeLa cells, and RAW 264.7 cells as evidenced by caspase activity and LDH release (W. Jiang, unpublished results). The apoptotic activity also correlates with the level of HMGB1 release. In studies by other investigators, melanoma cells induced to undergo apoptosis via TRAIL also showed release of HMGB1, providing further evidence for the release of this protein during apoptotic as well as necrotic cell death (27). In this regard, in the study of Scaffidi et al. (9), faint staining of HMGB1 was observed in Western blots of supernatants from apoptotic cells. These findings suggest that HMGB1 release does in fact occur during the process of apoptosis with HeLa cells, although the extent appears much less than that observed with necrotic cells.

In the current experiments, we have established a correlation between immune stimulation, HMGB1 release, and apoptosis. The ability of TLR ligands to induce apoptosis has been demonstrated both in vivo and in vitro (12, 28, 29), with this activity postulated to be important in host defense. Thus, the stimulation of macrophage death by a TLR ligand could represent a means of limiting tissue damage during an inflammatory reaction as well as a means of controlling infection (30). The induction of cell death in macrophages, for example, could operate with both viral and bacterial infections and curtail the multiplication of organisms in infected cells. Interestingly, certain viruses have developed a means of inhibiting apoptosis, suggesting a strategy to counteract this facet of host defense (31, 32).

Among the stimuli we tested, both LPS and poly(I:C) can induce apoptosis as well as cytokine production and cell proliferation. For LPS, the effects on apoptosis are complex and involve both proapoptotic and antiapoptotic actions that likely depend on the cell type, state of activation, and the role of transcription factors such as NF-κB (33–35). In our culture system, LPS clearly induced apoptosis for both RAW 264.7 cells as well as primary cell populations. Similarly, poly(I:C) promoted apoptosis, consistent with many observations on the effect of this agent, causing higher levels of death than LPS. Of note, CpG DNA failed to induce either HMGB1 release or apoptosis, confirming results of other studies (5, 36).

Although both LPS and poly(I:C) can cause HMGB1 release in vitro culture systems, the mediators involved may be different. Thus, for LPS, but not poly(I:C), inhibition of NO production prevents HMGB1 release. In contrast, for poly(I:C), HMGB1 release depends on IFN-α because an anti-IFN-α Ab prevents this process; for LPS, this Ab did not affect LPS-stimulated HMGB1 release. The current studies confirm these findings and demonstrate further that these mediators also promote apoptosis under the culture conditions used.

The signaling pathways involved in these activation and death processes are not precisely understood, although our findings point to a role of the MAPK family, including JNK. Although both LPS and poly(I:C) stimulate downstream pathways such as the MAPKs via a TLR, activation by poly(I:C) involves other signaling elements including double-stranded RNA-dependent protein kinase and retinoic acid-inducible gene I (37, 38). In the context of these experiments, we cannot determine which of the pathways induced by poly(I:C) is most relevant to HMGB1 release. We have previously suggested that Toll/IL-1R domain-containing adapting-inducing IFN-β (TRIF) may be important in stimulation of HMGB1 release because both LPS and poly(I:C) act through TRIF via a TLR, whereas CpG DNA, which does not stimulate HMGB1 release, does not act through TRIF (22, 39, 40). Studies are in progress to investigate the role of TRIF in both activation and apoptosis in this system.

Because, as shown herein, both NO and IFN can induce apoptosis, it is reasonable to propose that these mediators contribute to the death process as well as HMGB1 release. Indeed, blocking the effects of these mediators decreases death as well as HMGB1 release, consistent with a common action. Defining further the role of cell death in these processes is complicated by the effects of caspase inhibition. Although caspase inhibition would be expected to diminish cell death, previous studies have shown that the stimulation of macrophages in the presence of pancaspase inhibitors actually increases cell death by a process identified as necroptosis (41). The mechanism of this caspase-independent death process is not well understood, although it may relate to effects on the Nur77 orphan receptor whose activity can be modified by a caspase (42). In the context of the current studies, it would appear that an intervention that increases cell death (i.e., caspase inhibition in LPS-treated cells) augments HMGB1 release, supporting the role of macrophage cell death in the release process.

The current studies, by linking apoptosis and HMGB1 release, may clarify the generation of extracellular HMGB1 and facilitate interpretation of certain clinical findings. Thus, as shown recently, sepsis, a process in which HMGB1 acts prominently, is associated with increased levels of apoptosis (43). The current observation provides a direct mechanism for how apoptosis can lead to HMGB1 release (i.e., release by apoptotic cells). This mechanism contrasts with the postulates of Qin et al. (43) on an indirect pathway in which dead cells stimulate macrophages to release this protein. Such an indirect mechanism entails the stimulation of macrophages by apoptotic cells even though many studies suggest that apoptotic cells are anti-inflammatory. To the extent that HMGB1 released by apoptotic or secondary necrotic cells is immunologically active, our findings also would suggest that apoptotic cells, like necrotic cells, could stimulate inflammation via the released HMGB1. Future studies are therefore in progress to define the mechanisms of HMGB1 release in stimulated macrophages and distinguish the contribution of cells in various states of activation and death.

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References