Activation of Poly(ADP)-ribose Polymerase (PARP-1) Induces Release of the Pro-inflammatory Mediator HMGB1 from the Nucleus*

Dara Ditsworth, Wei-Xing Zong, and Craig B. Thompson

From the Abramson Family Cancer Research Institute, Department of Cancer Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Necrotic cells release inflammatory mediators that activate cytokine production from innate immune cells. One mediator of this activation is high mobility group box 1 protein (HMGB1). HMGB1 is normally a chromatin-associated protein and is sequestered at condensed chromatin during apoptosis. How it is released from chromatin during necrotic cell death is not known. Here we show that after DNA-alkylating damage, the activation of poly(ADP)-ribose polymerase (PARP) regulates the translocation of HMGB1 from the nucleus to the cytosol. This displaced HMGB1 is subject to release if the cell then loses plasma membrane integrity as a result of necrosis. Both full-length HMGB1 and a truncated form of HMGB1 lacking the highly conserved glutamate-rich C-terminal tail can induce macrophage activation and tumor necrosis factor-α production. However, displacement of HMGB1 from the nucleus following PARP activation requires the presence of the glutamate-rich C-terminal tail. Although the C-terminal tail is not the sole substrate for PARP modification of HMGB1, it appears to be required to destabilize HMGB1 association with chromatin following PARP-dependent chromatin modifications. These data suggest that PARP-dependent nuclear-to-cytosolic translocation of HMGB1 serves to establish the ability of cells to release this potent inflammatory mediator upon subsequent necrotic cell death.

It is generally thought that chemotherapeutic drugs induce tumor cell death through apoptosis. However, most cancer cells harbor defects in apoptotic signaling pathways (1) and are still effectively treated with DNA-damaging agents. By studying cells that are deficient in Bax and Bak, key regulators of apoptosis, we have previously shown that DNA-alkylating agents are effective inducers of non-apoptotic cell death (2). Furthermore, cell death in response to alkylating DNA damage is dependent on the activation of poly(ADP)-ribose polymerase (PARP) and displays features characteristic of necrosis, such as ATP depletion, plasma membrane disintegration, and the ability to stimulate inflammation.

PARP is a nuclear enzyme that catalyzes the transfer of ADP-ribose moieties from NAD+ to itself and other acceptor proteins in response to DNA damage (3). Of the many PARP family members, PARP-1 accounts for ~90% of the poly(ADP)-ribose-sylation reactions in the cell (4). Although PARP initially functions to alter chromatin structure and facilitate DNA repair following low levels of DNA damage, sustained PARP activity can lead to depletion of nuclear-cytoplasmic NAD+ and subsequent necrotic cell death. Parp-1−/− cells are initially protected from alkylating DNA damage but ultimately undergo apoptosis. Interestingly, following alkylating DNA damage, medium from parp-1−/− cells lacks the ability to stimulate inflammation (2).

High mobility group protein 1 (HMGB1) is a nuclear protein that when released into the extracellular space can elicit a potent inflammatory response (5). Originally identified as an architectural chromatin-associated protein, HMGB1 contains two HMG box domains and an acidic C terminus, binding DNA and nucleosomes without sequence specificity. Although other family members HMGB2 and HMGB3 are restricted to expression primarily during embryonic development, HMGB1 is nearly ubiquitously expressed and is one of the most abundant proteins in the nucleus (6, 7). In healthy cells, HMGB1 moves between chromatin bound and unbound states and serves many purposes in the nucleus, facilitating transcription factor binding, nucleosome remodeling (8), and DNA repair processes (9, 10). Following apoptosis, HMGB1 has been shown to be selectively retained in the nucleus by condensed chromatin (5). However, necrotic cells have been reported to release HMGB1 into the extracellular space. HMGB1 is thought to be a key mediator of inflammation because necrotic hmgb1−/− cells are unable to activate macrophages (5).

* This work was supported by grants from The Leukemia and Lymphoma Society, the NCI, National Institutes of Health and the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Present address: Dept. of Molecular Genetics and Microbiology, Stony Brook University, Stony Brook, NY 11794.
2 To whom correspondence should be addressed: Abramson Family Cancer Research Institute, 421 Curie Blvd., Philadelphia, PA 19104. Tel.: 215-746-5515; Fax: 215-746-5511; E-mail: craig@mail.med.upenn.edu.

3 The abbreviations used are: PAR, poly(ADP)-ribose; PARP, poly(ADP)-ribose polymerase; BMK, baby mouse kidney; DHIQ, 1,5-isoquinolinediol; HMGB1, high mobility group box 1 protein; IP, immunoprecipitate; LMB, leptomycin B; MEF, mouse embryonic fibroblast; MNNG, 1-nethyl-3-nitro-1-nitrosoguanidine; PI, propidium iodide; rh, recombinant human; TNF, tumor necrosis factor; FBS, fetal bovine serum; IL, interleukin; PI, propidium iodide; PBS, phosphate-buffered saline; DAPI, 4′,6-diamidino-2-phenylindole; biotin-NAD, 6-biotin-17-nicotinamide-adenine-dinucleotide; FL, full-length; TRITC, tetramethylrhodamine isothiocyanate; Bis-Tris, 2-(bis[2-hydroxyethyl]amino)-2-(hydroxymethyl)propane-1,3-diol.
PARP Signals HMGB1 Release during Necrosis

Once outside the cell, HMGB1 can mediate inflammation through binding a variety of receptors on innate immune cells (11). HMGB1 is one of a few endogenous danger signals that can alert the innate immune system of self-injury, among others such as uric acid and heat shock proteins (12). Extracellular HMGB1 directly binds the receptor for advanced glycation end products (RAGE) (13, 14) or signals through Toll-like receptors TLR2 and TLR4 (15, 16) to stimulate immune cell types (17, 18). In addition to release from necrotic cells, HMGB1 has been reported to be secreted by immune cells at delayed time points following stimulation (19, 20). In myeloid cells, HMGB1 secretion is thought to be regulated by acetylation and relocation to secretory lysosomes (21). Because of this paracrine feedback loop, extracellular HMGB1 greatly amplifies an inflammatory response. The clinical implications of blocking extracellular HMGB1 signaling have recently been appreciated as monoclonal antibodies against HMGB1 can protect against sepsis and other conditions in preclinical models (19, 22, 23).

Although HMGB1 release from cells undergoing necrosis is believed to occur through passive diffusion, the mechanism by which HMGB1 is released from chromatin during necrosis remains an open question. This study investigates the mechanism of HMGB1 release from cells following necrosis induced by DNA-damaging agents.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—Immortalized wild-type and parp-1<sup>−/−</sup> mouse embryonic fibroblasts (MEFs) and bax<sup>−/−</sup>bak<sup>−/−</sup> baby mouse kidney (BMK) cells were cultured as described previously (24, 25). RAW 264.7 cells were obtained from ATCC and cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% FBS (Gemini), 2 mM glutamine, 1% penicillin/streptomycin, and 20 mM Hapes.

Interleukin-3- (IL-3)-dependent bax<sup>−/−</sup>bak<sup>−/−</sup> hematopoietic cells and the murine pro-B cell line FL5.12 were cultured as described previously (2). Cells were grown in standard medium containing RPMI 1640 (Invitrogen), 10% FBS (Gemini), 2 mM glutamine, 1% penicillin/streptomycin, and 20 mM Hapes.

Interleukin-3- (IL-3)-dependent bax<sup>−/−</sup>bak<sup>−/−</sup> hematopoietic cells and the murine pro-B cell line FL5.12 were cultured as described previously (2). Cells were grown in standard medium containing RPMI 1640 (Invitrogen), 10% FBS (Gemini), 2 mM glutamine, 1% penicillin/streptomycin, and 20 mM Hapes.

Interleukin-3- (IL-3)-dependent bax<sup>−/−</sup>bak<sup>−/−</sup> hematopoietic cells and the murine pro-B cell line FL5.12 were cultured as described previously (2). Cells were grown in standard medium containing RPMI 1640 (Invitrogen), 10% FBS (Gemini), 2 mM glutamine, 1% penicillin/streptomycin, and 20 mM Hapes.

Interleukin-3- (IL-3)-dependent bax<sup>−/−</sup>bak<sup>−/−</sup> hematopoietic cells and the murine pro-B cell line FL5.12 were cultured as described previously (2). Cells were grown in standard medium containing RPMI 1640 (Invitrogen), 10% FBS (Gemini), 2 mM glutamine, 1% penicillin/streptomycin, and 20 mM Hapes.

Interleukin-3- (IL-3)-dependent bax<sup>−/−</sup>bak<sup>−/−</sup> hematopoietic cells and the murine pro-B cell line FL5.12 were cultured as described previously (2). Cells were grown in standard medium containing RPMI 1640 (Invitrogen), 10% FBS (Gemini), 2 mM glutamine, 1% penicillin/streptomycin, and 20 mM Hapes.

Transfection of Cells—Cells were transfected with plasmids using Geneporter2 (Gene Therapy Systems) or Lipofectamine 2000 (Invitrogen). Transfection in FL5.12 and IL-3-dependent bax<sup>−/−</sup>bak<sup>−/−</sup> cells was performed using the Nucleofector system (Amaxa). Single cell clones were generated by limited dilution following selection with Blasticidin (5 μg/ml).

Plasmids—All constructs were generated by reverse transcription-PCR of total RNA from FL5.12 cells. FLAG-tagged full-length HMGB1 (-FL) was created using forward primer 5’-CTGGATCCATGGACTACAAGGACGACGATGACAAGGCAAAGGAGATCTAAG-3’ and reverse primer 5’-GCGAATTTCTATTTCTATCATCATCATCTCT-3’, whereas truncated HMGB1(-ΔC), encoding amino acids 1–185, was generated using reverse primer 5’-GCGAATTTCTAATCTCTTTTCTTGCTCTT-3’. For protein expression, full-length and truncated HMGB1 were generated using reverse primers 5’-GCGAATCTCTATTTCTATCATCATCTCT-3’ for full-length HMGB1 or 5’-GCGAATCTTCTATTTCTTTTCTTGCTCTT-3’ for HMGB1-ΔC, with the same forward primer as above. All untagged constructs were expressed in the pEF6/V5-His-TOPO vector and then subcloned into the His<sub>N</sub>-terminal tagged expression vector pET16b (Novagen) using Xhol/BamHI sites for HMGB1. All constructs were verified by nucleotide sequencing.

Cell Lysis and Immunoblotting—Cells were lysed in radioimmune precipitation buffer (1% sodium deoxycholine, 0.1% SDS, 1% Triton X-100, 10 mM Tris at pH 8.0, 0.14 M NaCl) with protease inhibitor complex (Roche Applied Science). Protein concentration was determined using BCA protein assay reagents (Pierce). Samples were loaded onto precast 4–12% Bis-Tris NuPAGE gels (Invitrogen) at 10 or 20 μg/well, and Western blotting was performed with the following antibodies: HMGB1 (BD Biosciences or Abcam), PAR (BD Biosciences), PARP (clone C2-10, BD Biosciences), tubulin (Sigma), FLAG (M2 or rabbit, Sigma), actin (I-19, Santa Cruz Biotechnology), His probe (H-15, Santa Cruz Biotechnology), streptavidin (Upstate Biotechnology), and nucleolin (H-250, Santa Cruz Biotechnology).

Immunofluorescence—For immunofluorescence of suspension cells, at each experimental time point, 50,000 cells were placed onto multiple tissue culture grade coverslips in a 24-well plate and centrifuged for 5 min at 1500 rpm prior to fixation. For immunofluorescence of adherent cells, cells were seeded onto glass coverslips prior to experiment to fixation, and at each time point, they were transferred to a 24-well plate with medium containing propidium iodide for 5 min. After dipping coverslips in PBS, cells were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and washed in PBS containing 0.02% Triton, 1.5% FBS. For both cell types, permeabilized cells were incubated with antibodies against HMGB1 (1:500, Abcam) or FLAG (1:400, M2, Sigma). Cells were incubated with fluorescein isothiocyanate- or rhodamine-conjugated secondary antibodies (1:100, Jackson ImmunoResearch or Molecular Probes) or counterstained for actin using TRITC-phalloidin (1:500, J. H. J. M. and A. M. O. were the authors of the referenced study. They are both experts in cell biology and inflammation. Their previous research has focused on the mechanisms of necrosis and the role of HMGB1 in inflammation. They have also studied the effects of PARP inhibitors on cell survival during necrosis. The experiments described in this study were designed to investigate the role of PARP in the release of HMGB1 during necrosis. The results indicated that PARP inhibition prevented the release of HMGB1 during necrosis. This suggests that PARP may play a role in the regulation of HMGB1 release during necrosis. The findings have potential implications for the development of therapeutic strategies targeting PARP to prevent inflammation in necrotic cells.
JUNE 15, 2007 • VOLUME 282 • NUMBER 24  17847

PARP Signals HMGB1 Release during Necrosis

Chemicon). Nuclei were visualized by staining with 1 μg/ml DAPI. Cells were imaged using ×100 oil immersion lens on a Nikon Eclipse E800 microscope. For scoring, HMGB1 localizations was determined to be nuclear or diffuse regardless of PI staining, and mitotic cells were excluded. At least 300 cells were counted per sample in each experiment.

Cell Fractionation—Suspension cells (4 × 10^7 cells) or adherent cells (1 × 10^7 cells) were treated with MNNG for each time point. Cells were resuspended in five pellet volumes of hypotonic buffer A (10 mM Hepes-KOH, pH 7.4, 10 mM KCl, 1.5 mM MgCl, 0.5 mM EDTA, 0.5 mM EGTA, 1 μM protease inhibitors (Roche Applied Science)) on ice for 20 min. Cells were disrupted by passing through 23-gauge needles 20 times or until complete lysis as determined by trypan blue exclusion. Cells were centrifuged at 3000 rpm for 5 min at 4 °C. The supernatant was centrifuged at 14,000 rpm for 10 min at 4 °C and then saved as the cytosol fraction. The pellet was resuspended in two pellet volumes of buffer B (10 mM Hepes-KOH, pH 7.4, 0.42 M NaCl, 2.5% v/v glycerol, 1.5 mM MgCl, 0.5 mM EDTA, 0.5 mM EGTA, 1 μM dithiothreitol, 1 μM protease inhibitors) and incubated for 30 min at 4 °C while rotating at 60 rpm. The sample was centrifuged at 14,000 rpm for 10 min at 4 °C, and the supernatant was saved as the nuclear fraction. Samples were prepared with equivalent protein amounts for immunoblots.

Protein Expression and Purification—Bacterially produced His-tagged HMGB1 full-length and truncated proteins were expressed in BL21 Escherichia coli and purified according to Ref. 26. Purified His-Bcl-xL was generated by similar methods according to Ref. 27. Briefly, Luria broth containing carbencillin (50 μg/ml) was inoculated with a freshly transformed colony of E. coli. Cells were grown overnight at 37 °C with shaking. The culture was gradually increased to a volume of 1 liter and allowed to grow until reaching optical density A_595 ~ 0.7. Cultures were then induced with isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.5 mM and continued growth for 4 h at 37 °C with shaking. Cells were pelleted at 5000 rpm and lysed in a buffer containing 20 mM Tris, pH 8.0, 0.5 mM NaCl, 1% Nonidet P-40, 5 mM imidazole, 1 mM phenylmethylsulfonyl fluoride, 1 μM protease inhibitor cocktail. Purification of His_c-tagged HMGB1 proteins was performed using nickel-nitriotriacetic acid beads according to the QIAexpressionist protocol (Qiagen). His-tagged protein was eluted from the column using increasing volumes of buffer B (10 mM Hepes-KOH, pH 7.4, 0.42 M NaCl, 2.5% v/v glycerol, 1.5 mM MgCl, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol, 1 μM protease inhibitors) and incubated for 30 min at 4 °C while rotating at 60 rpm. The sample was centrifuged at 14,000 rpm for 10 min at 4 °C, and the supernatant was saved as the nuclear fraction. Samples were prepared with equivalent protein amounts for immunoblots.

In Vitro Poly(ADP)-riboseylation Assay—For in vitro poly(ADP)-ribosylation assays, we adapted methods from previous studies (28), using 6-biotin-17-nicotinamide-adenine-dinucleotide (biotin-NAD) instead of [³²P]NAD (29). Reactions included 0.1 μg each of purified recombinant His-HMGB1-FL, His-HMGB1-ΔC, or His-Bcl-xL or 1 μg of histone H1 (purified calf thymus, Calbiochem) as substrates. The final reaction was performed in a 50-μl mix containing buffer 50 mM Tris-HCl, pH 8.0, 25 mM MgCl₂, adding 5 μg of PARP-activated DNA (R&D), 20 μM biotin-NAD (R&D), or 1 μM NAD⁺ (Calbiochem) and 300 μM DHIQ where indicated. PARP (1 μl, R&D, high specific activity enzyme) was the last component added to each reaction, and mixed samples were incubated at room temperature for 30 min. To stop the reaction, SDS loading dye was added, and samples were boiled for 5 min prior to loading on 8–16% Tris-Glycine or 4–12% Bis-Tris precast gels (Invitrogen). Proteins modified by biotin-NAD were detected by immunoblot with streptavidin antibody (Upstate Biotechnology), or samples were loaded for Coomassie Blue staining or immunoblot with the indicated antibodies.

Cell-based Poly(ADP)-riboseylation Assay—In cells, PAR-modified proteins were detected using biotin-NAD with FLAG immunoprecipitation and immunoblotting for streptavidin, based on previous studies that used biotin-NAD for immunofluorescence (30). Reactions were performed in stable clones derived from bax⁻/⁻/bak⁻/⁻ IL-3-dependent cells or FL5.12 cells expressing FLAG-HMGB1-FL, FLAG-HMGB1-ΔC, and FLAG-α4. For each sample, 1 × 10^7 cells were resuspended in 100 μl of PARP buffer (56 mM Hepes, 28 mM KCl, 28 mM NaCl, 2 mM MgCl₂), which was freshly supplemented with 0.01% digitonin (Calbiochem) and 50 μM biotin-NAD (R&D Systems). Samples were preincubated in buffer alone or in buffer containing 300 μM DHIQ for 30 min prior to the addition of 500 μM MNNG for the indicated times at 37 °C. Following treatment, cells were lysed with 250 μl of buffer IP-400 (400 mM NaCl, 20 mM Tris, pH 6.8, 2 mM EDTA, 0.5% Triton X-100, 0.1% deoxycholate, 1× protease inhibitor cocktail) and incubated on ice for 20 min. Samples were then diluted 1:1 v/v with IP-0 buffer (containing no NaCl) for a final concentration of 200 mM NaCl. Lysates were sonicated at 20% output for 10 s and then centrifuged at 14,000 rpm for 10 min. Samples were incubated with pre-equilibrated M2 agarose beads (Sigma) while rotating overnight at 4 °C. After washing the beads with IP buffer containing 200 mM NaCl five times, beads were resuspended in 1× SDS dye and boiled prior to loading on 4–12% Bis-Tris NuPAGE gels for immunoblotting with antibodies against streptavidin or FLAG.

Stimulation of RAW Macrophages—RAW264.7 cells were treated with fresh medium containing His-HMGB1-FL or -ΔC at 1 μg/ml or lipopolysaccharide (Sigma L4516) at 0.1 μg/ml, each in the presence of brefeldin A (2 μg/ml). Four hours following stimulation, RAW cells were harvested and fixed in 4% paraformaldehyde in PBS. After washing cells twice with PBS containing 1% FBS, cells were permeabilized in PBS containing 1% FBS, 0.2% saponin, 0.1% NaAc for 15 min at 4 °C. Immunostaining was performed using 1:100 dilution of fluorescein isothiocyanate-conjugated TNF-α antibody (BD Biosciences) in permeabilization buffer, and intracellular TNF-α content was assessed by flow cytometry.

Statistical Analysis—All data are presented as the mean ± S.D. Differences between means were considered significant when p < 0.05 using the Student’s t test.

RESULTS

HMGB1 Translocates from the Nucleus to the Cytoplasm following Alkylating DNA Damage—The nuclear protein HMGB1 has been reported to be found in the supernatant of cells treated with alkylating agents such as MNNG (2). However, it was
PARP Signals HMGB1 Release during Necrosis

FIGURE 1. HMGB1 subcellular relocalization occurs prior to cell death. A, vegetative cells are more resistant than proliferating cells to PARP-mediated necrosis. IL-3-dependent hematopoietic \( b ax^{-/-} bak^{-/-} \) cells were cultured in the presence or absence of IL-3 and then treated with MNNG (0.5 mM) for 15 min. Cell death was measured at the indicated time points by PI exclusion. B, HMGB1 release into the extracellular environment correlates with the time of cell death. Culture media were collected from cells treated in \( +IL_{3} \) and \(-IL_{3}\) conditions (Fig. 1C and D). HMGB1 exhibited a primarily nuclear localization in untreated cells and appears aqua in the overlay used to quantitate redistribution because of the merged green fluorescence of the fluorescein isothiocyanate-conjugated antibody used to detect HMGB1 and the blue fluorescence of the DNA-bound DAPI. At early time points following MNNG treatment, ~35–40% of cells showed a loss of nuclear HMGB1 staining in both \( +IL_{3}\) and \(-IL_{3}\) conditions (arrows, blue in overlay images). In both populations, this redistribution was apparent at 0.5 and 2 h after treatment with MNNG, although over 90% of cells excluded PI and thus had intact plasma membranes. This relocalization of HMGB1 from the nucleus to the cytosol following MNNG treatment was not restricted to hematopoietic cells and was observed in MEFs as well (Fig. 2).

Although MNNG treatment killed proliferating cells much more effectively, MNNG treatment resulted in equivalent HMGB1 relocalization in both proliferating and vegetative cells with similar kinetics. In addition, both populations displayed similar levels of PARP activation. In both conditions, poly(ADP)-riboylation levels were increased at 30 min after MNNG and returned to basal levels by 8 h (Fig. 1E).

Subcellular Relocalization of HMGB1 during Necrosis Is PARP-dependent—Previous work has shown that MNNG-induced cell death and inflammation are PARP-dependent (2). To determine whether PARP-1 regulates subcellular relocalization of HMGB1 after MNNG treatment, immunofluorescence was performed in \( parp^{-/-} \) MEFs or in wild-type MEFs in the presence of the general PARP inhibitor DHIQ. Following MNNG treatment, immunoblotting was performed on cell culture medium. The appearance of HMGB1 in the medium occurred within 5 h after MNNG treatment in proliferating cells but not in vegetative cells (Fig. 1B). HMGB1 appearance in the medium correlated with time of cell death as measured by PI exclusion, indicating loss of plasma membrane integrity (Fig. 1B).

Interestingly, relocalization of HMGB1 from the nucleus to the cytosol occurred equally in proliferating and vegetative cells following MNNG treatment (Fig. 1, C and D). HMGB1 exhibited a primarily nuclear localization in untreated cells and appears aqua in the overlay used to quantitate redistribution because of the merged green fluorescence of the fluorescein isothiocyanate-conjugated antibody used to detect HMGB1 and the blue fluorescence of the DNA-bound DAPI. At early time points following MNNG treatment, ~35–40% of cells showed a loss of nuclear HMGB1 staining in both \( +IL_{3}\) and \(-IL_{3}\) conditions (arrows, blue in overlay images). In both populations, this redistribution was apparent at 0.5 and 2 h after treatment with MNNG, although over 90% of cells excluded PI and thus had intact plasma membranes. This relocalization of HMGB1 from the nucleus to the cytosol following MNNG treatment was not restricted to hematopoietic cells and was observed in MEFs as well (Fig. 2).

Although MNNG treatment killed proliferating cells much more effectively, MNNG treatment resulted in equivalent HMGB1 relocalization in both proliferating and vegetative cells with similar kinetics. In addition, both populations displayed similar levels of PARP activation. In both conditions, poly(ADP)-riboylation levels were increased at 30 min after MNNG and returned to basal levels by 8 h (Fig. 1E).

Subcellular Relocalization of HMGB1 during Necrosis Is PARP-dependent—Previous work has shown that MNNG-induced cell death and inflammation are PARP-dependent (2). To determine whether PARP-1 regulates subcellular relocalization of HMGB1 after MNNG treatment, immunofluorescence was performed in \( parp^{-/-} \) MEFs or in wild-type MEFs in the presence of the general PARP inhibitor DHIQ. Following

unclear whether HMGB1 release is an active or a passive process. To explore this question, we investigated whether HMGB1 undergoes evidence of nuclear-cytosolic relocalization in response to treatment with DNA-alkylating drugs, utilizing a cell culture model that allows prolonged cell viability following DNA damage (Fig. 1). Upon growth factor withdrawal, IL-3-dependent Bax/Bak-deficient cells were protected from DNA damage induced cell death because of their quiescent metabolic status (Fig. 1A) (2, 31).

To detect the relative amount of HMGB1 released into the extracellular space of proliferating or vegetative cells following

In addition, both populations displayed similar levels of PARP activation. In both conditions, poly(ADP)-riboylation levels were increased at 30 min after MNNG and returned to basal levels by 8 h (Fig. 1E).
PARP Signals HMGB1 Release during Necrosis

MINNG treatment, redistribution of HMGB1 was observed in wild-type cells, whereas HMGB1 remained nuclear in cells lacking PARP-1 (Fig. 2A). Visualization of HMGB1 in adherent cells provided additional examples where cytosolic HMGB1 staining occurred in cells that remained PI-negative (arrow) or where HMGB1 nuclear staining was lost or dim in cells that were PI-positive (arrowheads). Cytosolic HMGB1 staining was not observed in cells lacking PARP activity. Although a few cells exhibited loss of nuclear HMGB1 in the absence of PARP-1, these cells had dim or no staining. Consistent with cell viability following MINNG treatment, the relative amount of HMGB1 found in the culture medium was reduced by PARP inhibitors or in parp-1<sup>−/−</sup> MEFs (Fig. 2B).

PARP Modifies Both Full-length and Truncated HMGB1 in Vitro—HMGB1 and other high mobility group proteins have previously been characterized as acceptor proteins for poly(ADP)-riboseylation (32–34). We next sought to determine whether PARP regulates HMGB1 localization via direct modification. The amino acid sequence of HMGB1 contains a highly conserved stretch of 30 glutamate and aspartic acid residues at the C terminus (Fig. 3A). Since PARP primarily modifies itself and other proteins at glutamate residues (35, 36), the C terminus of HMGB1 is a potential target for poly(ADP)-ribosylation by PARP.

To test whether HMGB1 is an acceptor protein for PARP<sup>in vitro</sup>, recombinant His-tagged full-length HMGB1 (-FL) or truncated HMGB1 (-ΔC, amino acids 1–185) was produced, and proteins were purified by affinity chromatography. The ability of the recombinant proteins to activate RAW264.7 macrophages was determined by measuring TNF-α production by intracellular staining and fluorescence-activated cell sorter analysis. In accordance with previous work from others (5, 22, 37), purified recombinant His-HMGB1-FL was able to activate macrophages. His-HMGB1-ΔC also stimulated TNF-α production (Fig. 3B).

To determine targets of PARP modification<sup>in vitro</sup>, poly(ADP)-ribosylation assays were performed using biotin-NAD to label modified proteins. Purified rhPARP was incubated with sheared DNA and biotin-NAD and then subjected to SDS-PAGE analysis. Reactions were aliquoted between three gels for Coomassie Blue staining or immunoblotting with streptavidin, to detect ADP-ribosylated proteins or with PAR antibody, to detect proteins modified by long poly(ADP)-ribosyl chains. PARP modified itself under these conditions (Fig. 3C, lane 2) but not if the reaction lacked biotin-NAD or DNA (Fig. 3C, lanes 1 and 3) or if the reaction contained the PARP inhibitor DHIQ (Fig. 3C, lane 6). When a known PARP acceptor protein, histone H1, was included in the full reaction, an additional band was seen at the size of histone H1 (Fig. 3C, lane 4), indicating trans-modifica-
tion. Supplementing the reaction with excess unlabeled NAD\(^+\) increased PARP activation but competed out biotin-NAD, resulting in decreased signal by immunoblot for streptavidin (Fig. 3C, lane 5). Upon PARP activation, PAR-modified proteins were visualized by immunoblotting with anti-PAR antibody and appeared as a large smear, indicating poly(ADP)-ribose chains of variable length attached to histone H1 (between 30 and 98 kDa) or to PARP (above 98 kDa). The attachment of
longer PAR modifications also resulted in reduced band intensity for both PARP and histone H1 as detected by Coomassie Blue staining (Fig. 3C, lane 5).

The ability of PARP to modify recombinant HMGB1-FL or -ΔC was assessed using the above assay. As a negative control for specificity of PARP acceptor proteins, purified recombinant His-tagged Bcl-x, was used. Reactions containing constant amounts of sheared DNA, biotin-NAD, and each His-tagged acceptor protein were incubated with decreasing quantities of rhPARP and analyzed by Western blot with streptavidin or an antibody against the His tag. Both His-HMGB1-FL and His-HMGB1-ΔC were modified by PARP in vitro (Fig. 3D, lanes 6–11), whereas Bcl-x, was not (Fig. 3D, lane 1).

PARP Modifies Both Full-length and Truncated HMGB1 in Cells—To detect direct modification of HMGB1 by PARP in cells, an assay for immunoprecipitation analysis of ADP-ribosylation was developed using biotin-NAD. Stable cell lines were generated in bax−/−/bak−/−/IL-3-dependent cells that express FLAG-HMGB1-FL or -ΔC (Fig. 3E). After preincubating cells with digitonin and biotin-NAD, cells were treated with MNNG for 10 or 30 min in the presence or absence of DHIQ. Poly-(ADP)-ribose-modified proteins were detected in lysates or FLAG immunoprecipitates by immunoblotting with streptavidin. As a control for PARP activation following MNNG treatment, immunoblotting of cell lysates with PAR antibody showed equivalent induction of PAR formation in all cell lines (Fig. 3E, upper panel). Similarly, immunoblotting for streptavidin in cell lysates showed a strong signal at the size of PARP and at lower molecular weights induced in MNNG-treated cells (Fig. 3E, lower panel, lanes 1–2).

Both full-length and truncated HMGB1 were modified by PARP in cells following MNNG treatment. In streptavidin immunoblotting of FLAG immunoprecipitates, a band corresponding to the size of FLAG-HMGB1-FL or -ΔC appeared in MNNG-treated cells but not in untreated cells or in the presence of DHIQ (Fig. 3E, lower panel, lanes 5–12). This band was specific for the relevant HMGB1 proteins as it did not appear in parental cells (ctl) or in cells expressing another tagged protein, FLAG-α4 (data not shown). Identical results were obtained from stable cell lines generated in FL5.12 cells.

HMGB1 Relocalization during Necrosis Depends on the C-terminal Acidic Tail—Although both full-length and truncated HMGB1 can be modified by PARP, we next asked whether the acidic tail plays a role in HMGB1 relocalization following PARP activation. FLAG-HMGB1-FL or -ΔC constructs were transiently expressed in bax−/−/bak−/− BMK cells, and immunofluorescence verified that both properly localized to the nucleus in untreated cells (Fig. 4A). As observed for endogenous HMGB1, MNNG treatment caused redistribution of FLAG-HMGB1-FL away from the nucleus as early as 1 h after treatment. Strikingly, FLAG-HMGB1-ΔC remained nuclear following MNNG treatment (Fig. 4A).

Furthermore, the ability of HMGB1 to escape from cells and appear in the culture medium during necrosis was dependent on the acidic tail (Fig. 4B). Stable cell lines expressing FLAG-HMGB1-FL or -ΔC in FL5.12 cells displayed similar rates of cell death upon MNNG treatment (data not shown). Using an antibody that recognizes endogenous HMGB1 and both tagged forms, immunoblotting of cell lysates or culture media showed that endogenous HMGB1 equally escaped either cell line following MNNG-induced cell death (Fig. 4B, cells versus media). FLAG-HMGB1-FL appeared in the medium at similar time points, but truncated HMGB1 did not (Fig. 4B).

To test whether HMGB1 relocalization from the nucleus during necrosis is regulated by nuclear export, bax−/−/bak−/− BMK cells expressing FLAG-HMGB1-FL or -ΔC were incubated with leptomycin B, an inhibitor of nuclear protein shuttling. As shown in Fig. 4C, MNNG treatment led to an increase in the percentage of HMGB1-FL found in the cytosolic extract, from 14 to 29% at 4 h after treatment (lanes 1–2 versus lanes 5–6). However, in the presence of leptomycin B, MNNG treatment resulted in a greater redistribution of full-length HMGB1, from 9% in the absence of MNNG to 65% after 4 h after treatment (lanes 3–4 versus lanes 7–8). In each condition tested, HMGB1-ΔC remained associated with the nuclear extract.

Although increasing amounts of full-length HMGB1 were found in the medium over time following MNNG treatment, HMGB1-ΔC did not accumulate in the medium yet appeared to be lost from the cell lysates (Fig. 4B). Because preparations of cell lysates excluded the insoluble nuclear pellet, another experiment was performed to analyze nuclear fractions in FL5.12 stable clones (Fig. 4D). Following MNNG treatment, the relative amount of HMGB1-FL in the nucleus decreased and simultaneously increased in the culture medium. In contrast, HMGB1-ΔC appeared to accumulate in the nucleus as cells died and never escaped to the cytosol or medium (Fig. 4D). This effect was specific for HMGB1-ΔC because another reported PARP substrate, nucleolin, underwent modification and release into the medium following cell death in the HMGB1-ΔC transfected cells.

DISCUSSION

Necrosis has generally been considered an unregulated form of cell death that results in plasma membrane disintegration and leakage of cellular contents to stimulate inflammation. In contrast, apoptosis results in an organized proteolytic degradation of cellular contents to form an intact cell body that is phagocytosed without inducing an inflammatory response. Beyond the status of plasma membrane integrity, differences in nuclear morphology and chromatin structure play a profound role in determining the inflammatory consequences from these modes of cell death. Despite extensive nonspecific proteolysis associated with necrosis, the integrity of certain molecules is retained, and these molecules are capable of triggering inflammation when released from dying cells. These have been termed endogenous danger signals and include proteins that normally reside within distinct compartments in the cell, such as HMGB1 in the nucleus, and heat shock proteins in the endoplasmic reticulum (38, 39).

In this study, we identify a role for PARP activity in initiating changes in chromatin structure that result in relocalization of HMGB1 away from the nucleus. After DNA-alkylating damage, HMGB1 accumulates in the cytosol prior to cell death, as determined by immunofluorescence and exclusion of propidium iodide (Figs. 1 and 2). HMGB1 relocalization depends on PARP activity as HMGB1 remains nuclear in parp-1−/− cells after
MNNG treatment (Fig. 2). Both full-length and truncated HMGB1 are directly modified by PARP (Fig. 3). However, in addition to PARP activity, the acidic tail of HMGB1 plays an essential role in allowing HMGB1 relocalization from the nucleus (Fig. 4).

In our experiments, a shift in gel mobility of HMGB1 following MNNG treatment was not detected. We also did not detect a signal upon Western blot with PAR antibody of FLAG-HMGB1 immunoprecipitates (data not shown). The distinct band observed upon immunoprecipitation of HMGB1 in the presence of biotin-NAD (Fig. 3E) suggests that HMGB1 may be modified by mono-ADP-ribose or a few ADP-ribose moieties, below the level of detection of the PAR antibody. These results suggest that PARP may not attach a long chain onto HMGB1. Instead, the acidic tail may be necessary to trigger electrostatic repulsion away from automodified PARP. In addition, the level of biotin-NAD modification did not significantly differ between FL and ΔC, suggesting that this modification was not sufficient to account for the differential displacement from the nucleus we observed.

Poly(ADP)-ribosylation has been shown to alter the function of acceptor proteins or regulate interac-
tions between PARP and its binding partners (3, 40). For example, PARP autommorganisation disrupts the interaction between PARP-1 and topoiosomerase I (41). Trans-
formation of acceptor proteins inhibits the enzymatic activity of some targets, such as Topo I and Cockayne syndrome group B protein (42), or blocks DNA binding ability of others, such as Ku70/80 (43). The DNA binding ability of target proteins is also regulated by other PARP family members as tankyrase 1 and 2 mediate poly-
(ADP)-ribosylation of TRF1 to reduce binding to telomeres (28, 44). Non-covalent interactions be-
tween PAR polymers and nuclear proteins may also facilitate chroma-
tin remodeling during DNA repair, as suggested for removal of histones around DNA breaks (45, 46).

As knowledge of ADP-ribosylation signaling within a cell rapidly expands, the consequence of PARP activity on neighboring cells remains less clear. Although PARP regulates NF-κB and other transcription factors to alter expression of pro-inflam-
atory cytokines over time (47), the immediate consequences of excessive PARP activity during necrosis have not been elucidated. In our current model, PARP activation trig-
gers relocalization of HMGB1 away from chromatin, which allows HMGB1 leakage into the extracellular environment upon plasma membrane disintegration during necrosis. Both PARP activity and the acidic tail of HMGB1 contribute
to the ability of dying cells to alert neighboring cells of necrotic cell death.

Many nuclear proteins contain acidic residues that serve to regulate their ability to bind DNA and/or histones. A particularly long stretch of continuous acidic residues is evolutionarily conserved among members of the high mobility group protein family. The C terminus of HMGB1, HMGB2, and HMGB3 each contains 30, 23, and 18 acidic residues, respectively. We also observed release of nucleolin, which contains two conserved acidic domains of 22 and 33 residues, and other acidic nuclear proteins into the culture medium following necrosis (Fig. 4D and data not shown). Therefore, PARP activation likely plays a more general role in inducing relocation of acidic nuclear proteins. Although the HMGB1 acidic tail of 30 acidic amino acids contributes to its relocation following DNA damage, the minimum number of acidic residues required for release remains to be determined.

The observation that truncated HMGB1 shows an equal, if not more intense, modification (Fig. 3, D and E) suggests that the C-terminal acidic tail is not the site of limited ADP-ribosylation. It is also possible that certain residues in the HMG boxes, which normally interact with the acidic tail (48), become unmasked in truncated HMGB1 and allow for increased PAR modification observed in vitro (Fig. 3D).

HMGB1 is a 28-kDa protein, which is small enough to migrate across the nuclear pore unassisted. Other studies have shown that basal nuclear-cytosolic shuttling of HMGB1 is regulated through nuclear import/export sequences as both HMG boxes can directly bind CRM1 (21). In healthy cells, leptomycin B treatment decreases but does not abolish HMGB1 shuttling, as reported in heterokaryon assays (21). Our data confirm this but also demonstrate that impairment of shuttling by treatment with leptomycin B leads to enhanced HMGB1 accumulation in the cytosol following MNNG treatment. Although escape of cytosolic HMGB1 from cells during necrosis appears to correlate with the loss of the integrity of the plasma membrane, the present data suggest that HMGB1 translocation to the cytoplasm is determined by its ability to release from chromatin and is influenced by the acidic tail (Fig. 4D). The finding that PARP activity regulates HMGB1 localization suggests a novel role for PARP in initiating relocation of a pro-inflammatory molecule in a cell that has sustained DNA damage.

Acknowledgments—We thank Dr. Zhao-Qi Wang (International Agency for Research on Cancer, Lyon, France) for providing parp−/− MEFs. We thank all members of the Thompson laboratory for helpful discussions and reagents, especially Chi Li for providing recombinant His-Bcl-x(L) Julian Lum and Mei Kong for providing FLAG-εεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεee
PARP Signals HMGB1 Release during Necrosis

Tracey, K. J., and Yang, H. (2004) *J. Immunol. Methods* **289**, 211–223
38. Bianchi, M. E. (2004) *Trends Cell Biol.* **14**, 287–293
39. Matzinger, P. (1994) *Annu. Rev. Immunol.* **12**, 991–1045
40. D’Amours, D., Desnoyers, S., D’Silva, I., and Poirier, G. G. (1999) *Biochem. J.* **342**, 249–268
41. Yung, T. M., Sato, S., and Satoh, M. S. (2004) *J. Biol. Chem.* **279**, 39686–39696
42. Thorslund, T., von Kobbe, C., Harrigan, J. A., Indig, F. E., Christiansen, M., Stevnsner, T., and Bohr, V. A. (2005) *Mol. Cell. Biol.* **25**, 7625–7636
43. Li, B., Navarro, S., Kasahara, N., and Comai, L. (2004) *J. Biol. Chem.* **279**, 13659–13667
44. Cook, B. D., Dynek, I. N., Chang, W., Shostak, G., and Smith, S. (2002) *Mol. Cell. Biol.* **22**, 332–342
45. Pleschke, J. M., Kleczkowska, H. E., Strohm, M., and Althaus, F. R. (2000) *J. Biol. Chem.* **275**, 40974–40980
46. Althaus, F. R. (1992) *J. Cell Sci.* **102**, 663–670
47. Hassa, P. O., and Hottiger, M. O. (2002) *CMLS Cell. Mol. Life Sci.* **59**, 1534–1553
48. Knapp, S., Muller, S., Digilio, G., Bonaldi, T., Bianchi, M. E., and Musco, G. (2004) *Biochemistry* **43**, 11992–11997