High mobility group box 1 (HMGB1) protein plays multiple roles in transcription, replication, and cellular differentiation. HMGB1 is also secreted by activated monocytes and macrophages and passively released by necrotic or damaged cells, stimulating inflammation. HMGB1 is a novel antigen of anti-neutrophil cytoplasmic antibodies (ANCA) observed in the sera of patients with ulcerative colitis and autoimmune hepatitis, suggesting that HMGB1 is secreted from neutrophils to the extracellular milieu. However, the actual distribution of HMGB1 in the cytoplasm of neutrophils and the mechanisms responsible for it are obscure. Here we show that HMGB1 in neutrophils is post-translationally mono-methylated at Lys42. The methylation alters the conformation of HMGB1 and weakens its DNA binding activity, causing it to become largely distributed in the cytoplasm by passive diffusion out of the nucleus. Thus, post-translational methylation of HMGB1 causes its cytoplasmic localization in neutrophils. This novel pathway explains the distribution of nuclear HMGB1 to the cytoplasm and is important for understanding how neutrophils release HMGB1 to the extracellular milieu.

High mobility group box 1 (HMGB1) protein is one of the most abundant nonhistone chromosomal proteins in eukaryotic organisms. The primary sequences of HMGB1 in various higher organisms, from birds to mammals, show more than 90% homology with each other (1). The protein has multiple roles in transcription, replication, and cellular differentiation (2, 3). HMGB1 interacts with several transcription factors, thereby allowing them to perform their cellular roles. The phenotype of Hmgb1 knock-out mice confirmed the functional importance of HMGB1 as a regulator of transcription: they die shortly after birth and show a defect in the transcriptional control exerted by the glucocorticoid receptor (4). The subcellular distribution of the protein is tissue-specific: HMGB1 is located in both the nuclei and the cytoplasm of different tissues, such as lymphoid tissue, testis, neurons, and hepatocytes (5). Wang et al. (6) identified HMGB1 as a late mediator of endotoxin lethality in mice and showed that monocytes and macrophages stimulated by lipopolysaccharide (LPS), tumor necrosis factor (TNF) or interleukin-1 (IL-1) secrete HMGB1 in a delayed response. Patients with sepsis show an increased serum level of HMGB1, which is correlated with the severity of infection (7). Moreover, HMGB1 in monocytes and macrophages is extensively acetylated upon activation by LPS, causing localization of the protein to the cytosol (8). Cytosolic HMGB1 is then concentrated into secretory lysosomes and secreted when the cells receive an appropriate second signal (9). The recent discovery of extracellular HMGB1 as a proinflammatory mediator has been supported by a number of studies. In addition, HMGB1 is passively released from the nucleus to the extracellular milieu by cells that die as a result of necrosis or damage (10).

Our previous studies showed that HMGB1 and HMGB2 are novel antigens of anti-neutrophil cytoplasmic antibodies (ANCA), which are observed in the sera of the majority of patients with rheumatic diseases (11), ulcerative colitis (UC) (12, 13), and autoimmune hepatitis (14), suggesting the cytoplasmic distribution of HMGB1 in neutrophils. Subsequently, we found that autoantibodies in sera from patients with UC hardly recognize HMGB1 in neutrophils. However, they are able to recognize HMGB1 in lymphocytes, as well as HMGB2, as judged by immunoblot analysis (15). The anti-HMGB1 and -HMGB2 monoclonal antibody FBH7, recognizing a conformation of 52–56 residues of HMGB1, shows a similar profile to the antibodies in patient sera (15). These results suggest that HMGB1 in neutrophils is different in conformation from that in lymphocytes at the epitope regions of patient sera and FBH7 and that HMGB1 is secreted from neutrophils to the extracellular milieu. However, the actual distribution of HMGB1 in the cytoplasm of neutrophils and the mechanisms responsible are obscure.

Here we show that HMGB1 in neutrophils is post-translationally mono-methylated at lysine 42 (Lys42). The methylation alters the conformation of HMGB1 and weakens its DNA binding activity, causing it to become largely distributed in the cytoplasm by passive diffusion out of the nucleus. Thus, post-trans-
lational methylation of HMGB1 causes its cytoplasmic localization in neutrophils. This novel pathway explains the distribution of nuclear HMGB1 to the cytoplasm and is important for understanding how neutrophils release HMGB1 to the extracellular milieu to produce ANCA against HMGB1 in serum of affected patients.

EXPERIMENTAL PROCEDURES

Preparation of HMGB1 and Recombinant Proteins—HMGB1 was prepared from pig thymus as described previously (16). Percollary acid (PCA) extract fractions were prepared from neutrophils and lymphocytes of human peripheral blood as described (13, 17). Recombinant epitope-tagged, including 6× histidine and Xpress tags, HMGB1 box A fragment B1A (amino acid residues 1–76, wt) and its mutants B1A-R23L (R23L), B1A-K27L (K27L), B1A-K28L (K28L), and B1A-K42L (K42L), and B1A-K29L (amino acid residues 1–76, wt) and its mutants B1A-R23L (R23L), B1A-K27L (K27L), B1A-K28L (K28L), and B1A-K42L (K42L), and AIB fragment B1AIB (amino acid residues 1–164; box A + linker + box B, wt) and its mutants B1AIB-R23L (R23L), B1AIB-K27L (K27L), B1AIB-K28L (K28L), B1AIB-K29L (K29L), and B1AIB-K42L (K42L) were overexpressed in Escherichia coli (JM109) cells transformed with the pTrcHisA plasmid carrying the corresponding cDNA sequences, and purified as described previously (15). FLAG-tagged HMGB1 (fHMGB1-wt) and its mutants fHMGB1-K29L, fHMGB1-K42L, fHMGB1-K42A (K42A), fHMGB1-K29L/K42L, fHMGB1-ΔA (a lack of box A domain), and fHMGB1-R109E (R109E) were transiently expressed in HeLa S3 cells by transfection with the pCI-neo plasmid carrying the corresponding cDNA sequences using X-tremeGENE Q2 transfection reagent (Roche Applied Science). Likewise, Aequorea coerulescens green fluorescence protein (GFP) and the fusion protein (GFP-NEs) of GFP with the nuclear export signal (NES) sequence of human immunodeficiency virus type 1 (HIV-1) Rev protein (LPPLERTL) was expressed.

Cell Culture—HL-60 cells and HeLa S3 cells were maintained in Roswell Park Memorial Institute (RPMI) medium 1640 (Sigma) containing 10% (v/v) fetal bovine serum and Iscove’s modified Dulbecco’s medium (IMDM, Sigma) supplemented with 2 mM glutamine (Invitrogen) containing 10% (v/v) fetal bovine serum, respectively. HL-60 cells were treated with 1 μM all-trans retinoic acid (Wako) in RPMI medium 1640 for the indicated number of days to allow their differentiation into neutrophil-like cells. The rates of cell differentiation were measured by Wright-Giemsa staining (18) and NBT reduction (19). The supernatant of the cell pellet was treated with PCA (final 5% (w/v)) and rotated at 4 °C for 1 h. The 5% PCA precipitate obtained was processed as described previously (7).

Antibodies—Mouse anti-HMGB1 monoclonal antibody KS1 recognizing HMGB1, mouse anti-HMGB1, and -HMGB2 monoclonal antibody F8H7 recognizing HMGB1 and HMGB2 conformations, and rabbit anti-HMGB polyclonal antibody (pAb) 1 were used. Affinity-purified rabbit antibody against mono-methylated K42 peptide (NFSEFSKmKCSER, B1K42me) of HMGB1 (αB1K42me) was prepared by Japan Bioservices Co., Ltd. Anti-Xpress epitope tag antibody and anti-FLAG M2 antibody were purchased from Invitrogen and Sigma, respectively. The secondary antibodies conjugated with horseradish peroxidase (HRP) and Alexa Fluor 488 and 568 were purchased from Promega and Molecular Probes, respectively.

ELISA and Immunostaining—Two synthetic peptides were prepared for ELISA. One peptide was B1K42me, and the other was its unmethylated peptide (NFSEFSKmKCSER, B1unmod.) corresponding to amino acid residues 36–47 of HMGB1.

An aliquot of 5 μg/ml of the peptides and purified pig HMGB1 in phosphate-buffered saline, pH 7.4, was adsorbed to the wells of a 96-well assay plate (Corning). HRP-conjugated secondary antibody and 3,3’,5,5’-tetramethylbenzidine (TMB) substrate (Sub) were used, and then the absorbance at 450 nm was measured with a microplate reader (Bio-Rad).

Respective HL-60 cells, lymphocytes, and neutrophils were cytopspun onto microscope slides, and fixed with 4% paraformaldehyde for 10 min at room temperature. HeLa cells seeded onto an 8-well chamber slide (Lab-Tek) were transfected with the pCI-fHMGB1 and its mutants, and cultured for 48 h. The cells were immunostained and observed with an Axiovert 200 fluorescence microscope (Zeiss) and LSM510 META confocal microscope (Zeiss). For competitive immunostaining, αB1K42me was preincubated with 0.5 μg/μl B1K42me or B1unmod. peptide for 30 min at room temperature.

MALDI-TOF MS Analysis—HMGB1 in PCA extract fractions prepared from neutrophils and lymphocytes was separated by SDS-PAGE. The protein in the gel piece was reduced with dithiothreitol and then hydrolyzed by trypsin or chymotrypsin. The peptides were extracted in 60% (v/v) acetonitrile containing 1% (v/v) trifluoroacetic acid. The extracts were concentrated and desalted with ZipTip C-18 (Millipore), and mixed with saturated α-ciano-4-hydroxycinnamic acid in 50% acetonitrile containing 0.1% trifluoroacetic acid for analysis using a REFLEX III mass spectrometer (Bruker Daltonics).

DNA Binding Assays—Southwestern analysis and electrophoretic mobility shift assay (EMSA) were carried as described previously (20, 21).

Subcellular Fractionation of Neutrophils—Nuclear and cytoplasmic fractionation experiments were performed with minor modifications as described previously (22, 23). To prepare the nuclear and cytoplasmic fractions, neutrophils (1 × 106 cells) obtained from human peripheral blood were suspended in 50 μl of fractionation buffer (4.05 mM Na2HPO4, 0.74 mM KH2PO4, 68.5 mM NaCl, 1.34 mM KCl, and 1 mM phenylmethylsulfonyl fluoride) containing 100 μg/ml digitonin, and then stood on ice for 5 min. After the permeabilization treatment, the lysate was centrifuged for 5 min. The pellet and supernatant were collected as the nuclear and the cytoplasmic fractions, respectively.

RESULTS

Differences in Conformation of HMGB1 between Lymphocytes and Neutrophils—Previously, we found that autoantibodies in serum samples from patients with UC hardly recognize HMGB1 in neutrophils, whereas they are able to recognize HMGB1 in lymphocytes, as well as HMGB2, as judged by immunoblot analysis (15). Anti-HMGB1 and -HMGB2 monoclonal antibody F8H7 showed a similar profile to the antibodies in patient sera, while anti-HMGB1 monoclonal antibody KS1 revealed the presence of HMGB1 in neutrophils. To elucidate...
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the difference in immunoreactivity between neutrophil HMGB1 and lymphocyte HMGB1, we mapped the epitopes for FBH7 and KS1 using several methods. The results showed that the epitope of FBH7 is located at amino acid residues 52–56 of HMGB1, and that the intact conformation of HMGB1 box A or the combined structure of the α-helices is important for FBH7 recognition. In contrast, the epitope of KS1 is located at amino acid residues 70–72 of HMGB1, and the conformation of box A is not necessary for its recognition (Fig. 2, B and C). These results suggested that HMGB1 in neutrophils is different in conformation from that in lymphocytes at the epitope regions of patient sera and FBH7. Mapping of the epitope showed that FBH7 recognizes the conformation of 52–56 residues of HMGB1 and HMGB2, and suggested that the epitope region or its peripheral structure in neutrophil HMGB1 is conformationally changed, resulting in a decrease of recognition by the antibodies (Fig. 1A) (15). This conformational alteration of HMGB1 may confer new biochemical properties on the protein. Therefore, PCA extract fractions containing mainly HMGB1 and HMGB2, and histone H1 were prepared from lymphocytes and neutrophils. The amounts of HMGB1 in the two cell types were similar, judging from their Coomassie and Amido Black staining profiles (Fig. 1B). Southwestern blotting analysis for neutrophil HMGB1 probed with 32P-labeled DNA showed a weaker band in comparison with lymphocytes. These data suggested that neutrophil HMGB1 has less DNA binding activity than lymphocyte HMGB1 because of a possible change in conformation. Despite the difference of HMGB1 between lymphocytes and neutrophils, we did not detect any HMGB1 variant in these cells (data not shown).

HMGB1 in Neutrophils Is Post-translationally Mono-methylated at Lys42—One of the primary factors inducing conformational changes in proteins is post-translational modification. Then, HMGB1 in neutrophils may be post-translationally modified. To identify the probable modification of HMGB1, HMGB1 in the SDS gel band of the PCA extract fraction from neutrophils was subjected to trypsin digestion, followed by MALDI-TOF mass spectrometry. Acetylated, phosphorylated, or glycosylated peptide was not found in neutrophil HMGB1. On the other hand, in addition to the peptide peaks of the predicted masses, peaks with additional masses of 14 and 28 Da corresponding to one and two methyl group adducts, were observed in repeated experiments (Table 1). Notably, a peak corresponding to the mono-methylated 30–42 peptide of HMGB1 (m/z 1478.7080) was most frequently observed in neutrophil HMGB1 digests, but not in lymphocyte HMGB1 digests (Fig. 2A). This result indicated that Lys42 in HMGB1 of neutrophils is post-translationally mono-methylated, and that Arg23, Lys27, Lys28, and Lys29 are the possible sites of methylation (Fig. 2B, C, and E). The Lys42 mono-methylated peak was also reproducibly observed by MALDI-TOF mass spectrometry in chymotrypsin digests of neutrophil HMGB1 (supplemental Table S1). No modification of Lys44 and Lys46 in neutrophil HMGB1, which are located within the epitope of FBH7, was detected. In addition, none of the lysine and arginine residues in lymphocyte HMGB1 were methylated. The limited amount and partial methylation of neutrophil HMGB1 hampered further MS/MS analysis. Therefore, to verify mono-methylation of Lys42, an affinity-purified rabbit antibody against mono-methylated K42 peptide (αB1K42me) was prepared. ELISA and immunoblotting were conducted with αB1K42me in order to examine the specificity of the antibody. As shown in Fig. 2D, B1K42me peptide and neutrophil HMGB1 were recognized by αB1K42me, but B1unmod. peptide, pig, and lymphocyte HMGB1 were not. In addition, neutrophils were stained by αB1K42me, indicating that Lys42 of HMGB1 in neutrophils, but not that in lymphocytes, is mono-methylated (Fig. 2, E and F). Interestingly, most of the Lys42-methylated HMGB1 seemed to be localized in the cytoplasm, while total HMGB1 recognized by KS1 was distrib-
uted in both the nucleus and the cytoplasm. The cytoplasmic immunostaining profile obtained with H9251/B1K42me was consistent with that obtained with KS1 in the magnified view (Fig. 2G), and was completely abolished in the presence of B1K42me peptide as a competitor, but not by B1unmod. peptide (Fig. 2H). These observations demonstrated that a proportion of HMGB1 molecules in neutrophils are post-translationally mono-methylated at Lys42.

Subcellular Distribution of HMGB1 in Lymphocytes and Neutrophils—Fig. 3A shows a profile of neutrophil HMGB1 obtained from lymphocyte (L) and neutrophil (N) HMGB1. B and C, potential methylated residues (in black boxes) in neutrophil HMGB1 obtained from TOF-MS analysis. (B, amino acid sequence; l, linker region; j, joiner region; C, NMR structure of box A, Protein Data Bank ID 1AAB.) The epitopes of KS1 and FBH7 are indicated by underlining (B) and a blue line (C). D, specificity of anti-mono-methylated Lys42 of HMGB1 antibody (H9251/B1K42me). B1unmod., B1K42me peptide, and pig HMGB1 were absorbed to wells of a 96-well plate and assayed by ELISA (upper panel) (mean ± S.D.; n = 3). Five percent PCA extracts prepared from lymphocytes and neutrophils were separated by SDS-PAGE and then analyzed by immunoblotting with H9251/B1K42me (bottom panel). E–H, detection of Lys42-mono-methylated HMGB1 in neutrophils. Neutrophils (E) and lymphocytes (F) were fixed and coimmunostained with H9251/B1K42me (green) and KS1 (red). G, magnified views of E, H, competitive immunostaining using B1unmod. (left panel) and B1K42me peptide (right panel). Nuclei were counterstained with Hoechst 33258 (blue), and merged (E–G). The cells were observed using a LSM510 META confocal microscope. Bar represents 20 μm (E, F, and H) or 5 μm (G).
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Fig. 3B, indicating that most of the cytoplasmic HMGB1 is methylated. The staining profile of lymphocytes was consistent with that obtained using Hoechst 33258, showing that HMGB1 is localized only in the nucleus. Together, the data demonstrate that most Lys42-mono-methylated HMGB1 is localized in the cytoplasm of neutrophil, whereas non-methylated HMGB1 exists in the nucleus.

Extranuclear and Extracellular Release of HMGB1 from HL-60 Cells during Neutrophil-like Cell Differentiation—The above results suggested that Lys42 mono-methylation of HMGB1 occurs during the process of differentiation into neutrophil. HL-60 cells are a useful model of neutrophils for investigating the processes involved, because they can be induced to differentiate into neutrophil-like cells by culture in the presence of all-trans retinoic acid. Wright-Giemsa staining and NBT reduction assay showed that about 80% of HL-60 cells had differentiated into neutrophil-like cells at 6 days, i.e. the final period or termination of their differentiation process (Fig. 4A).

The reactivity of FBH7 with HMGB1 in cell lysates was decreased after 6 days in comparison with that of KS1 (Fig. 4B), suggesting that the Lys42 post-translational methylation of neutrophil HMGB1 was accompanied by cell maturation. We then investigated whether HL-60 cells release HMGB1 to the extracellular milieu in the process of differentiation. Immunoblotting analysis with KS1 detected increasing amounts of HMGB1 in the cell culture supernatants depending on the differentiation process, whereas immunoblotting with FBH7 did not, suggesting that the released HMGB1 must be methylated (Fig. 4C). About 10% of HMGB1 protein in HL-60 cells was released at the end of the differentiation process (Fig. 4D). In addition, both the nuclei and the cytoplasm of differentiated HL-60 cells were stained with KS1, whereas only the nuclei were stained with FBH7 (Fig. 4E), showing a staining profile similar to that of

FIGURE 3. Subcellular distribution of HMGB1. A, neutrophils and lymphocytes were fixed and immunostained with KS1 or FBH7 (center, red). Nuclei were counterstained with Hoechst 33258 (left, blue), and merged images are shown (right). Bar represents 20 μm. B, the nuclear (ppt) and the cytoplasmic (sup) fractions prepared from neutrophils were separated by SDS-PAGE, and analyzed by immunoblotting with KS1 and FBH7. Black and white arrowheads indicate HMGB1 and HMGB2, respectively.

FIGURE 4. Extranuclear and extracellular release of HMGB1 from HL-60 cells during neutrophilic differentiation. A, the differentiation rate of HL-60 cells cultured in the presence of retinoic acid measured by Wright-Giemsa staining (solid circles) and NBT reduction assay (clear circles). B, immunoblotting with KS1 and FBH7 for cell lysates from differentiating HL-60 cells. C, immunoblotting with KS1 and FBH7 for the supernatant of HL-60 cell culture. D, immunoblotting with KS1 for the cell lysates (cell) of differentiating HL-60 cells and the supernatant (sup) of culture at 0 and 6 days after retinoic acid treatment. A black arrowhead indicates HMGB1. E, undifferentiated HL-60 cells and HL-60 cells allowed to differentiate for 6 days were fixed and immunostained with KS1 or FBH7 (center, red). Nuclei were counterstained with Hoechst 33258 (left, blue), and merged images are shown (right). Bar represents 20 μm.
neutrophils (Fig. 3A). These data indicated that the conformational change of box A, the cytoplasmic distribution, and the release of HMGB1 accompanied by Lys42 mono-methylation must occur during the process of differentiation into neutrophils.

HMGB1 Mutation Analyses of Immunoreactivity, DNA Binding Activity, and Subcellular Distribution—Results of Southwestern blotting analysis for neutrophil HMGB1 (Fig. 1B) suggested a mechanism for the cytoplasmic distribution of Lys\(^{42}\)-methylated HMGB1. Epitope-tagged B1AIB mutants were incubated with DNA (pBluescript II KS (+)) at a protein/DNA molar ratio of 250, and analyzed by EMSA (left panel). Epitope-tagged B1A mutants were incubated with DNA in the presence of anti-Xpress antibody, and analyzed by the supershift on EMSA (right panel).

To determine by fluorescence microscopy whether the mimic of Lys\(^{42}\)-methylated HMGB1 shows a cytoplasmic distribution, the FLAG-tagged HMGB1 (fHMGB1-wt) and its mutants including fHMGB1-K42L were transiently expressed in HeLa cells (Fig. 6A). The fHMGB1-K42L and fHMGB1-K29L/K42L showed higher distributions in both the cytoplasm and the nucleus, whereas fHMGB1-wt and fHMGB1-K29L were localized mainly in the nucleus. Control fHMGB1-H9004A showed cytoplasmic localization. Box B in HMGB1 plays an important role in the association with DNA. The Arg\(^{109}\) residue in box B has direct electrostatic interaction with DNA to bring about the correct array of the box on DNA into which the side chain of Phe\(^{102}\) intercalates. R109E mutation resulted in loss of DNA binding activity of box B and a decrease in that of AlB domain without any conformational change (21). Further investigation of the relationship between the decrease of DNA binding activity and cytoplasmic localization of proteins showed that the signal of fHMGB1-R109E by anti-FLAG antibody was detectable in both the nucleus and the cytoplasm (Fig. 6A). These results clearly indicated that the DNA binding activity of HMGB1 is a potent factor for its nuclear localization.
HMGB1 presumably has an obscuring non-classical NES, and is exported to the cytoplasm depending on both chromosomal region maintenance 1 (CRM1) exportin and passive diffusion (8). CRM1-dependent nuclear export can be inhibited by leptomycin B (LMB), which blocks the interaction between CRM1 and NES (24). To make sure this, a control experiment was conducted. The fusion protein of GFP-NES was exclusively distributed in the cytoplasm, whereas GFP protein was in the nuclei and the cytoplasm of HeLa cells (Fig. 6B). In addition, the cytoplasmic distribution of GFP-NES protein was changed in the distribution into the nuclei and the cytoplasm by the treatment with LMB. On the other hand, the cytoplasmic distribution of fHMGB1-K42L and fHMGB1-ΔA was not affected by the treatment with LMB. These results showed that the nuclear export of fHMGB1 mutants is CRM1-independent. Because the nuclear pore complexes function as aqueous channels with a diameter of about 10 nm, they must allow passive diffusion of proteins with a molecular mass of up to 40–60 kDa (25). fHMGB1-K42L and fHMGB1-R109E were imported to the nucleus, as well as fHMGB1-wt, in an interspecies heterokaryon assay (supplemental Fig. S1). Thus, HMGB1, which has lost all or part of its DNA binding activity by post-translational modification as well as mutation, may pass from the nucleus to the cytoplasm by simple diffusion.

**DISCUSSION**

Several investigations on the post-translational modifications of HMGB1 have been conducted. The acidic tail of HMGB1 from the dipterous insects Chironomus and Drosophila, which has a single HMG box, was phosphorylated by casein kinase II in vivo (26, 27). The phosphorylation of HMGB1 increased its conformational and metabolic stabilities, and reduced its binding affinity for four-way junction DNA, whereas its strength of binding to linear DNA was unchanged, and its nuclear translocation was inhibited. The phosphorylation of HMGB1 in vertebrate cells has not been reported. Another modification profile of HMGB1 is the acetylation of Lys2 and Lys11 (28, 29), and of only Lys2 (30) in the N-terminal region of the protein from Guerin ascites tumor cells by sodium butyrate treatment. The binding affinity of acetylated HMGB1 for UV-damaged, cis-platinated, and four-way junction DNA is significantly higher than that of the unmodified protein. HMGB1 is hyperacetylated in monocytic cells, and hyperacetylation of the

![FIGURE 6. Cytoplasmic distribution of HMGB1.](image-url)
HMGB1 NLS region in macrophages causes relocation of the protein to the cytosol due to inhibition of nuclear import (8). No glycosylation or methylation of HMGB1 in vitro and in vivo has been reported. To our knowledge, this is the first report to indicate that HMGB1 in neutrophils is mono-methylated at Lys^{22} (Fig. 2 and Table 1). Subsequently, we demonstrated that the DNA binding activity of methylated HMGB1 is significantly reduced by the conformational change in box A caused by the methylation (Figs. 1B and 5, A and B). The global folding of HMG box, which comprises three α-helices arranged in an L-shape, is well conserved. The structure is stabilized by extensive hydrophobic interaction formed by the conserved aromatic residues Phe^{18}, Trp^{48}, and Phe^{59} in box A (31). The difference in locations between the FBH7 epitope region and the Lys^{22} methylation site suggests that the methylation affects the formation of the hydrophobic core by altering the trajectory of α-helix II. Because the amino acid residue corresponding to position 42 in HMGB1 is highly conserved in vertebrate HMGB1 and HMGB2, and in the HMG box family proteins including HMG-D, HMGT, NHP6a, hMtTF1, ABF2, SRY, SOX, T160, and SSRP1, the residue must be important for the conformation of HMG boxes. In addition, the position of Lys^{22} does not face the DNA backbone in the DNA binding structure of box A. In this respect, Lys^{22} may be targeted for methylation in neutrophils.

HMGB1 in neutrophils is localized to both the nucleus and the cytoplasm, while the protein in other cell types is localized mainly in the nucleus (Figs. 2, E and F and 3A). The protein is extremely mobile and can quickly shuttle between the nucleus and the cytoplasm (32). The nuclear proteins are translocated into the nucleus through the nuclear pore complexes on the nuclear membrane after translation in the cytoplasm (25). Because the nuclear pore complexes function as aqueous channels with a diameter of about 10 nm, they must allow passive diffusion of proteins with molecular masses of up to 40 – 60 kDa (25). On the other hand, many nuclear proteins may actively enter the nucleus regardless of their molecular size (33). The nuclear localization signal (NLS) and NES in HMGB1 have not been identified, and the nucleocytoplasmic transport mechanism has remained unclear, despite several studies (20, 34–36). However, HMGB1 and some HMG-related proteins containing HMG box(es) have been shown to become localized in the nucleus by active transport (34–36). Our previous study showed that HMGB2-β-galactosidase fusion protein is actively translocated into the nucleus and that basic regions interspaced with the long DNA binding sequence are necessary for the nuclear accumulation of HMGB2. The close configuration of basic regions at both ends of the sequence in the tertiary structure may function as the NLS (20). This NLS feature may be different from typical ones such as the single or bipartite basic cluster formed in many nuclear proteins (37). Because human HMGB1 has 79% identity at the amino acid level with HMGB2, the NLS in HMGB1 is considered to be consistent with that in HMGB2. The nuclear export machinery for HMGB1 and HMGB2 has also been unclear, and in addition the proteins have no typical NES such as a leucine-rich signal (24). To determine whether the Lys^{22} methylation of HMGB1 affects its subcellular distribution, the FLAG-tagged HMGB1-K42L mutant was transiently expressed in HeLa cells. In accordance with our prediction, the mutant was distributed in the cytoplasm (Fig. 6A). In addition, Lys^{22}-mono-methylated HMGB1 was localized mainly to the cytoplasm in neutrophils (Figs. 2C and 3). The FLAG-tagged HMGB1-R109E mutant, which has significantly decreased DNA binding activity without a conformational change in the HMG box, actually exhibited a cytoplasmic distribution (Fig. 6A). These mutants showed no change in cytoplasmic distribution upon treatment with LMB, and were imported into the nucleus of COS-7 cells in the heterokaryon (Fig. 6B and supplemental Fig. S1). Therefore, the diffusion of methylated HMGB1 from the nucleus to the cytoplasm must be caused by a decrease in affinity for chromosomal DNA.

Our previous studies showed that HMGB1 is a novel antigen of ANCA, which are observed in the sera of patients with several diseases (11–14). These facts indicate that HMGB1 may be exported from the nucleus to the cytoplasm in neutrophils, and then released into the extracellular milieu. However, the mechanisms responsible are obscure. It has also remained unclear how HMGB1 is secreted from monocytes and other competent-type cells: the protein possesses no signal peptide which would direct it to the endoplasmic reticulum (ER). This lack of a secretion leader peptide is a feature shared with a small number of secreted proteins, such as the cytokine IL-1β (38). Studies of murine erythroleukemia (MEL) cells have shown that extracellular export of HMGB1 is not dependent on the ER and the Golgi complex, but promoted by an increase in intracellular Ca^{2+} and possibly by the activation of a Ca-dependent protein kinase C isoform (39). There is a limited amount of information on cytoplasmic HMGB1, which is packaged into a specific population of secretory lysosomes present in hematopoietic cells (40), before being released extracellularly (9, 41). It is still unclear how HMGB1 is loaded into secretory lysosomes and whether HMGB1 plays a role in the cytoplasm. The secretion of HMGB1 from monocytes or macrophages occurs in response to inflammatory stimuli, such as LPS, or cytokines such as TNF-α, IL-1β, and INF-γ (6). HMGB1 is passively released from necrotic cells as a soluble molecule and evokes inflammatory responses both in vitro and in vivo (42). HMGB1 released from necrotic cells triggers the production of TNF-α from monocytes, and blockade of HMGB1 in vivo reduces leukocyte recruitment in acute hepatic necrosis. These findings suggest that HMGB1 contributes to the inflammation elicited by necrosis (42). Neutrophils, which are also known as polymorphonuclear leukocytes, constitute the first line of defense against infectious agents or non-self substances. Mature neutrophils contain cytoplasmic granules and a lobulated chromatin-dense nucleus with no nucleolus, and their cell proliferation is suppressed. In this respect, the fundamental roles of HMGB1 in transcription and replication in actively proliferating cells may not be played in neutrophils, and it seems reasonable to assume that the cytoplasmic distribution accompanied by the methylation of neutrophil HMGB1 is necessary for release into the extracellular milieu. In addition, methylated HMGB1 may not be necessary for the differentiation of HL-60 cells as an intracellular pro-differentiation factor, because HMGB1 was conformationally changed and appeared in the extracellular...
lar milieu during the final period, or upon termination, of the differentiation of HL-60 cells into neutrophil-like cells (Fig. 4).

We therefore conclude that Lys\textsuperscript{42} in HMGB1 is post-translationally methylated at the end of the process of neutrophilic differentiation in myelocytic cells. The methylation of Lys\textsuperscript{42} alters the conformation of box A containing the epitope formed from amino acid residues 52–56, which is intrinsically recognized by the antibody FBH7 as well as patient sera. Methylation of HMGB1 weakens its ability to bind to DNA as a result of conformational change. Thus, the methylated HMGB1 becomes distributed in the cytoplasm through passive diffusion out of the nucleus. The cytoplasmic HMGB1 in neutrophils may be released from the cells when an appropriate activation signal is received. It is of interest to clarify the possible function of the large amount of methylated HMGB1 present in the cytoplasm of neutrophils. It will also be important to understand whether and how neutrophils release methylated HMGB1 into the extracellular milieu during the final period, or upon termination, of the differentiation of HL-60 cells into neutrophil-like cells (Fig. 4).

Differentiation of HL-60 cells into neutrophil-like cells (Fig. 4).

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