Colon Cancer Cell-Derived High Mobility Group 1/Amphoterin Induces Growth Inhibition and Apoptosis in Macrophages

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High mobility group (HMGB)1/amphoterin is a multifunctional cytokine involved in invasion and metastasis of cancer and in inflammation. To investigate HMGB1/amphoterin effects on macrophages, U937 human monocytic leukemia cells and rat peritoneal and human alveolar macrophages were examined. U937 cells expressed low levels of an HMGB1/amphoterin receptor, receptor for advanced glycation end-products (RAGE), whereas RAGE production was induced in differentiated phorbol 12-myristate 13-acetate (PMA)-U937 cells. Treatment with cultured medium of HMGB1/amphoterin-secreting WiDr human colon cancer cells showed growth inhibition of both U937 and PMA-U937 cells and apoptosis in PMA-U937 cells. The number of PMA-U937 cells was markedly decreased by co-culture with WiDr cells exposed to HMGB1/amphoterin sense S-oligodeoxynucleotide (ODN) in spheroids or monolayers. In contrast, PMA-U937 cells co-cultured with WiDr cells exposed to HMGB1/amphoterin anti-sense S-ODN were preserved in number. PMA-U937 cells exposed to RAGE anti-sense S-ODN were insensitive to WiDr-cultured medium. Recombinant human HMGB1/amphoterin induced growth inhibition in thioglycollate-induced rat peritoneal macrophages, PMA-U937 cells, and human alveolar macrophages, an effect that was abrogated by absorption with anti-HMGB1 antibody. Phosphorylation of JNK and Rac1 was induced in PMA-U937 cells treated with HMGB1/amphoterin. These results suggest that HMGB1/amphoterin induces growth inhibition and apoptosis in macrophages through RAGE intracellular signaling pathway. (*Am J Pathol* 2005, 166:751–759)

Apoptosis of macrophages is a specialized event, which is involved in the acceleration of inflammatory processes and in atherosclerosis.^{1,2} Several mechanisms underlying apoptosis in macrophages have been reported. In sepsis, endotoxin (lipopolysaccharide, LPS) plays a key role in macrophage-associated inflammation.^{1,3} Endotoxin is a strong activator of macrophages-derived nitric oxide (NO) production.⁴ Extremely high NO levels induce apoptosis and necrosis in macrophages.^{5,6} In atherosclerosis, cholesterol accumulation in the endoplasmic reticulum evokes an unfolded protein response in macrophages infiltrating the arterial intima.² Unfolded protein response triggers the apoptotic process in macrophages and accelerates atherosclerosis. In addition to NO and cholesterol accumulation, various factors including cell wall lipoprotein of phagocytosed bacteria,⁷ heavy metals,⁸ extracellular adenosine triphosphate,⁹ and ceramide from cell membrane sphingomyelin,⁵ induce apoptosis in macrophages.

In cancer, the activation of macrophages is suppressed, and tumor-promoting activity induced by the production of vascular endothelial growth factor¹⁰ and platelet-derived endothelial cell growth factor.¹¹ We reported that macrophage infiltration is significantly reduced in metastatic colon cancer, which produces high mobility group 1 (HMGB1)/amphoterin.¹² HMGB1/amphoterin plays dual roles as both a chromatin structural protein (HMGB1) and a cytokine (amphoterin).^{1,13} Secreted HMGB1/amphoterin induces cancer cell growth, motility, and invasion, and accelerates cancer metastasis via activation of a specific membrane receptor (RAGE, receptor for advanced glycation end products) with predominant intracellular signaling via Rac1/Cdc42.^{14–18} HMGB1/amphoterin also acts as anti-apoptotic factor in cancer cells by inducing the expression of Bcl-2.¹⁹ In the present study, we report a proapoptotic role of HMGB1/

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amphoterin in macrophages, which has an inhibitory effect on cancer cells. These findings contribute to our understanding of the mechanism of cancer cell escape from host immunity and macrophage-associated acceleration of inflammation.

Materials and Methods

Cell Culture

WiDr colon carcinoma cells were obtained from the Japanese Cancer Research Resources Bank. U937 monocytic leukemia cell line was purchased from Dainihon Pharmaceutical Co., Tokyo, Japan. All cell lines were maintained in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) containing 10% fetal bovine serum (FBS) (Sigma Chemical Co.) under conditions of 5% CO₂ in air at 37°C. Macrophage differentiation of U937 cells was induced by incubation with 10 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma Chemical Co.)²⁰ for 5 days, after which floating cells were removed by rinsing with phosphate-buffered saline (PBS). Differentiated U937 cells (PMA-U937 cells) attached to the dishes were used in further studies.

Anti-Sense Phosphorothioate (S)-Oligodeoxynucleotide Assay

An 18-mer S-oligodeoxynucleotide (ODN) composed of the anti-sense sequence of nucleotides 6 to 23 of RAGE cDNA (GenBank AB036432) and an 18-mer S-ODN composed of the anti-sense sequence of nucleotides 1 to 18 of HMGB1/amphoterin cDNA (GenBank X12597) were synthesized and purified by reverse-phase high-performance liquid chromatography (Espec Oligo Service, Tsukuba, Japan). The sequence of the RAGE anti-sense ODN was 5'-CTG CTT CCT TCC AGG GTC-3', and the sequence of the HMGB1/amphoterin anti-sense ODN was 5'-AGG ATC TCC TTT GCC CAT-3'. Sense sequence 18-mers were used as negative controls. Cells were pretreated with 3 μ mol/L anti-sense or sense S-ODN for 6 days, with medium exchange and addition of anti-sense or sense S-ODN every 2 days. The cells were then used in experiments.

Preparation of Conditioned Medium

For immunoblot analysis, cells were cultured in RPMI 1640 medium containing 10% FBS for 2 days. The conditioned medium was filtered through a 0.2- μ m filter (Becton-Dickinson Labware, Bedford, MA), and precipitated with acetone. The pellet was dissolved in lysis buffer [50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 5 mmol/L ethylenediaminetetraacetic acid (EDTA), 50 μ g/ml phenylmethyl sulfonyl fluoride, 1 μ g/ml aprotinin, 0.5% Nonidet P-40] to a concentration 50-fold that of the original medium.

Immunoblot Analysis

Whole-cell lysates were prepared as described previously.²¹ Fifty- μ g of lysates were subjected to immunoblot analysis in 12.5% sodium dodecyl sulfate-polyacrylamide gels followed by electrotransfer to nitrocellulose filters. The filters were incubated with primary antibody and then with peroxidase-conjugated IgG antibody (Medical and Biological Laboratories, Nagoya, Japan). An α -tubulin antibody was used to assess the levels of protein loaded per lane (Oncogene Research Products, Cambridge, MA). The immune complex was visualized with an enhanced chemiluminescence-Western blot detection system (Amersham, Aylesbury, UK). Primary antibodies included anti-RAGE antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-HMGB1 antibody (Upstate Biotechnology, Inc., Lake Placid, NY), and anti-phospho-Rac1 and anti-phospho-JNK antibodies (Santa Cruz Biotechnology). For semiquantitative analysis, specific signals on immunoblotted membranes were digitized and quantified with NIH Image computer software (National Institutes of Health, Bethesda, MD).

Cell Growth

U937 cells were harvested from floating cultures. PMA-U937 cells were harvested from monolayer cultures by brief treatment with 0.1% trypsin and 0.1% EDTA (Sigma Chemical Co.). U937 cells were seeded at a density of 10,000 cells per well in 12-well tissue culture plates. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma Chemical Co.] was added to the culture medium at a concentration of 25 μ g/ml at 1 hour before harvest. Harvested cell pellets were lysed with 1 ml of dimethyl sulfoxide, and 200 μ l of the lysate were examined at 540 nm. PMA-U937 cells were seeded at a density of 2000 cells per well in 96-well tissue culture plates. After cells were exposed to MTT for 1 hour before harvest, chromogenic granules from the cells were dissolved in dimethyl sulfoxide and subjected to examination at 540 nm. The experiments were performed in triplicate.

Spheroid Infiltration Assay

WiDr cells (5×10^5) treated with HMGB1/amphoterin anti-sense or sense S-ODN were grown in six-well plates coated with 1% agarose (Sigma Chemical Co.) and incubated at 37°C in 5% CO₂.²² After 24 hours, multicellular spheroids (300 to 500 μ m in diameter) were co-cultured with PMA-U937 cells, surface-labeled with PKH26 chemifluorescent dye (Zynaxis, Inc., Malvern, PA). Infiltration of labeled cells was observed at 480 nm with a fluorescence microscope. Non-HMGB1/amphoterin-secreting IEC6 cells were used as a negative control.

Monolayer Co-Culture

WiDr cells (5×10^4) treated with HMGB1/amphoterin anti-sense or sense S-ODN were mixed with PMA-U937 cells (1 times 10^4) surface-labeled with PKH26 chemi-

fluorescent dye (Zynaxis, Inc.) and seeded in each well of a six-well tissue culture plate. Cells were observed at 480 nm with a fluorescence microscope.

Monolayer Attachment Assay

WiDr cells treated with HMGB1/amphoterin anti-sense or sense S-ODN were seeded at a density of 5×10^4 cells per well in six-well tissue culture plates. When the cells were subconfluent, 1×10^4 PMA-U937 cells surface-labeled with PKH26 chemifluorescent dye (Zynaxis, Inc.) were added. Cells were observed at 480 nm with a fluorescence microscope. In co-culture assays mentioned above, each experiment was repeated three times. Density of PMA-U937 cells was quantitated by the chemifluorescence (Fluoroimage Analyzer; Fujifilm, Tokyo, Japan).

Transwell Cell Layer Infiltration Assay

A modified Boyden chamber with a type IV collagen-coated insert (pore size, 3 μ m; diameter, 5 mm; Becton-Dickinson Labware) was used for macrophage infiltration assay. In the upper chamber (insert), WiDr cells treated with HMGB1/amphoterin anti-sense or sense S-ODN were seeded at a density of 2×10^4 cells per well and cultured overnight to form a multicell layer on the bottom membrane. PMA-U937 or U937 cells (2×10^4 cells per well) surface-labeled with PKH26 chemifluorescent dye (Zynaxis, Inc.) were then seeded in the upper chamber. After 12 hours, fluorescence-positive cells in the lower chamber were counted by an autocytofluorometer (Sysmecs, Kobe, Japan). Fluorescence-positive cells also were detected by fluorescence microscope in cell smear specimens.

Assessment of Apoptosis

Apoptotic cells were detected by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labeling (TUNEL method). Paraffin sections were dewaxed in xylene followed by rinsing in a graded ethanol series in PBS. The sections were digested by proteinase K (20 mg/ml) (Sigma Chemical Co.) for 30 minutes at 37°C followed by three rinses with PBS. dUTP-biotin (Boehringer-Mannheim, Mannheim, Germany) was labeled by terminal deoxynucleotidyl transferase reaction (Boehringer-Mannheim) in cacodylate buffer for 60 minutes at 37°C followed by three rinses with PBS. After blocking with 5% normal horse serum and 1% normal goat serum, the sections were incubated with a 1:50 dilution of a fluorescein isothiocyanate-conjugated streptavidin (DAKO Corp., Carpinteria, CA) for 30 minutes at room temperature. Cell nuclei were counterstained with propidium iodide. Staining was observed by fluorescence microscopy. The percent frequency of TUNEL-positive cells was calculated from the ratio of positive nuclei to 200 examined nuclei.

Extraction of HMGB1/Amphoterin

HMGB1/amphoterin was extracted from WiDr cells according to the method of Wagner and colleagues.²³ Harvested WiDr cells were suspended in hypotonic buffer

[0.1 mmol/L piperazine-*N,N'*-bis(2-ethane sulfonic acid) (PIPES), pH 6.5, 5 mmol/L CaCl_2 , 5 mmol/L dithiothreitol, 0.5% Triton X-100, 0.5% Nonidet P-40], and centrifuged at $1000 \times g$. Cell pellets were resuspended in extraction buffer [0.35 mol/L NaCl, 20 mmol/L Tris-HCl, pH 7.2, 12 mmol/L MgCl_2 , 5 mmol/L ethylene glycol bis(2-aminoethyl ether)tetraacetic acid (EGTA), 5 mmol/L dithiothreitol] and centrifuged at $10,000 \times g$ to collect the supernatant. The supernatant was passed through a PBE94 column (Amersham Biosciences Corp., Piscataway, NJ) equilibrated with equilibration buffer (20 mmol/L Tris-HCl, pH 7.5, 0.35 mol/L NaCl, 5 mmol/L dithiothreitol) and eluted with elution buffer (20 mmol/L Tris-HCl, pH 7.5, 5 mmol/L dithiothreitol) with a NaCl gradient from 0.35 mol/L to 1 mol/L. Eluates were dialyzed against PBS overnight. For examination of inhibitory effect on macrophages, extracted HMGB1 (0.75 mol/L salt eluate) was concentrated to 20 μ g/ml for 72 hours. We also used recombinant human HMGB1 tagged with glutathione S-transferase (GST) (rhHMGB1; Abnova Corp., Taiwan, ROC). The nucleotide and amino acid sequences of human HMGB1 was referred to GenBank BC003378 and UniGene Hs.434102, respectively. Protein was generated by wheat germ cell-free *in vitro* expression system with GST tag (CellFree Sciences Co. Ltd., Japan) and purified by Glutathione Sepharose 4 Fast Flow.

Neutralization of HMGB1/Amphoterin

For absorption of HMGB1/amphoterin, WiDr-conditioned medium (500 μ l) or extracted HMGB1/amphoterin (50 μ g) were mixed with 10 μ g of anti-HMGB1 antibody (Upstate Biotechnology, Inc.), and incubated at 37°C for 1 hour. HMGB1-antibody complexes were removed by A/G agarose (Santa Cruz Biotechnology, Inc.). Rabbit serum (DAKO) was used as a control.

Rat Peritoneal Macrophages

Six-week-old male, Fisher 344 rats (Japan SLC, Inc., Shizuoka, Japan) were injected intraperitoneally with 3 ml of 10% thioglycollate (Becton-Dickinson Microbiology Systems, Sparks, MD). After 3 days, the rats were killed, and the peritoneal cavities were washed with cold PBS to collect infiltrated macrophages. The lavages were centrifuged, and the pellets were resuspended in 10% FBS-supplemented RPMI 1640 medium. Suspended cells were cultured overnight. Adherent cells were reseeded at 5×10^4 cells per well in 24-well dishes.

Human Alveolar Macrophages

After obtaining their informed consent, bronchoalveolar lavage was performed on healthy nonsmoking volunteers.²⁴ Briefly, after anesthetizing the oral cavity and the upper airway with lidocaine spray, the tip of an Olympus fiberoptic bronchoscope (1T20; Olympus Co., Tokyo, Japan) was wedged into one of the segments of the right middle lobe. The lung was washed with 50 ml of sterilized saline (0.9% NaCl) prewarmed to 37°C, and the fluid was

gently sucked out with a 50-ml syringe. This procedure was repeated three times. A total of 150 ml of saline was instilled, of which ~65% was recovered. The lavage cells were passed through sterilized gauze and were washed twice with RPMI 1640 medium. The yield of human alveolar macrophages from normal volunteers was $\sim 1.8 \times 10^7$ viable cell/wedge segment. More than 93% were viable, as determined by trypan blue dye exclusion, and more than 89% of the obtained cells were alveolar macrophages, as revealed by DiffQuik staining. The cells were resuspended in RPMI medium supplemented with 10% FBS. The cells (5×10^4 /well) were plated for 1 hour in 96-well Microtest III plates, and then nonadherent cell were removed by washing with PBS. At this point, >99% of the adherent cells were identified to be alveolar macrophages, as judged by their morphology, and were used as alveolar macrophages monolayer. Proliferation of alveolar macrophages was measured by the MTT dye reduction method.²⁵ Purified AM (5×10^4)/50 μ l plated into 96-well plates in RPMI medium supplemented with 10% FBS were added samples (50 μ l) and incubated for 71 hours. Fresh RPMI medium supplemented with 10% FBS (100 μ l) was added to the culture. After this, 50 μ l of stock MTT solution (2 mg/ml; Sigma) was added and incubated for 1 hour. The medium containing MTT solution was removed and the residual dark blue crystals dissolved in 100 μ l of dimethyl sulfoxide. Absorbance was measured using an MTP-120 microplate reader (Corona Electric, Ibaraki, Japan) at test and reference wavelengths of 550 and 630 nm, respectively.

Mobility Shift Assay

A nuclear factor (NF) κ B-consensus oligonucleotide (5'-AGT TGA GGG GAC TTT CCC AGG C-3', Santa-Cruz) was end-labeled with γ -³²P-ATP by polynucleotide kinase. Nuclear extract (5 μ g), poly dI-dC (1 μ g), and labeled oligonucleotide (0.5 ng) were incubated in reaction buffer (10 mmol/L Tris-HCl, pH 7.5, 50 mmol/L NaCl, 1 mmol/L dithiothreitol, 1 mmol/L EDTA, 5% glycerol) for 20 minutes at room temperature. To detect an antibody supershift, prepare reaction mixture as described above, adding 2 μ l of anti-NF- κ Bp65 antibody (Santa-Cruz) per 20 μ l of reaction volume and incubate at room temperature for 45 minutes. DNA-protein complexes were resolved by electrophoresis through a 4% polyacrylamide gel containing 50 mmol/L Tris, pH 7.5, 0.38 mol/L glycine, and 2 mmol/L EDTA. The gel was dried and autoradiographed.

Inhibition of iNOS

PMA-U937 cells or thioglycollate-induced rat peritoneal macrophages were seeded at 2×10^4 cells per well in 24-well culture dishes. Cells treated with 20 μ g/ml of the 0.75 mol/L salt fraction were co-treated with *N* ω -nitro-L-arginine methyl ester (L-NAME, Sigma Chemical Co.) at 0, 4, 8, and 25 μ mol/L in triplicate. As a control, cells were treated with the same amount of dimethyl sulfoxide without L-NAME. After 72 hours, cell numbers were quantified.

Figure 1. Production of RAGE and HMGB1/amphoterin in U937 cells and PMA-U937 cells. Protein expression of RAGE was examined in U937 and PMA-U937 cell lysates, by immunoblotting (**top**). α -Tubulin was used as a loading control. HMGB1/amphoterin in culture medium was examined by immunoblotting (**bottom**). Coomassie blue staining served as a loading control. WiDr HMGB1/amphoterin-AS, WiDr cells exposed to HMGB1/amphoterin anti-sense S-ODN; PMA-U937 RAGE-AS, PMA-U937 cells exposed to RAGE anti-sense S-ODN.

Nitrite Concentration

For assessing NO production by cells, 1×10^6 cells were seeded with 1 ml of regular medium and treated with reagents described in the Results section for 24 hours. For positive control, LPS (Sigma) was used for treatment. Cultured medium was mixed with the same volume of Griess solution (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, 2.5% phosphoric acid). As a control, 100 μ mol/L of sodium nitrite (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was diluted serially. After a 30-minute incubation at room temperature, the optical density of each mixture was measured at 540 nm. The nitrite concentration was calculated from a standard curve of serially diluted nitrite. The detectable range of this method is 2.0 to 250 μ mol/L. Each sample was tested in triplicate.

Statistical Analysis

Statistical significance was examined by the two-tailed Fisher's exact test, the two-tailed χ^2 test, and the two-tailed, unpaired Mann-Whitney test by using InStat software (Graphpad Software, Los Angeles, CA). Statistical significance was defined as a two-sided *P* value of less than 0.05.

Results

Expression of RAGE and HMGB1/Amphoterin in U937 and PMA-U937 Cells

RAGE protein was expressed in PMA-U937 cells, whereas U937 cells produced very low levels of RAGE (Figure 1). HMGB1/amphoterin secretion into the culture medium was not detected in U937 cells, whereas PMA-U937 cells secreted HMGB1/amphoterin at low levels. WiDr colon cancer cells produced RAGE and secreted HMGB1/amphoterin. HMGB1/amphoterin secretion by WiDr cells was suppressed by exposure to HMGB1/am-

Figure 2. Effect of WiDr colon cancer cell culture medium on U937 and PMA-U937 cells. **a** and **b**: U937 and PMA-U937 cells were treated with cultured media of WiDr cells (WiDr-CM) or WiDr cells exposed to HMGB1/amphotericin anti-sense S-ODN (HMGB1-AS WiDr-CM) or HMGB1/amphotericin sense S-ODN (HMGB1-S WiDr-CM). U937 or PMA-U937 cells exposed to RAGE anti-sense S-ODN (RAGE-AS U937 or RAGE-AS PMA-U937) or RAGE sense S-ODN (RAGE-S U937 or RAGE-S PMA-U937) were also treated with cultured media. Error bars indicate SD. **c**: Effect of WiDr-cultured medium on growth of U937 and PMA-U937 cells. Cells were treated with WiDr-cultured medium for 72 hours (CM). Cells were also treated with WiDr-cultured medium pretreated with anti-HMGB1 antibody (Ab). Rabbit serum was used as a negative control for neutralizing antibody assay (serum). **d**: Apoptosis of PMA-U937 cells treated with WiDr-cultured medium were detected by Giemsa staining and the TUNEL method. **e**: The ratio of apoptotic cells was calculated for PMA-U937 cells treated with cultured media of WiDr cells exposed to HMGB1/amphotericin anti-sense S-ODN (anti-sense) or HMGB1/amphotericin sense S-ODN (sense). Error bars indicate SD.

photerin anti-sense S-ODN. RAGE production by PMA-U937 cells was suppressed by exposure to RAGE anti-sense S-ODN.

Effect of WiDr-Conditioned Medium on Growth of U937 and PMA-U937 Cells

As shown in Figure 2a, U937 cells treated with WiDr-conditioned medium showed inhibition of cell growth (28% the level of untreated cells). Inhibition was reversed by decreasing the HMGB1/amphotericin in the conditioned medium by treatment of WiDr cells with HMGB1/amphotericin anti-sense S-ODN or by repression of RAGE in U937 cells with RAGE anti-sense S-ODN. PMA-U937 cells, which show more RAGE expression than do U937 cells, showed reduced cell growth by treatment with WiDr-conditioned medium (Figure 2b). Next, U937 and PMA-U937 cells were treated with WiDr-conditioned medium pretreated with or without anti-HMGB1/amphotericin antibody (Figure 2c). WiDr-conditioned medium without

Figure 3. Effect of co-culture of WiDr cells and U937 cells or PMA-U937 cells. **a-d**: Multicellular spheroids consisting of chemiluminescence-labeled PMA-U937 or U937 cells mixed with WiDr cells exposed to HMGB1/amphotericin anti-sense S-ODN (HMGB1-AS) or HMGB1/amphotericin sense S-ODN (HMGB1-S) were observed by fluorescence microscopy. **e** and **f**: Monolayer co-culture of PMA-U937 cells with WiDr cells. Chemiluminescence-labeled PMA-U937 cells mixed with WiDr cells exposed to HMGB1/amphotericin anti-sense S-ODN (HMGB1-AS) or HMGB1/amphotericin sense S-ODN (HMGB1-S) were seeded onto culture dishes. **g** and **h**: Monolayer attachment assay of PMA-U937 cells with WiDr cells. Chemiluminescence-labeled PMA-U937 cells were seeded onto a monolayer of WiDr cells exposed to HMGB1/amphotericin anti-sense S-ODN (HMGB1-AS) or HMGB1/amphotericin sense S-ODN (HMGB1-S). **i-m**: Transwell cell layer infiltration assay. Chemiluminescence-labeled PMA-U937 cells or U937 cells were seeded onto multiple cell layers of WiDr cells on the bottom of the upper chamber of a modified Boyden chamber. HMGB1-AS, WiDr cells exposed to HMGB1/amphotericin anti-sense S-ODN; HMGB1-S, WiDr cells exposed to HMGB1/amphotericin sense S-ODN. **i-l**: Smears of chemiluminescence-labeled PMA-U937 cells or U937 cells were observed by fluorescence microscopy. **m**: Chemiluminescence-positive cells infiltrating beyond the multiple cell layer were counted. Bright cells indicate chemiluminescence-labeled cells. Indicated numbers in **a-h** were fluorescent intensities. Fluorescent intensities of cells treated with anti-sense S-ODN were set to 100%. Average $\pm$ SD.

pretreatment provided growth inhibition in both U937 and PMA-U937 cells. In contrast, WiDr-conditioned medium pretreated with anti-HMGB1/amphotericin antibody did not show relevant growth inhibition.

PMA-U937 cells treated with WiDr-conditioned medium showed many apoptotic changes (Figure 2d). The reduction of PMA-U937 cell growth was reversed by pretreatment of WiDr cells with HMGB1/amphotericin anti-sense S-ODN or pretreatment of PMA-U937 cells with RAGE anti-sense S-ODN. The numbers of apoptotic PMA-U937 cells in cultures treated with conditioned medium of WiDr cells treated with HMGB1/amphotericin sense S-ODN were four times greater than those of cells treated with conditioned medium of WiDr cells treated with HMGB1/amphotericin anti-sense S-ODN (Figure 2e).

Co-Culture of PMA-U937 and WiDr Cells

We examined the effect of physical association between PMA-U937 or U937 cells and WiDr cells. We first examined spheroid co-culture of PMA-U937 or U937 cells with WiDr cells (Figure 3; a to d). The number of chemiluminescence-labeled PMA-U937 cells was significantly decreased in spheroids with HMGB1/amphotericin sense S-ODN-treated WiDr cells ($P < 0.0001$), whereas the number was retained in spheroids with HMGB1/amphotericin anti-sense S-ODN-treated WiDr cells. In contrast,

U937 cells, which express lower levels of RAGE than do PMA-U937 cells, showed less of a decrease in spheroids with HMGB1/amphotericin sense S-ODN-treated WiDr cells than that of PMA-U937 cells ($P = 0.7057$; Figure 3, c and d).

Monolayer co-culture of PMA-U937 cells with WiDr cells and co-culture of PMA-U937 cells on a WiDr cell monolayer (monolayer attachment assay) showed a significant decrease in chemiluminescence-positive PMA-U937 cells ($P = 0.0003$ and $P = 0.0001$, respectively) (Figure 3, f and h). In contrast, PMA-U937 cells co-cultured with WiDr cells exposed to HMGB1/amphotericin did not show a decrease (Figure 3, e and g). The number of remaining PMA-U937 cells in monolayer co-culture and in the monolayer attachment assay with WiDr cells exposed to HMGB1 sense S-ODN were 27% and 16%, respectively, of those in monolayer with WiDr cells exposed to HMGB1 anti-sense S-ODN.

Infiltration of PMA-U937 cells into a WiDr cell layer was examined by transwell cell layer infiltration assay. Chemiluminescence-labeled PMA-U937 cells infiltrated into the lower chamber through a multiple cell layer of WiDr cells formed on a type IV collagen-coated membrane in the bottom of the upper chamber (Figure 3; i to m). Chemiluminescence-positive cells infiltrating through WiDr cells exposed to HMGB1/amphotericin sense S-ODN were markedly decreased compared to those infiltrating through WiDr cells exposed to HMGB1/amphotericin anti-sense S-ODN (Figure 3, i and j). The number of PMA-U937 cells infiltrating WiDr cells exposed to HMGB1/amphotericin anti-sense S-ODN was 568 ± 48 (Figure 3m). In contrast, the number of PMA-U937 cells infiltrating WiDr cells exposed to HMGB1/amphotericin sense S-ODN was 6582 ± 443 ($P < 0.0001$). A decrease in infiltrating U937 cells through WiDr cells exposed to HMGB1/amphotericin sense S-ODN was also found compared to those infiltrating WiDr cells exposed to HMGB1/amphotericin anti-sense S-ODN ($P < 0.05$; Figure 3; k, l, and m).

Biological Effect of Extracted HMGB1/Amphotericin

We extracted HMGB1/amphotericin from U937 cells according to the method of Wagner and colleagues.²³ The HMGB1/amphotericin content in each fraction of each salt concentration was examined by immunoblotting (Figure 4a). In the 0.75 mol/L salt fraction, a strong HMGB1/amphotericin signal was detected. In the 0.7 mol/L salt fraction, a weak signal was detected, showing an intensity 8% of that of the 0.75 mol/L salt fraction. From the results, the 0.75 mol/L salt fraction was the major fraction of HMGB1/amphotericin, similar to the results of Wagner and colleagues.²³

Extracts of each salt concentration were concentrated to 20 $\mu\text{g/ml}$ and used to examine the inhibitory effect on macrophage growth (Figure 4b). Cells treated with the 0.75 mol/L salt fraction showed maximal suppression of cell growth. The numbers of U937 and PMA-U937 cells were 52% and 26% of those of untreated cells, respectively. The 0.7 mol/L salt fraction showed weak inhibitory

Figure 4. Effect of extracted HMGB1/amphotericin on PMA-U937 cells and thioglycollate-induced rat peritoneal macrophages. **a:** HMGB1/amphotericin content in nuclear extracts from U937 cells. Each fraction of the nuclear extract eluted by a salt gradient was subjected to Western blot analysis with anti-HMGB1 antibody. Coomassie blue staining served as a control for loading. **b:** Effect of each fraction of the nuclear extract on cell growth of U937 and PMA-U937 cells. Cells were treated with 20 $\mu\text{g/ml}$ of each fraction for 72 hours. The cell number was standardized to that of nontreated U937 or PMA-U937 cells at 100%. Error bars indicate SD. **c:** Effect of various concentrations of the 0.75 mol/L salt fraction of nuclear extract on growth of U937 and PMA-U937 cells. Cells were treated with the indicated concentrations of the 0.75 mol/L fraction for 72 hours. The cell number was standardized to that of untreated U937 or PMA-U937 cells at 100%. Error bars indicate SD. **d:** Western blot analysis of GST-tagged recombinant human HMGB1 (rhHMGB1). **Lane 1**, 0.75 mol/L salt fraction; **lane 2**, rhHMGB1. **e:** Effect of various concentrations of the 0.75 mol/L salt fraction of nuclear extract and rhHMGB1 on growth of thioglycollate-induced rat peritoneal macrophages. Cells were treated with 0.75 mol/L salt fraction or rhHMGB1 of indicated concentrations for 72 hours. The cell number was standardized to that of untreated rat peritoneal macrophages at 100%. Error bars indicate SD. **f** and **g:** Effect of rhHMGB1 on growth of PMA-U937 cells and human alveolar macrophages. Cells were treated with various concentration of rhHMGB1 for 72 hours. Cells were also treated with rhHMGB1 pretreated with anti-HMGB1 antibody (Ab). Error bars indicate SD.

effects (92% in U937 cells and 77% in PMA-U937 cells). Various concentrations of the 0.75 mol/L salt fraction were used to examine the macrophage inhibitory effect of HMGB1/amphotericin (Figure 4c). In both U937 and PMA-U937 cells, decreased cell numbers were found in a concentration-dependent manner.

We also used rhHMGB1 protein to confirm the macrophage inhibitory effect of HMGB1/amphotericin (Figure 4, d and e). Thioglycollate-induced rat peritoneal macrophages were treated with various concentrations of the 0.75 mol/L salt fraction or rhHMGB1. After 72 hours, the number of treated cells showed cell growth inhibition with

a dose-dependent manner. PMA-U937 cells and human alveolar macrophages were also treated with rhHMGB1 (Figure 4, f and g). PMA-U937 cells and human alveolar macrophages showed cell growth inhibition with a dose-dependent manner. Human alveolar macrophages were more sensitive to rhHMGB1. Neutralization of rhHMGB1 by pretreatment with anti-HMGB1 antibody abrogated growth inhibition by rhHMGB1.

Effect of HMGB1/Amphotericin on Phosphorylation of the MAPK Signal Pathway and NO Production in PMA-U937 Cells

HMGB1/amphotericin stimulates the MAPK pathway through RAGE. We examined the phosphorylation status of JNK and Rac1 in PMA-U937 cells treated with the 0.75 mol/L salt fraction (20 μ g/ml) (Figure 5a). The level of phosphorylated Rac1 was increased by treatment with the 0.75 mol/L salt fraction with a peak at 15 minutes after treatment (287% of control). The level of phosphorylated JNK was increased by treatment with the 0.75 mol/L fraction, with a peak at 60 minutes after treatment (336% of control). In contrast, total protein levels of JNK and Rac1 were not affected by the treatment. In U937 cells, increased phosphorylation of JNK and Rac1 was albeit, at lower levels than that in PMA-U937 cells. Protein levels of NF- κ B were not affected by HMGB1/amphotericin treatment in PMA-U937 or U937 cells. In contrast, mobility shift assay using NF- κ B consensus oligonucleotides showed increment of DNA-binding NF- κ B in rhHMGB1-treated PMA-U937 cells (Figure 5b).

To explore one possible mechanism, we tested the concept that HMGB1-mediated macrophage apoptosis via NO. In Figure 5c, we examined the effect of HMGB1/amphotericin on NO production. HMGB1/amphotericin induced NO production in PMA-U937 cells and thioglycollate-induced rat peritoneal macrophages. The nitrite concentration in the cultured medium showed low levels of increase in rat peritoneal macrophages treated with HMGB1/amphotericin in a dose-dependent manner. HMGB1/amphotericin induced less effect of NO product in PMA-U937 cells than that in rat peritoneal macrophages. HMGB1/amphotericin-induced NO production was significantly lower than that induced by LPS in both PMA-U937 cells and rat peritoneal macrophages. L-NAME treatment did not abrogate cell growth inhibition in HMGB1/amphotericin-treated PMA-U937 cells or rat peritoneal macrophages (Figure 5d). These data indicate that likely NO-independent mechanisms are responsible for the HMGB1 effect on macrophage cell death.

Discussion

In the present study, we showed induction of apoptosis by HMGB1/amphotericin in macrophages. HMGB1/amphotericin was recently recognized as a cytokine that participates in cancer progression and inflammation. The fact that HMGB1/amphotericin induces apoptosis in macrophages provides a novel association of macrophages with cancer.

Figure 5. Effect of HMGB1/amphotericin on phosphorylation of intracellular signaling pathways and NO production. **a:** Cell lysates of U937 and PMA-U937 cells were subjected to Western blot analysis to show the total and phosphorylation levels of Rac1 and JNK, and NF- κ B protein. α -Tubulin was used as a loading control. **b:** Mobility shift assay of NF- κ B oligonucleotide. Nuclear extracts of PMA-U937 cells treated with or without rhHMGB1 were incubated with NF- κ B consensus oligonucleotide and electrophoresed in 4% polyacrylamide gel. Incubation added by anti-NF- κ Bp65 antibody showed a super shift band (lane 3, arrow). **c:** Nitrite concentration in cultured media of PMA-U937 cells or thioglycollate-induced rat peritoneal macrophages treated with HMGB1/amphotericin or LPS. Cells (1×10^6) were treated with the indicated concentration of 0.75 mol/L fraction or LPS for 24 hours and the nitrite concentration of the cultured media was measured by the Griess method. Error bars indicate SD. **d:** PMA-U937 cells and thioglycollate-induced rat peritoneal macrophages treated with 20 μ g/ml of the 0.75 mol/L fraction were co-treated with various concentrations of L-NAME for 72 hours. Cell numbers are represented as the percentage of nontreated cells. Error bars indicate SD.

When PMA-U937 cells were treated with HMGB1/amphotericin, phosphorylation levels of Rac1 and JNK were increased. Rac1/Cdc42 is reported to be a major intracellular signaling pathway of RAGE.¹⁸ In cancer cells or outgrowing neurites, Rac1/Cdc42 phosphorylation provides integration of cytoskeleton and formation of cytomembranous ruffling, resulting in activated cell mobility.^{18,26} Rac1/Cdc42 is also known to participate in cell survival and in apoptosis.²⁷ The bidirectional role of Rac1/Cdc42 depends on cell type. In fibroblasts, endothelial cells, and epithelial cells, Rac1/Cdc42 acts in an anti-apoptotic manner,^{28–32} whereas Rac1/Cdc42 induces apoptosis in myoblasts.³³ In U937 cells, a dominant-negative mutant of Rac1 abrogates tumor necrosis

factor- α -induced apoptosis.³⁴ Rac1/Cdc42 is reported to associate with activation of p38MAPK and JNK/SAPK.²⁷ p38MAPK and JNK/SAPK transmit multifunctional signals. However, JNK/SAPK is involved in Fas/tumor necrosis factor and ceramide-induced apoptotic pathways.³⁵ Apoptosis is induced by ceramide in macrophages through the JNK/SAPK pathway.⁵ In myoblasts or Rac1-overexpressing NIH3T3 cells, JNK/SAPK is associated with apoptotic signals transmitted by Rac1/Cdc42.^{33,36} In the present study, we confirmed that phosphorylation levels of Rac1 and JNK/SAPK were increased in PMA-U937 cells treated with HMGB1/amphotericin-containing WiDr-conditioned medium.

We also examined NF- κ B protein levels and the DNA binding in HMGB1-treated PMA-U937 cells. NF- κ B protein level in whole cell lysate of HMGB1-treated PMA-U937 cells was not significantly different from that in untreated cells. In contrast, binding of NF- κ B consensus oligonucleotide with NF- κ B protein in the nuclear extract was increased by HMGB1 treatment. Because NF- κ B is known to generate a survival signal to cells, HMGB1 provides contradirectional effects on cells; proapoptotic effect via Rac1/Cdc42 or JNK, and anti-apoptotic effect via NF- κ B. Total effect might be affected by the balance of proapoptotic and anti-apoptotic signals, and other stimulants involving cytokines and growth factors.

NO produced by activated macrophages injures macrophages themselves and induces cell death.⁶ We confirmed the effects of NO produced in HMGB1/amphotericin-treated macrophages. In both PMA-U937 cells and thioglycollate-induced rat peritoneal macrophages, the nitrite concentration in the conditioned media was not high. HMGB1/amphotericin-treated PMA-U937 cells and rat peritoneal macrophages produced significantly lower levels of the nitrite in LPS-treated cells (Figure 5). Moreover, iNOS inhibition by L-NAME did not reverse growth inhibition of these cells in response to HMGB1/amphotericin treatment. These findings suggest that HMGB1/amphotericin is a weak stimulant for NO production in macrophages and does not induce cell death by NO.

HMGB1/amphotericin expression is up-regulated in cancer cells.^{15–18} HMGB1/amphotericin-induced macrophage apoptosis might affect host immunity against cancer. We previously found that macrophage infiltration into cancers is suppressed in HMGB1/amphotericin-producing colon cancer.¹² This finding indicates that cancer cell-produced HMGB1/amphotericin induces apoptosis in macrophages infiltrating into cancers. Thus, HMGB1/amphotericin may provide cancer cells the advantages of cancer progression and suppression of host immunity. Further examination of the roles of macrophage apoptosis induced by HMGB1/amphotericin in cancer may provide novel therapeutic targets in these diseases.

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