HMGB1 is secreted by immunostimulated enterocytes and contributes to cytomix-induced hyperpermeability of Caco-2 monolayers

Shiguang Liu, Donna B. Stolz, Penny L. Sappington, Carlos A. Macias, Meaghan E. Killeen, Jyrki J. Tenhunen, Russell L. Delude, and Mitchell P. Fink. HMGB1 is secreted by immunostimulated enterocytes and contributes to cytomix-induced hyperpermeability of Caco-2 monolayers. Am J Physiol Cell Physiol 290: C990–C999, 2006. First published November 9, 2005; doi:10.1152/ajpcell.00308.2005.—High-mobility group box 1 (HMGB1), a cytokine-like proinflammatory protein, is secreted by activated macrophages and released by necrotic cells. We hypothesized that immunostimulated enterocytes might be another source for this mediator. Accordingly, Caco-2 cells or primary mouse intestinal epithelial cells (IECs) were incubated with “cytomix” (a mixture of TNF, IL-1β, and IFN-γ) for various periods. HMGB1 in cell culture supernatants was detected by Western blot analysis and visualized in Caco-2 cells with the use of fluorescence confocal and immunotransmission electron microscopy. Caco-2 cells growing on filters in diffusion chambers were stimulated with cytomix for 48 h in the absence or presence of anti-HMGB1 antibody, and permeability to fluorescein isothiocyanate-labeled dextran (average molecular mass, 4 kDa; FD4) was assessed. Cytomix-stimulated Caco-2 cells secreted HMGB1 into the apical but not the basolateral compartments of diffusion chambers. Although undetectable at 6 and 12 h after the start of incubation with cytomix, HMGB1 was present in supernatants after 24 h of incubation. HMGB1 secretion by Caco-2 monolayers also was induced when the cells were exposed to FSL-1, a Toll-like receptor (Tlr)-2 agonist, or flagellin, a Tlr5 agonist, but not lipopolysaccharide, a Tlr4 agonist. Cytomix also induced HMGB1 secretion by primary IECs. Cytoplasmic HMGB1 is localized within vesicles in Caco-2 cells and is secreted, at least in part, associated with exosomes. Incubating Caco-2 cells with cytomix increased FD4 permeation, but this effect was significantly decreased in the presence of anti-HMGB1 antibody. Collectively, these data support the view that HMGB1 is secreted by immunostimulated enterocytes. This process may exacerbate inflammation-induced epithelial hyperpermeability via an autocrine feedback loop.

EXOCYTE; TOXIN; TOLL-LIKE RECEPTOR; FLAGELIN

HIGH-MOBILITY GROUP PROTEINS are small DNA binding proteins that serve an important role in transcriptional regulation (7). One of these proteins, high-mobility group box-1 (HMGB1), has been identified as late-acting mediator of lipopolysaccharide (LPS)- (38) or sepsis-induced (41) lethality in mice. HMGB1 also has been implicated as a mediator of LPS- (1) and hemorrhagic shock–induced acute lung injury in mice (16). In addition, our laboratory showed that the addition of recombinant HMGB1 to the culture medium for Caco-2 human enterocyte-like monolayers increases the permeability of the epithelial barrier to a fluorescent macromolecule, fluorescein isothiocyanate-labeled dextran with an average molecular mass of 4,000 kDa (FD4) (30). Moreover, we showed that injecting mice with HMGB1 induces gut mucosal hyperpermeability to FD4 and promotes bacterial translocation to mesenteric lymph nodes (30). Additional studies (4) have documented that HMGB1 is a cytokine-like molecule that can promote TNF release from mononuclear cells.

HMGB1 also has been implicated in the pathogenesis of human disease. In the original report describing HMGB1 as a mediator of LPS-induced lethality, Wang et al. (1) reported that circulating levels of this protein are increased in patients with severe sepsis. Shortly thereafter, Ombrellino et al. (23) described a patient with high circulating levels of HMGB1 after an episode of hemorrhagic shock. More recently, increased levels of HMGB1 mRNA have been detected in whole blood samples from patients with septic shock, particularly among nonsurvivors (24). Similarly, persistently high serum levels of HMGB1 protein have been detected in patients with septic shock (34).

HMGB1 is actively secreted by immunostimulated macrophages (6, 11, 28, 38), natural killer cells (33), and puitocytes (39). This protein is also released by necrotic, but not apoptotic, cells (31). Because HMGB1 has been shown to modulate intestinal epithelial barrier function (30), we hypothesized that active secretion by enterocytes might be yet another source for this cytokine-like protein. Herein, we report that stimulating either Caco-2 cells or primary cultures of murine enterocytes with cytomix, a mixture of interferon (IFN)-γ, interleukin (IL)-1β, and tumor necrosis factor (TNF), induced secretion of HMGB1. In addition, we show that the addition of a neutralizing polyclonal anti-HMGB1 antibody partially blocked the increase in permeability caused by incubating Caco-2 monolayers with cytomix. The data also indicate that HMGB1 is secreted in soluble form as well as a particulate form that is sequestered within exosomes. These data support the view that enterocyte-derived HMGB1 may be an autocrine amplifier of derangements in epithelial barrier function initiated by other proinflammatory stimuli.

MATERIALS AND METHODS

The research protocol complied with the regulations in the National Institutes of Health publication Guide for the Care and Use of Laboratory Animals and was approved by the Institutional Animal Care and Use Committee. All animals were housed and handled in accordance with the National Institutes of Health publication Guide for the Care and Use of Laboratory Animals. Infected animals were euthanized by an intraperitoneal injection of sodium pentobarbital, and the lungs were removed and immediately placed in 30% buffered formalin. Sections were stained with hematoxylin and eosin. HMGB1 in the cytoplasmic, nuclear, and extracellular compartments was visualized by immunohistochemistry. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Animals. C57BL/6 mice (4 – 8 wk old; ∼25 g) were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained at the University of Pittsburgh Animal Research Center with a 12:12-h light-dark cycle and free access to standard laboratory chow and water. The animals were not fasted before the experiments.

Materials. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Dulbecco’s modified Eagle medium (DMEM) and phosphate-buffered saline (PBS) were from Bio-Whittaker (Walkersville, MD). Fetal bovine serum was from Hyclone (Logan, UT). Flagellin (LPS content < 125 EU/ml) and a synthetic Toll-like receptor (Tlr)-2 agonist, FSL-1 (Pam2CGDPKHPKSF), were from Invivogen (San Diego, CA). Highly purified (TLRgrade) Salmonella minnesota R595 (Re) LPS was from Alexis Biochemicals (San Diego, CA).

Caco-2 cells. Caco-2 cells were obtained from ATCC (Manassas, VA) and routinely maintained on collagen I-coated Biocoat tissue culture dishes (Becton-Dickinson, Bedford, MA) at 37°C in a 5% CO2 humidified atmosphere. In DMEM supplemented with 10% fetal bovine serum, penicillin G (100 U/ml), streptomycin (100 g/ml), pyruvate (2 mmol/l), L-glutamine (4 mmol/l), and nonessential amino acids. For some studies, the cells were seeded onto 24-well plastic plates (100,000 cells/well). After being cultured for 7 days, some wells were stimulated with cytokinx, medium containing medium containing cytokinx, and supernatants were harvested at the time points indicated in RESULTS.

For other experiments, Caco-2 cells (50,000 cells/well) were seeded onto permeable filters in 12-well transwell bicanimal chambers (COSTAR, Corning, NY) and fed biweekly. After 21 days, some wells were stimulated with cytokinx, and supernatants were harvested after 24 and 48 h.

To measure changes in the permeability of Caco-2 monolayers, the cells were plated onto permeable filters in 12-well Transwell chambers (105 cells/well) and fed biweekly. Permeability studies were performed using confluent monolayers between 21 and 28 days after being seeded. The permeability probe was fluorescein isothiocyanate-labeled dextran (average molecular mass = 4 kDa; FD4). A sterile stock solution of FD4 (25 mg/ml) was prepared by dissolving the compound in HEPES-buffered DMEM (pH 6.8) and passing it through a filter (0.45-μm pore size). For permeability studies, the medium was aspirated from the apical and basolateral sides of the Transwell chambers. FD4 solution (200 μl) was added to the apical compartments. The medium on the basolateral side of the Transwell chambers was replaced with 500 μl of control medium, medium containing cytokinx, medium containing medium containing cytokinx, and supernatants were harvested after 24 h of incubation. 30 μl of medium were aspirated from the basolateral compartments for spectrofluorometric determination of FD4 concentration as previously described (21). The permeability of monolayers was expressed as a clearance with the units (nl·cm−2·h−1), which was calculated as previously described (30).

Isolation and culture of primary murine intestinal epithelial cells. C57BL/6 black mice were euthanized, and the small intestine was washed twice with ice-cold Ca2+- and Mg2+-free HBSS and resuspended in DMEM/F12 culture medium (BioWhittaker), which was supplemented with 5% FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin, 1× insulin-transferrin-selenium (Invitrogen), 20 mM HEPES, pH 7.4, 0.25 μg/ml amphotericin B, 1 μg/ml fibronectin, and 1 μg/ml hydrocortisone. Six-well plates were coated with collagen I and poly-L-lysine 1 h before the cells were seeded. The plates were seeded with 500 crypts/well and incubated at 37°C under a 5% CO2 atmosphere. Cellular viability was >95% by Trypan blue exclusion assay. Three days after being seeded, the cells were stimulated with cytokinx, and supernatants were harvested after 24 and 48 h.

Lactate dehydrogenase release assay. Cells were incubated in the absence or presence of cytokinx for 24 or 48 h. Samples of medium were collected, and the amount of lactate dehydrogenase (LDH) in the medium was measured with an assay kit (TOX-7) from Sigma, by following the protocol suggested by the manufacturer. To control for slight differences among wells in the total number of cells, we then lysed the cells, using two freeze-thaw cycles, and measured the total amount of LDH in each well. Results are expressed as the quantity of LDH in culture supernatants (before the freeze-thaw cycles) divided by the total amount of LDH in each well after cell lysis was induced by the freeze-thaw cycles.

Western blot analysis. Equal volumes of cell-culture supernatant were mixed with 6X loading buffer [375 mM Tris, pH 6.8, 50% glycerol, 0.03% bromophenol blue, and 10% SDS]. After being boiled, the samples were subjected to 10% SDS-PAGE electrophoresis. The resolved proteins were transferred to PVDF membrane (Amersham Pharmacia Biotech, Leicestershire, UK) and blocked with Blotto buffer (1× PBS, 5% nonfat milk, 0.05% Tween 20, and 0.2% NaNO3) for 1 h. The membrane was then incubated with rabbit polyclonal anti-HMGB1 antibodies (BD Pharmingen, San Jose, CA) 1:2,000 diluted in blocking buffer overnight at 4°C. After being washed three times in 1× PBS with 0.3% Triton X-100, pH 7.4 (PBST), immunoblots were exposed for 1 h at room temperature to a 1:20,000 dilution of the HRP-conjugated goat anti-rabbit secondary antibody. After being washed three times with 1× PBST, the membrane was illuminated with the ECL reagents (Amersham Pharmacia Biotech) and X-ray film exposed, according to the manufacturer’s instructions. HMGB1 expression was quantified densitometrically with the use of GelExpert version 3.5 software (Nucleotek, San Mateo, CA) and comparison to a standard curve generating by carrying out Western blot analysis with known quantities of recombinant human HMGB1.

Exosome isolation from culture medium. Caco-2 cells were grown in 75 cm2 culture flasks until confluent. The cells were incubated under control conditions or stimulated with cytokinx for 24 h. The medium was collected and centrifuged at 400 g at 4°C for 5 min to remove debris. The supernatants were subjected to ultracentrifugation at 100,000 g at 4°C for 1 h to pellet exosomes. The supernatant was collected and assayed for the presence of HMGB1 by Western blot analysis and the pellet was washed with 1× PBS and pelleted again by ultracentrifugation as described above. The final pellet was resuspended in 200 μl of PBS for immuno-transmission electron microscopy (TEM) and Western blot analysis.

Immunofluorescence confocal microscopy. Caco-2 cells were grown on filter inserts as described and fixed in 2% paraformaldehyde in PBS for 1 h. Cells were washed three times in PBS, then three times (5 min each) with PBS supplemented with 0.5% bovine serum albumin (PBB). Cells were permeabilized in 0.1% Triton X-100 in PBB for 30 min, then washed once with PBB. Cells were blocked with 2% bovine serum albumin in PBS for 40 min then washed once with PBB. Rabbit anti-HMGB1 antibodies (1:2,000) were added to cells in PBB incubated at room temperature for 1 h. Cells were washed four times in PBB, then secondary antibodies (goat anti-rabbit Alexa 488; 1:500 dilution; Molecular Probes, Eugene, OR), rhodamine-phalloidin
sections were washed two times in PBS, and then washed six times in PBG and labeled with goat anti-rabbit antibody conjugated normal goat serum in PBG. Sections were labeled with rabbit anti-HMGB1 for 1 h at room temperature. Cells were washed three times with PBS, and then three more times with PBG, followed by a 30-min blocking incubation with 5% normal goat serum in PBG. Sections were situated on ultracryotome stubs that were cross-sectioned, frozen with liquid nitrogen, and stored in liquid nitrogen until use. Ultrathin sections (70 nm) were cut with the use of a Reichert Ultracut U ultramicrotome with a FC4S cryo-attachment, lifted on a small drop of 2.3 M sucrose in PBS, and mounted on Formvar-coated copper grids. Sections were washed three times with PBS, then three times with PBG, followed by a 30-min blocking incubation with 5% normal goat serum in PBG. Sections were labeled with rabbit anti-HMGB1 (1:100 dilution in PBG) for 1 h. Sections were washed four times in PBG and labeled with goat anti-rabbit antibody conjugated with 5 nm of colloidal gold by 10.220.33.3 on April 10, 2017 http://ajpcell.physiology.org/ Downloaded from

Immunoelectron microscopy. Caco-2 cells were grown on filter inserts as described and fixed in cryofix (2% paraformaldehyde, 0.01% glutaraldehyde in 0.1 M PBS) for 1 h. Cells on filters were coated on both the top and bottom of the filter with 3% gelatin in PBS for 1 h at 37°C. The gelatin was solified at 4°C for 1 h, and then fixed for an additional 15 min in cryofix. Washed exosomal pellets were likewise encapsulated in 3% gelatin. Gelatin-cell filter and gelatin-exosome pellet blocks were cryoprotected in polyvinylpyrrolidone cryoprotectant (25% polyvinylpyrrolidone, 2.3 M sucrose, 0.055 M Na2CO3, pH 7.4) overnight at 4°C, as previously described (35). Blocks were situated on ultracryotome stubs that were cross-sectioned, frozen with liquid nitrogen, and stored in liquid nitrogen until use. Ultrathin sections (70 nm) were cut with the use of a Reichert Ultracut U ultramicrotome with a FC4S cryo-attachment, lifted on a small drop of 2.3 M sucrose in PBS, and mounted on Formvar-coated copper grids. Sections were washed three times with PBS, then three times with PBG, followed by a 30-min blocking incubation with 5% normal goat serum in PBG. Sections were labeled with rabbit anti-HMGB1 (1:100 dilution in PBG) for 1 h. Sections were washed four times in PBG and labeled with goat anti-rabbit antibody conjugated with 5 nm of colloidal gold by 10.220.33.3 on April 10, 2017 http://ajpcell.physiology.org/ Downloaded from

Exosome staining. Copper grids (200 mesh) were coated with 0.125% Formvar (Ted Pella, Redding, CA) in chloroform. Washed exosomal pellets (1–10 μl of suspension) were loaded onto grids by centrifugation in an Airfuge Ultracentrifuge (Beckman, Palo Alto, CA) using 2% paraformaldehyde in PBS for 10 min, washed in PBS, and then negatively stained with 1% aqueous uranyl acetate. For immunostaining, exosomes were fixed with 2.5% glutaraldehyde in PBS for 10 min, washed in PBS, and then negatively stained with 1% aqueous uranyl acetate. For immunostaining, exosomes were fixed with 2% paraformaldehyde in PBS for 5 min. The exosomes were washed three times with PBS, and then three more times with PBS. Some samples were permeabilized with 0.1% Triton X-100 in PBG for 5 min, followed by 30-min incubation with 5% normal goat serum in PBG. Exosomes were labeled with rabbit anti-HMGB1 for 1 h at room temperature. Exosomes were washed four times with PBG and then labeled with 5 nm of colloidal gold by being incubated with gold-conjugated goat anti-rabbit antibody at 1:25 dilution at room temperature for 1 h. Sections were washed three times in PBG and three times in PBS. The sections were fixed in 2.5% glutaraldehyde in PBS for 5 min, washed two times in PBS, and then washed six times in ddH2O. The grid was placed on a drop of 0.45 μm filtered 2% phosphotungstic acid, pH 6.0 in milli-Q H2O for 30–60 s. Excess stain was wicked away and the samples were viewed with a JEM 1210 TEM at 80 kV.

Statistical methods. Clearance data from the Transwell experiments using FD4 are expressed as means ± SE. Data were analyzed by ANOVA, followed by Fisher’s protected least-significant difference test. Significance was declared for P < 0.05.

RESULTS

Cytomix-stimulated Caco-2 cells secrete HMGB1 in a polarized fashion. Caco-2 cells growing on permeable membranes in bicameral diffusion chambers were incubated for 24 h in the absence or presence of cytomix. Irrespective of whether cytomix was added to the apical or the basolateral compartments of the chambers, stimulating the cells with the cytokine cocktail resulted in release of HMGB1 into the apical (but not the basolateral) culture medium (Fig. 1A). Viability of Caco-2 cells, as assessed by exclusion of the vital dye, Trypan blue, was >98% both before and after stimulation with cytomix. Furthermore, incubation of Caco-2 cells with cytomix for 24 or 48 h did not change the release of the intracellular enzyme LDH (Fig. 1C). Thus the release of HMGB1 from immunostimulated Caco-2 cells was not due to cytomix-induced cell death.

Cytomix-induced secretion of HMGB1 increases over time. Caco-2 cells growing in plastic wells were incubated in the absence or presence of cytomix. Samples of the supernatants were obtained from the cytomix-stimulated cultures after graded periods of time, ranging from 6 to 48 h of incubation. HMGB1 was undetectable by Western blot analysis in supernatants from the unstimulated cells and in supernatants from controls cultured for 6 or 12 h with cytomix (Fig. 1B). However, HMGB1 was clearly detectable in the cells after 24 h of exposure to cytomix, and the quantity of the protein released into the supernatant was greater still after 48 h of incubation with the cytokine mixture.

Cytomix-stimulated Caco-2 cells growing on filters in diffusion chambers or on plastic plates release similar quantities of HMGB1. By generating a standard curve with known amounts of recombinant human HMGB1, we used Western blot analysis to quantify the release of HMGB1 from control and immunostimulated Caco-2 cells. Irrespective of whether the cells were grown on filters in diffusion chambers or in plastic wells, stimulation with cytomix resulted in the release of similar amounts of HMGB1 (Fig. 1D). For example, after 24 h of incubation with cytomix, the cells released ~800 ng of HMGB1 per 10⁶ cells. For comparison, murine peritoneal macrophages stimulated with IFN-γ (100 or 1,000 U/ml) for 16 h release ~100 ng of HMGB1 per 10⁶ cells (28).

Tlr2 and Tlr5 agonists induce HMGB1 secretion by Caco-2 cells. Previous studies indicate that several human enterocyte-like cells lines (including Caco-2) express pathogen-associated molecular pattern (PAMP) recognition receptors, including Tlr2 (9) and Tlr5 (5). Under normal conditions, expression of Tlr4 is downregulated in Caco-2 cells (3, 22) but expression of this PAMP is increased when these cells are stimulated with IFN-γ and/or TNF (2). Prompted by these observations, we carried out studies to determine whether LPS (Tlr4 agonist), FSL-1 (synthetic Tlr2 agonist), or flagellin (Tlr5 agonist) can stimulate Caco-2 cells to secrete HMGB1. Accordingly, Caco-2 cells growing in 24-well plastic plates were incubated under control conditions or stimulated with graded concentrations of LPS, FSL-1, or flagellin. After 48 h, supernatants were harvested and assayed for HMGB1 by Western blot analysis. Both FSL-1 (Fig. 2A) and flagellin (Fig. 2B) clearly stimulated
HMGB1 in a concentration-dependent fashion, whereas highly purified LPS (0.5 to 10 μg/ml) was without effect (data not shown).

Primary murine IECs secrete HMGB1. Caco-2 cells are transformed human enterocyte-like cells. Although these cells recapitulate many features of normal small intestinal absorptive epithelial cells (13, 26), they are nonetheless cancer cells. Cultured WiDr human colon cancer cells were recently reported to release HMGB1 (18). Accordingly, having shown that immunostimulated Caco-2 cells secrete HMGB1, we sought to determine whether primary nontransformed IECs also can be stimulated to secrete HMGB1 by incubating the cells with cytomix. When culture supernatants from primary murine IEC cultures, which had been stimulated with cytomix for 24 or 48 h, were assayed by Western blot analysis, HMGB1 was clearly detectable (Fig. 3). In contrast, HMGB1 was not present in culture supernatants from unstimulated primary murine IEC cultures.

**HMGB1 is associated with multivesicular bodies in Caco-2 cells and exosomes in Caco-2 supernatants.** Caco-2 cells were grown on inserts in 6- or 24-well diffusion chambers and then incubated for 24 h in the absence or presence of cytomix. In both control (Figs. 4, A–C, and 5A) and cytomix-stimulated (Figs. 4, D–F, and 5B) Caco-2 cells, confocal immunofluorescence microscopy revealed that HMGB1 presented a very heterogeneous labeling pattern, and was localized to the nucleus, the cytoplasm, as well as under and on the apical membrane. Although the nuclei of some cells were brightly stained, nuclear staining in other cells was much less intense or totally absent. In the cytoplasm, HMGB1 immunofluorescence was often punctate, suggesting compartmentalization of the protein within vesicles both within and on the surface of the cell.
cells. In addition, punctuate HMG1 staining was observed adherent to the external surface of the Caco-2 plasma membrane and microvilli, concentrating at areas of cell-cell contact.

To obtain more detailed information about the localization of HMG1, Caco-2 cells were grown on filters and then processed for immuno-TEM. Consistent with the findings of earlier investigators (17, 19), our images showed that Caco-2 cells contain multivesicular bodies (MVBs) as well as smaller membrane-bound vesicles (Fig. 6A). Electron-dense gold particles, indicative of binding of the anti-HMG1 antibody, were localized within MVBs and smaller vesicles within the cell (Fig. 6A), as well as on the cell surface associated with the microvilli (Fig. 6B, inset), and in the nucleus (not shown). Labeling was often concentrated in vesicles that had dense material within the lumen (Fig. 6A, inset).

Exosomes are 30- to 90-nm membrane-bound vesicles that are secreted by numerous cell types, including reticulocytes (14), platelets (12), B lymphocytes (27), dendritic cells (42), and epithelial cells (37). Exosomes are formed when MVBs fuse with the plasma membrane, releasing the vesicles into the extracellular compartment (36). Because we observed that HMG1 was localized within MVBs in Caco-2 cells, we sought to determine whether HMG1 is associated with exosomes secreted by unstimulated or cytokine-stimulated Caco-2 cells. Therefore, apical supernatants were collected from control and cytokine-treated cells grown on filters in Transwell chambers. The supernatants were subjected to ultracentrifugation, and, after the pellet was washed and resuspended in PBS, the exosome-containing fraction was centrifuged onto grids for extraction and negative staining, as described in MATERIALS AND METHODS. Immuno-TEM images revealed HMG1 associated with exosomes from immunostimulated (Fig. 7) Caco-2 cells. HMG1 was also found associated with material from nonstimulated Caco-2 cells, but at much lower frequency (data not shown). Interestingly, the greatest signal was observed when exosome preparations were extracted with 0.1% Triton X-100 (Fig. 7B, inset), indicating that some of the HMG1 may be inside the vesicles or otherwise complexed with other secreted material.

To evaluate HMG1 localization within the pellet components without extraction that was necessary to observe the labeling in the negative staining protocol, we ultracytosectioned the 100,000 g pellets and stained ultrathin sections for HMG1. Consistent with the data presented in Fig. 7, HMG1 was found within exosomal vesicles (Fig. 8, arrows), and was associated with other material external to the exosomes (arrowhead in Fig. 8).

To obtain biochemical evidence that HMG1 is secreted in exosomes, we cultured Caco-2 cells growing on inserts in Transwell diffusion chambers for 48 h in the absence or presence of cytokin. Apical supernatants were collected and ultracentrifuged. The resulting supernatant was collected and assayed for the presence of HMG1 by Western blot analysis. The pellets were washed extensively before being assayed for HMG1 and β-actin. Although both unstimulated and immunostimulated Caco-2 cells released HMG1, Western blots of supernatants (before ultracentrifugation) revealed that the cultures incubated with cytokinin released a great deal more of this protein (see the gels labeled “whole medium” in Fig. 9). This finding recapitulates the data presented in Fig. 1. When the particulate fractions from ultracentrifuged Caco-2 supernatants were assayed by Western blot analysis, the samples from unstimulated and cytokinin-stimulated cells contained similar amounts of the cytoskeletal protein, β-actin (see the gels labeled “pellet” in Fig. 9). However, the exosome-containing 100,000 g particulate phase from the cytokinin-stimulated cultures contained large amounts of HMG1, whereas this protein was just barely detectable in the pellet samples from unstimulated Caco-2 cultures. When the soluble fractions from ultracentrifuged culture supernatants were assayed, the samples from cytokinin-stimulated cells contained considerably more HMG1 than did the samples from control cells, but HMG1 was nonetheless clearly present even in the samples from unstimulated cultures (see the gels labeled “supernatant” in Fig. 9). Collectively, these data support the view that under basal conditions, Caco-2 cells release small quantities of HMG1 predominantly in a soluble form, whereas immunostimulated cultures release large quantities of HMG1 both in a soluble form and associated with β-actin-containing particles (presumably exosomes).
HMGB1 secreted by immunostimulated Caco-2 cells amplifies cytomix-induced hyperpermeability in an autocrine fashion. Our laboratory previously reported that HMGB1 is capable of increasing the permeability of Caco-2 monolayers in vitro (30). Accordingly, we hypothesized that HMGB1 released by cytomix-stimulated Caco-2 cells might exacerbate the increase in epithelial permeability induced by the mixture of TNF, IL-1β, and IFN-γ. To test this hypothesis, we incubated Caco-2 monolayers under control conditions or with graded concentrations of polyclonal anti-HMGB1 antibody or with cytomix or with cytomix in the presence of graded concentrations of anti-HMGB1 antibody. As expected, incubating the cells with cytomix significantly increased the permeability of the epithelial sheet to the fluorescent probe, FD4 (Fig. 10). In the absence of cytomix, anti-HMGB1 antibody was without effect, but treatment with the antibody significantly attenuated the development of hyperpermeability induced by the mixture of TNF, IL-1β, and IFN-γ.

DISCUSSION

An important new line of investigation was opened by the seminal discoveries of Wang et al. (38) and Andersson et al. (4), who showed that HMGB1 is not only a DNA binding protein but also a cytokine-like proinflammatory molecule. Because the primary amino acid sequence of HMGB1 is devoid of a signal peptide, secretion of this protein presumably occurs via a nonclassic secretory pathway. Indeed, when monocytes are activated by exposure to LPS, HMGB1 relocalizes from the nucleus into cytoplasmic organelles that belong to the endolysosomal compartment (11). A quantitative analysis carried out Gardella et al. (11) revealed that 65% of HMGB1 is confined to the nucleus in resting monocytes, but only 26% of HMGB1 is nuclear and 74% appears associated with cytoplasmic organelles in LPS-stimulated monocytes. In activated monocytes, the transfer of HMGB1 from the nucleus to the cytoplasm is mediated by hyperacetylation of critical lysine clusters that are components of nuclear localization signals (6).

In the present work, we extended the investigation of HMGB1 secretion by immunostimulated cells to a different cell type, namely enterocytes. Although Kuniyasu et al. (18) recently reported that WiDr human colon cancer cells constitutively release HMGB1 into culture supernatants, we observed only very low levels of this protein in the media of unstimulated Caco-2 transformed human enterocyte-like cells. However, after stimulation of the cells with cytomix, the synthetic Tlr2 ligand, FSL-1, or the Tlr5 ligand, flagellin, we observed a large increase in the amount of HMGB1 released.
into the culture media. Because it is known that HMGB1 is released by necrotic (but not apoptotic) cells (31), it is important that we were able to document that incubating Caco-2 cells with cytomix for 48 h was neither associated with an increase in the number of cells taking up the vital dye, Trypan blue, nor increased release of the intracellular enzyme LDH. These observations confirm findings previously reported by our laboratory, wherein we showed that incubation of Caco-2 cells

Fig. 7. Immunolocalization of HMGB1 to isolated exosomes from the apical medium of 24 h stimulated Caco-2 cells. Washed exosomes were ultracentrifuged onto coated grids and processed for immuno-TEM, as described in MATERIALS AND METHODS. Most of the label is observed on structures the size and shape of exosomes (arrows, inset) but also on material that does not appear to be vesicular (arrowheads). Label is also seen most frequently on samples that have been extracted with 0.1% Triton X-100 (B, inset) vs. those samples not extracted (A).

Fig. 8. Immuno-ultracyro-TEM of 100,000 g pellets isolated from apical medium of 24-h stimulated Caco-2 cells. High-speed pellets were processed for immuno-TEM as described. Sections were stained for HMGB1, followed by 5-nm gold secondary antibodies. Sections of the pellets showed that some HMGB1 was found inside vesicles (V, arrows) as well as on material nonvesicular in nature (arrowheads). This finding is consistent with the result shown in Fig. 7.
with cytomix fails to increase staining with the fluorescent dye, ethidium homodimer-1, which penetrates only into dead cells (29).

Because Kuniyasu et al. (18) showed that colon cancer cells release HMGB1 and Caco-2 cells are also cancer cells, it is noteworthy that we showed that cytomix-stimulated (but not resting) primary murine enterocyte cultures release HMGB1. We isolated and cultured murine enterocytes, using the approach described by Whitehead et al. (40). The cells grown using this method previously were identified as epithelial cells (not fibroblasts) (32, 40). Thus we believe that our findings support the view that immunostimulated enterocytes (and not just colon cancer cells) secrete HMGB1, and the release of this protein by these cells is the result of an active process rather than secondary to cell death.

Data obtained by Gardella et al. (11) support the notion that the secretion of HMGB1 by stimulated monocytes occurs when secretory lysosomes undergo exocytosis. While our data cannot exclude that a similar mechanism is responsible for the secretion of HMGB1 by Caco-2 cells, our findings support the view that one pathway for the release of HMGB1 from these cells depends on the release of exosomes into the extracellular environment on exocytic fusion of multivesicular endosomes with the cell surface. The data supporting this view are biochemical, immunohistochemical, and immuno-ultrastructural in nature. Specifically, we showed that when supernatants from immunostimulated Caco-2 cultures are subjected to ultracentrifugation, HMGB1 can be detected in the exosome-containing pellet, even after being washed extensively. By the same token, HMGB1 can also be detected in the exosome-depleted supernatant from this ultracentrifugation process, suggesting that the protein may be released from Caco-2 cells by more than one process.

Using confocal fluorescence microscopy, we visualized immunoreactive HMGB1 within cytoplasmic vesicles, which were predominantly localized toward the apical ends of cells near regions of cell-cell contact. We also observed punctate surface staining for HMGB1, especially in cells exposed to cytomix. Using immuno-TEM, we showed that Caco-2 cells contain MVBs, a finding that confirms previously reported observations (17, 19). Electron-dense gold particles, indicative of binding of the anti-HMGB1 antibody, were localized within MVBs and smaller vesicles within the cells. Immuno-TEM images also revealed HMGB1 associated with exosomes from cytomix-stimulated Caco-2 cells. Because electron-dense gold particles were most apparent after extracting the exosome preparations with 0.1% Triton X-100, a process that renders the membrane-bound particles permeable to the anti-HMGB1 antibody, our findings suggest that HMGB is probably localized inside the vesicles. The immunohistochemical and immuno-ultrastructural studies, however, clearly revealed the presence of extracellular HMGB1 that was not associated with exosomes. This finding is consistent with Western blot analysis, which shows the presence of HMGB1 in the soluble fraction of ultracentrifuged culture supernatants from cytomix-stimulated Caco-2 cells.

Enterocyte-like cells, including Caco-2 cells, are known to secrete a number of cytokines. In some cases, secretion of these mediators is polarized. For example, the secretion of CC and CXC chemokines by stimulated HT-29, T84, or Caco-2 enterocyte-like cells is predominantly basolateral (10, 15, 20). In this study, we observed that the secretion of HMGB1 by stimulated Caco-2 cells was also polarized, favoring release into the apical rather than the basolateral environment. Because key receptors for HMGB1, such as Tlr2 and Tlr4, are localized to the apical surface of enterocytes (8, 25), our observation that HMGB1 was secreted apically supports the idea that release of this protein might serve an autocrine role to amplify the activation of enterocytes by other factors. This notion is supported by our previously reported (30) observation that HMGB1 is promotes activation of the proinflammatory tran-
sorption factor, NK-κB, in Caco-2 cells and also increases the permeability of Caco-2 monolayers. To specifically test this hypothesis, we stimulated Caco-2 monolayers in the absence or presence of a polyclonal neutralizing anti-HMG1 antibody added to the apical compartment of Transwell chambers. Although failing to completely block the increase in epithelial permeability, the anti-HMG1 antibody significantly blunted the cytokin-induced development of hyperpermeability. Thus secretion of HMG1 may be an important positive feedback phenomenon that promotes the development of intestinal epithelial barrier dysfunction due to inflammation. HMG1 appears to modulate intestinal epithelial barrier function by decreasing the expression of key tight junction proteins, including zonula occludens-1 and occludin (Liu S, Sappington PL, and Fink MP, unpublished observations).

To our knowledge, the data presented here are the first to indicate that exposing (both transformed human and primary murine) enterocyte-like epithelial cells to a variety of proinflammatory stimuli leads to active secretion of HMG1. Our data suggest that the release of HMG1 associated with exosomes is a key mechanism for the secretion of this protein by Caco-2 cells. Finally, our data support the view that HMG1 secretion by IECs may have functional significance, serving to amplify the derangements in barrier function induced by other proinflammatory stimuli.

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