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Histone Deimination As a Response to Inflammatory Stimuli in Neutrophils

Indira Neeli, Salar N. Khan, and Marko Radic

Posttranslational modifications, such as the deimination of arginine to citrulline by peptidyl arginine deiminase (PAD4), change protein structure and function. For autoantigens, covalent modifications represent a mechanism to sidestep tolerance and stimulate autoimmunity. To examine conditions leading to histone deimination in neutrophils, we used Abs that detect citrullines in the N terminus of histone H3. Deimination was investigated in human neutrophils and HL-60 cells differentiated into granulocytes. We observed rapid and robust H3 deimination in HL-60 cells exposed to LPS, TNF, lipoteichoic acid, f-MLP, or hydrogen peroxide, which are stimuli that activate neutrophils. Importantly, we also observed H3 deimination in human neutrophils exposed to these stimuli. Citrullinated histones were identified as components of extracellular chromatin traps (NETs) produced by degranulating neutrophils. In contrast, apoptosis proceeded without detectable H3 deimination in HL-60 cells exposed to staurosporine or camptothecin. We conclude that histone deimination in neutrophils is induced in response to inflammatory stimuli and not by treatments that induce apoptosis. Our results further suggest that deiminated histone H3, a covalently modified form of a prominent nuclear autoantigen, is released to the extracellular space as part of the neutrophil response to infections. The possible association of a modified autoantigen with microbial components could, in predisposed individuals, increase the risk of autoimmunity.


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tones acquire a number of posttranslational modifications (1). One intriguing histone posttranslational modification is the deimination of specific arginines in histones H2A, H3, and H4 (2–4). The conversion of arginine to citrulline, an atypical amino acid that lacks arginine’s positive charge, is accomplished by a member of the peptidylarginine deiminase (PAD) family of enzymes, PAD4 (5). Although PAD4-mediated modifications of histones are involved in regulating chromatin structure and gene expression in a variety of cell types (6–8), PAD4 may assume an additional role in eosinophil and neutrophil responses to infections. In these cells, PAD4 is an abundant component of cytoplasmic granules (9) and its activation leads to widespread histone deimination. However, a granulocyte-specific role of PAD4 has not yet been defined.

Interest in PAD4 intensified following the identification of genetic polymorphisms that enhance the expression of PAD4 and constitute a risk factor for rheumatoid arthritis (10). PAD4 polymorphisms are associated with rheumatoid arthritis susceptibility in Asian and North American populations (11), and thus may provide insights into the pathogenesis of this important autoimmune disease (12). Inflammation in the joints of rheumatoid arthritis patients was demonstrated to involve PAD4 activation in the infiltrating leukocytes and the in situ generation of deiminated proteins (13). In animal models of rheumatoid arthritis, PAD4 expression was correlated with the induction or progression of disease (14). However, the conditions that lead to PAD4 activation in the inflamed synovium have not been clearly identified.

The activity of PAD4 has been examined in HL-60 cells that, following treatment with all-trans retinoic acid (ATRA), acquire properties of mature neutrophils. Addition of calcium ionophore to ATRA-treated HL-60 cells induces rapid histone deimination (2, 15). Because elevated intracellular calcium levels promote apoptosis (16), it was concluded that histone deimination is an early step in apoptosis. In this study, we examine conditions that favor production of citrullinated histones in HL-60 cells and human blood neutrophils. By establishing that PAD4 activation is an inflammatory response that does not require caspase activity, we define the relationship between granulocyte apoptosis and histone deimination. Our results indicate that deimination of histones, rather than constituting an essential component of neutrophil apoptosis, is a convergence point for diverse signals that trigger the neutrophil response to infections.

Materials and Methods

Abs and supplies

Anti-citrullinated histone H3 rabbit Abs (ab 5103, lot no. 122699) were obtained from Abcam, anti-phospho-H2B (Ser14) rabbit Abs (07-191) from Upstate Biotechnology, and anti-poly(ADP-ribose) polymerase (PARP) IgG1 mouse mAb (clone 42) from BD Biosciences. ATRA, calcium ionophore (A23187), staurosporine, camptothecin, f-MLP, LPS, cycloheximide, and HRP-conjugated secondary Abs to rabbit or mouse Ig were purchased from Sigma-Aldrich. The pan-caspase inhibitor, z-VAD-fmk, was obtained from Calbiochem. Goat anti-rabbit AF648, goat anti-rabbit AF488, annexin V AF488, and Sytox orange were obtained from Invitrogen Life Technologies. H2O2 was obtained from Fisher Scientific. Recombinant human TNF was a gift of Dr. L. Pfeffer (University of Tennessee Center of Excellence for Microbial Pathogenesis, the Center of Excellence for Diseases of Connective Tissue, and the University of Tennessee Center of Excellence for Microbial Pathogenesis, the Center of Excellence for Diseases of Connective Tissue, and the University of Tennessee Center of Excellence for Microbial Pathogenesis, the Center of Excellence for Diseases of Connective Tissue, and the University of Tennessee Center of Excellence for Microbial Pathogenesis, the Center of Excellence for Diseases of Connective Tissue, and the University of Tennessee Center of Excellence for Microbial Pathogenesis, the Center of Excellence for Diseases of Connective Tissue, and the University of Tennessee Center of Excellence for Microbial Pathogenesis, the Center of Excellence for Diseases of Connective Tissue, and the University of Tennessee Center of Excellence for Microbial Pathogenesis, the Center of Excellence for Diseases of Connective Tissue, and the University of Tennessee Center of Excellence for Microbial Pathogenesis, the Center of Excellence for Diseases of Connective Tissue, and the University of Tennessee Center of Excellence for Microbial Pathogenesis, the Center of Excellence for Diseases of Connective Tissue, and the University of Tennessee Center of Excellence for Microbial Pathogenesis, the Center of Excellence for Diseases of Connective Tissue, and the University of Tennessee Center of Excellence for Microbial Pathogenesis, the Center of Excellence for Diseases of Connective Tissue, and the University of Tennessee Center of Excellence for Microbial Pathogenesis, the Center of Excellence for Diseases of Connective Tissue, and the University of Tennessee Center of Excellence for Microbial Pathogenesis, the Center of Excellence for Diseases of Connective Tissue, and the University of Tennessee Center of Excellence for Microbial Pathogenesis, the Center of Excellence for Diseases of Connective Tissue, and the University of Tennessee Center of Excellence for Microbial Pathogenesis, the Center of Excellence for Diseases of Connective Tissue, and the University of Tennessee Center of Excellence for Microbial Pathogenesis, the Center of Excellence for Diseases of Connective Tissue, and the University of Tennessee Center of Excellence for Microbial Pathogenesis, the Center of Excellence for Diseases of Connective Tissue, and the University of Tennessee Center of Excellence for Microbial Pathogenesis, the Center of Excellence for Diseases of Connective Tissue, and the University of Tennessee Center of Excellence for Microbial Pathogenesis, the Center of Excellence for Diseases of Connective Tissue, and the University of Tennessee Center of Excellence for Microbial Pathogenesis, the Center of Excellence for Diseases of Connective Tissue, and the University of Tennessee Center of Excellence for Microbial Pathogenesis, the Center of Excellence for Diseases of Connective Tissue, and the University of Tennessee Center of Excellence for Microbial Pathogenesis, the Center of Excellence for Diseases of Connective Tissue, and the University of Tennessee Center of Excellence for Microbial Pathogenesis, the Center of Excellence for Diseases of Connective Tissue, and the University of Tennessee Center of Excellence for Microbial Pathogenesis, the Center of Excellence for Diseases of Connective Tissue, and the University of Tennessee Center of Excellence for Microbial Pathogenesis, the Center of Excellence for Diseases of Connective Tissue, and the University of Tennessee Center of Excellence for Microbial Pathogenesis, the Center of Excellence for Diseases of Connective Tissue, and the University of Tennessee Center of Excellence for Microbial Pathogenesis, the Center of Excellence for Diseases of Connective Tissue, and the University of Tennessee Center of Excellence for Microbial Pathogenesis, the Center of Excellence for Diseases of Connective Tiss...
health Science center, Memphis, TN) and additional quantities were obtained from BioSource International. Lipoteichoic acid (LTA) was a gift from Dr. D. Hasty (Veterans Affairs, Memphis, TN).

**Cell culture**

HL-60 cells (CCL240; American Type Culture Collection) were grown in Iscove’s modified DMEM (Invitrogen Life Technologies) supplemented with 20% FBS (Gemini Bio-Products). HL-60 cells were differentiated into granulocytes by culturing the cells in medium containing 1 μM ATRA for 48 h.

**Treatments of HL-60 cells**

ATRA-differentiated HL-60 granulocytes were treated with 4 μM calcium ionophore in medium containing 2 mM CaCl₂ or left untreated and incubated for various times at 37°C. To inhibit apoptosis, cells were treated with z-VAD-fmk (100 μM) for 15 min before addition of stimuli. After the treatments, cells were washed once with ice-cold PBS, and cell lysates were prepared in SDS-buffer (2% SDS in 62.5 mM Tris (pH 6.8), supplemented with 5% 2-ME, and 10% glycerol). Alternatively, HL-60 granulocytes were treated with 1 μM staurosporine or 2 μM camptothecin in medium containing 1 or 2 mM CaCl₂ for 5 h to induce apoptosis, and cell lysates were prepared as earlier described. Triplicate samples were used to analyze histone H3 deimination, histone H2B phosphorylation, and PARP cleavage by Western blotting. In separate experiments, HL-60 granulocytes were incubated at 37°C for 2 h with various concentrations of TNF, fMLP, LPS, LTA, or H₂O₂. To inhibit apoptosis, cells were treated with z-VAD-fmk (100 μM) for 15 min before addition of stimuli. After the treatments, cells were washed once with ice-cold PBS, and cell lysates were prepared in SDS-buffer (2% SDS in 62.5 mM Tris (pH 6.8), supplemented with 5% 2-ME, and 10% glycerol). Alternatively, HL-60 granulocytes were treated with 1 μM staurosporine or 2 μM camptothecin in medium containing 1 or 2 mM CaCl₂ for 5 h to induce apoptosis, and cell lysates were prepared as earlier described. Triplicate samples were used to analyze histone H3 deimination, histone H2B phosphorylation, and PARP cleavage by Western blotting.

**Neutrophil isolation**

Neutrophils were obtained from Lifeblood Biological Services in accord with protocols approved by the University of Tennessee Institutional Review Board and isolated following modified methods of Wang et al. (17) and Sergeant et al. (18). Briefly, neutrophils were purified at room temperature from the EDTA-anticoagulated blood of healthy donors. Neutrophils were enriched in the supernatant of a dextran sedimentation and in the pellet of an isopyknic density gradient (Gallard Schlesinger) under endotoxin-free conditions. Polypolyethylene tubes containing the cell pellets were transferred to ice, the remaining erythrocytes were lysed in ice-cold hypotonic (0.2%) sodium chloride solution for 30 s, and the solution was rendered physiologic saline by addition of hypertonic (1.6%) sodium chloride solution. At this point, neutrophil viability was typically >98%, as assessed by trypan blue dye exclusion. Neutrophils were suspended at a density of 2 × 10⁷/ml in PBS without Ca²⁺ or Mg²⁺, but with 0.1% glucose and 0.5% heat-inactivated human serum. Neutrophils were stimulated with increasing concentrations of TNF, fMLP, LPS, LTA, or H₂O₂ by preparing extracts at various times up to 5 h after addition of either stimulus. In parallel, we examined the requirement for caspases by preincubating the cells in the presence of z-VAD-fmk before stimulation. Following incubation, cell lysates were prepared, and the lysates were tested for histone H3 deimination and PARP cleavage by Western blotting.

**Neutrophil culture**

Neutrophils were cultured to analyze histone H3 deimination, histone H2B phosphorylation, and PARP cleavage by Western blotting. Cell lysates were separated on 12 or 15% SDS-PAGE, and proteins were blotted to nitrocellulose membranes. Membranes were blocked for 1 h at room temperature with 5% BSA or 5% milk in TBST (25 mM Tris (pH 7.2), 150 mM NaCl, and 0.1% Tween 20) and rinsed before overnight incubation at 4°C with a dilution of primary Abs in TBST. Subsequently, membranes were washed and incubated with goat anti-rabbit secondary Ab conjugated to HRP for 1 h at room temperature, washed three times with TBST and twice with TBS alone. The HRP activity was detected by using chemiluminescence reagent plus (PerkinElmer Life Sciences).

**Confocal microscopy**

Neutrophils were allowed to settle for 30 min onto glass coverslips that were precoated with 0.001% poly-L-lysine and placed in wells of 6-well tissue-culture plates at 37°C in 5% CO₂. The cells were treated with LPS, alternative stimuli in the presence or absence of various inhibitors, or left untreated in the absence of calcium, and incubated for 1 h at 37°C. The coverslips were washed inside the wells with ice-cold HBSS, the cells were fixed with 6% paraformaldehyde in HBSS for 15 min at room temperature and blocked overnight with blocking solution (HBSS with 10% FBS, 1% BSA, 0.05% Tween 20, and 2 mM EDTA) at 4°C. The next morning, the coverslips were washed with wash buffer (HBSS with 3% FBS), incubated with rabbit anti-citrullinated histone H3 Abs (diluted 1/100 in wash buffer) for 30 min at 4°C, washed again, and incubated with goat anti-rabbit IgG coupled with AF647, Sytox orange, and annexin V coupled to AF488 for 30 min at 4°C. Coverslips were washed, mounted on slides in wash buffer containing 50% glycerol, and analyzed by confocal microscopy, as previously described (19).

**Results**

Deimination of histones in ATRA-differentiated HL-60 cells treated with calcium ionophore is independent of caspase activation

Ca²⁺ ionophore treatment of ATRA-differentiated HL-60 cells resulted in readily detectable histone deimination, with citrullines in histone H3 being observed at 15 min after addition of ionophore and increasing in abundance until reaching a plateau at 2 h (Fig. 1A). Thus, as reported previously (2, 15), an increase in intracellular calcium results in rapid and sustained histone H3 deimination.

To test whether ionophore treatment induces apoptosis, we probed for a product of cleavage by caspase 3 (Fig. 1B). PARP, an enzyme that acts on DNA and chromatin at sites of DNA damage, is cleaved by caspase 3 (20). We used a mAb to detect both the full-length protein (116 kDa) and the cleaved N terminus (24 kDa) of PARP (see Materials and Methods). Lysates of ionophore-treated HL-60 cells contained both forms of PARP (Fig. 1B), although cleaved PARP could not be detected before 3 h after addition of ionophore (Fig. 1B). Thus, even though calcium influx into HL-60 cells induces caspase activation, cleavage of PARP was detected much later than histone H3 deimination following addition of A23187 ionophore. Each lane on the gel contained equivalent amounts of total protein, as indicated by reprobing the blot shown in Fig. 1A with Abs to β-actin (Fig. 1C).

To determine whether caspase activity contributes to the observed histone deimination in cells treated with ionophore, we pre-treated HL-60 cells with 100 μM z-VAD-fmk, a pan-caspase inhibitor. In the presence of z-VAD, histone H3 deimination followed a similar time course and reached a similar magnitude as in the absence of the caspase inhibitor (compare Fig. 1, A with D). The extent of deimination was somewhat greater at 15 min and 1 h in the presence of the inhibitor, and deimination reached a plateau at least 1 h earlier (Fig. 1D). The effective inhibition of caspase 3 by z-VAD was confirmed by an analysis of PARP cleavage in these samples (Fig. 1E). These data suggest that histone H3 deimination in response to calcium ionophore does not require a significant contribution from caspases.

To examine the effect of the calcium ionophore on the morphology of HL-60 cells, we treated cells as for the experiments described and used them in confocal microscopy (Fig. 1, G–I). We stained the nucleus with Sytox orange, a DNA dye, and we visualized cell membranes by the binding of annexin V. In response to the ionophore, we noted that the binding of annexin V to the plasma membrane was increased (Fig. 1H) relative to untreated cells (Fig. 1G), suggesting that membrane asymmetry is compromised by the integration of the ionophore into the plasma membrane. However, other morphologic hallmarks of apoptosis, such as chromatin condensation and nuclear fragmentation, were not readily observed in ionophore-treated cells. In addition, we noted that internal membranes were more reactive with annexin V in cells treated with calcium ionophore (Fig. 1H), suggesting a redistribution of phosphatidylserine from the plasma membrane to the internal cell membranes. Incubation with z-VAD before ionophore treatment decreased the staining of cells with annexin V (Fig. 1I), suggesting that the loss of membrane asymmetry and phospholipid redistribution may, at least in part, be a consequence of cell death.
of caspase activity. These experiments suggest that ionophore treatment does not result in typical morphologic changes associated with apoptosis and caution against the sole use of annexin V staining in the analysis of cellular events induced by ionophore treatment of neutrophils.

**Deimination of histones is not a feature of HL-60 cell apoptosis**

To test whether histone H3 deimination is induced in response to standard treatments that promote apoptosis, we exposed cells to 2 μM camptothecin or 1 μM staurosporine for 5 h. This time frame is sufficient to observe morphological changes indicative of apoptosis in these cells. Whereas HL-60 cells are relatively round in culture and contain a lobed nucleus after differentiation with ATRA (Fig. 2A), cells treated with camptothecin (Fig. 2B) or staurosporine (Fig. 2C) for 5 h exhibit chromatin condensation, nuclear fragmentation, and displacement of nuclear fragments to blebs at the cell surface. These morphologic changes are associated with the enzymatic activity of caspase 3, as shown by substantial PARP cleavage in camptothecin- or staurosporine-treated cells (Fig. 2D). Immunoblotting of cell lysates detected both intact PARP (116 kDa) and its cleavage product (24 kDa). At 5 h following induction of apoptosis, intact PARP was greatly reduced relative to PARP from undifferentiated or untreated cells, whereas the PARP cleavage product was readily detected (Fig. 2D). Caspase cleavage was not affected by varying the calcium concentration from 1 to 2 mM during the incubation.

Despite the effective induction of apoptosis, deiminated histone H3 could not be detected in HL-60 cells exposed to camptothecin or staurosporine (Fig. 2E). The lack of H3 deimination was not simply a consequence of insufficient Ca²⁺, as the presence of 1 or 2 mM Ca²⁺ in medium during the incubation period failed to enhance deimination (Fig. 2E). In contrast, treatment with Ca²⁺ ionophore induced readily detectable histone deimination.

To examine whether our conditions for inducing apoptosis allow other histone modifications to occur, the HL-60 lysates were analyzed for phosphorylation of Ser¹⁴ in histone H2B (Fig. 2F). This chromatin modification is induced in apoptosis (21). Phosphorylation of Ser¹⁴ was increased in lysates prepared from camptothecin-treated HL-60 cells relative to controls, whereas staurosporine-treated cells contained no detectable phosphoserine at position 14 of histone H2B. The reduced extent of H2B phosphorylation indicates that staurosporine, a well-known kinase inhibitor, also inhibits phosphorylation of Ser¹⁴ in histone H2B (21). A low but detectable amount of Ser¹⁴ phosphorylation was observed in untreated HL-60 cells, and that amount increased during ATRA treatment, indicating slightly increased apoptosis in cells treated with ATRA (Fig. 2F).

These experiments suggested that induction of apoptosis in ATRA-differentiated HL-60 cells proceeds without detectable histone H3 deimination, even though classical morphological features of apoptosis as well as caspase 3 activation, PARP cleavage,
and H2B phosphorylation are readily observed. These results indicated that histone deimination is not induced by the execution of apoptosis in ATRA-differentiated HL-60 cells.

**Stimuli that induce histone H3 deimination in HL-60 cells**

Because the induction of apoptosis in HL-60 cells could be dissociated from histone H3 deimination, we searched for alternative and more physiological stimuli that result in H3 deimination. Mature neutrophils are exquisitely sensitive to a variety of signals that arise in the course of infections (22). Many of these signals cause a transient increase in intracellular calcium levels or induce calcium signaling. To explore whether inflammatory stimuli lead to histone H3 deimination, we selected a set of treatments that activate or cause degranulation of neutrophils. Each of the selected stimuli ligates different cellular receptors (23–27), yet leads to elevation of intracellular calcium. Because calcium is required for PAD4 activity, we considered these stimuli as likely candidates for the physiological induction of histone deimination.

TNF is arguably the quintessential proinflammatory cytokine because it plays a role in neutrophil attachment to substrate, migration, priming, and degranulation (28). Neutrophils also produce TNF, thereby amplifying the inflammatory response. To test whether histone deimination is induced as part of the neutrophil response to TNF, we exposed ATRA-treated HL-60 cells to various concentrations of TNF over a period of 2 h and detected histone H3 deimination in cell lysates by immunoblotting (Fig. 3A). We observed a gradual increase in histone deimination in response to increasing concentrations of TNF over the range from 2 to 32 ng/ml (Fig. 3A).

To determine whether histone deimination is induced in HL-60 cells treated with other stimuli associated with an infection, we examined the response of HL-60 cells to f-MLP, a peptide that is generated by proteolysis of bacterial polypeptides and induces neutrophil chemotaxis and degranulation. We observed increased histone deimination as the concentration of f-MLP was raised from 0.25 to 1 nM (Fig. 3B). Similarly, exposure of HL-60 cells to LPS concentrations ranging from 1 ng/ml to 2 μg/ml resulted in increasing levels of deiminated histones, with levels approaching...
those observed with ionophore treatment (Fig. 3C). We also tested the response of HL-60 cells to H$_2$O$_2$, a substance produced during the respiratory burst in neutrophils, and observed a dose response over concentrations ranging from 0.1 to 10 mM (Fig. 3D). These concentrations were similar to concentrations that stimulate a calcium signal in granulocytes, suggesting that physiological stimuli that induce an inflammatory response also result in histone deimination.

To test whether the physiological stimuli that we used activate caspases and whether caspase activity is required for histone deimination, we assayed the time course of PARP cleavage and histone deimination in HL-60 cell lysates. We examined the extent of H3 deimination following different times of incubation in the presence of 100 ng/ml LPS and observed that deimination gradually increases as incubation times are extended from 1 to 5 h (Fig. 3E). Conversely, our analysis revealed no evidence of PARP cleavage even after 5 h of LPS treatment (Fig. 3F). This analysis was conducted with each of the stimuli, and only H$_2$O$_2$ incubation showed histone deimination (Fig. 3G) as well as PARP cleavage (Fig. 3H). Induction of caspases by treatment with reactive oxygen species has previously been observed (29). Inhibition of caspase 3 with z-VAD-fmk did not appreciably alter the extent of histone H3 deimination (Fig. 3G), although PARP cleavage was substantially reduced (Fig. 3H). These data indicate that inflammatory stimuli do not require activation of caspases to induce robust histone deimination.

**Stimuli that induce histone deimination in human blood neutrophils**

To extend our observations of histone deimination to human blood neutrophils, we purified polymorphonuclear granulocytes from the blood of healthy human donors and incubated them with stimuli that were effective at inducing histone deimination in HL-60 cells. Histone deimination increased as LPS concentration was raised from 0.1 to 1000 ng/ml (Fig. 4A). This result established that the LPS dose response, as measured by the detection of deiminated histone H3, mirrors other well-known neutrophil responses to LPS (30, 31). To assess the time dependence of this response, we monitored the extent of histone deimination at different time points up to 4 h after addition of 100 ng/ml LPS (Fig. 4B). Deimination could be detected as early as 30 min after LPS addition and the signal increased until reaching a plateau at 3 h after addition of the stimulus (Fig. 4B).

In addition, we established the neutrophil dose response to varying concentrations of TNF and f-MLP. We observed a gradual increase in histone deimination as TNF was increased from 0.5 to 8.0 ng/ml (Fig. 4C). For f-MLP, histone deimination could be detected starting at 0.1 nM and increasing up to 100 nM. The extent of histone H3 deimination at 1 µM was less compared with the level observed at 100 nM (Fig. 4D). By increasing the dose of the deaminase, deamination was increased. A dose response for LTA, a component of Gram-positive bacterial cell walls, was observed by raising the LTA concentration from 0.5 to 8.0 µg/ml and detecting increased H3 deimination (Fig. 4E). To test whether products of the oxidative burst induce histone deimination, we varied the H$_2$O$_2$ concentration from 1 µM to 10 mM to test the optimal response to peroxide. Maximal histone H3 deimination was observed at ~100 µM H$_2$O$_2$, although there was a fairly broad range of optimal stimulatory concentrations (Fig. 4F).

The signal intensity was compared with signals observed in the absence of stimulation and to the extent of histone H3 deimination induced by A23187 ionophore (Fig. 4F). We noted that a low level of deimination could be detected in cells incubated in buffer alone, although deimination in response to the various stimuli was invariably greater and easily approached the levels observed following ionophore treatment.

**Chromatin released by degranulating neutrophils contains deiminated histone H3**

Conflicting reports have indicated that PAD4 is either a component of cytoplasmic granules in neutrophils (9) or is a nuclear protein (15). The data indicating granule association were obtained by using blood neutrophils (9), whereas nuclear localization of PAD4 was observed in transfected HeLa cells (15). The need to assay localization of PAD4 in heterologous systems reflects, in part, the difficulty of transfecting neutrophils, and in part, the difficulty of detecting endogenous PAD4 by immunofluorescence (15). Because of the reported difficulty of observing PAD4 in neutrophils, we limited our study to the detection of its most abundant reaction product, the deiminated core histone H3.

To examine the cellular distribution of deiminated histone H3, we used Abs to the citrullinated terminus of H3 in confocal microscopy (Fig. 5). We detected nuclear DNA by its binding to Sytox orange, a sensitive and highly specific DNA dye. The plasma membrane was visualized by binding of fluorescent annexin V. Neutrophils incubated in the absence of proinflammatory stimuli exhibited no detectable histone H3 deimination (Fig. 5A). However, within 1 h of adding f-MLP to neutrophils, Ab binding to cytoplasmic sites (Fig. 5B, open arrowhead) and smaller, less numerous nuclear foci (Fig. 5B, solid arrowhead) produced intense fluorescence. Cytoplasmic staining alone, or a combination of both cytoplasmic and nuclear staining (Fig. 5B), was present in ~10–20% of treated cells. One possibility to account for our observations is that, as reported by another study (9), PAD4 accumulates...
in cytoplasmic granules and that histone H3 is subject to deimination either concurrently with, or immediately following, translation, but before incorporation into chromatin. Alternatively, the nuclear fluorescence may indicate that deimination also occurs at specific sites in the nucleus. However, the diminished or absent binding to citrullinated H3 in the nucleus compared with the binding in the cytoplasm suggests that most H3 deimination occurs in the cytoplasm.

One difficulty in precisely describing the events surrounding histone deimination in neutrophils is that deimination overlaps in time with a massive redistribution of chromatin that accompanies the production of “neutrophil extracellular traps” (“NETs”) (32, 33). In response to the same stimuli that we used to induce histone deimination, neutrophils disintegrate their nuclei and release chromatin from the cell. Such extracellular chromatin may serve to immobilize bacteria and prevent the spread of the infection (33). Histone deimination may be linked to this chromatin redistribution, as our data illustrate (Fig. 5C). One hour after addition of f-MLP to the cells, we observed neutrophils in which Abs to deiminated H3 band to foci in the cytoplasm (Fig. 5C, solid arrowhead), additional neutrophils in which Ab binding was distributed throughout the interior of the cell (Fig. 5C, open arrowhead), and several neutrophils that had released chromatin from the cell. Binding of Ab showed deiminated histone H3 in association with the dispersed, extracellular chromatin NETs, as fluorescence signals for DNA (blue) and for the citrullinated H3 (red) closely overlapped. Integration of these and other data from a timed series of images suggests that histone deimination mainly occurs in the cytoplasm during or shortly after histone biosynthesis. The data also suggest that deiminated histones associate with chromatin inside the nucleus, after the release of chromatin into the cytoplasm, or both. Following NET release, deiminated histones remain attached to extracellular chromatin.

Neutrophils exposed to LPS undergo the full spectrum of sequential morphologic changes (Fig. 5D). Following 1 h of incubation with LPS, cells that displayed intact nuclei contained deiminated H3 within cytoplasmic foci (Fig. 5D), whereas cells that had intermixed their nuclear and cytoplasmic compartments showed more uniform Ab binding extending throughout the cell (Fig. 5D). In general, the chromatin released into NETs had an irregular distribution. However, a fortuitous bundling of NETs (Fig. 5D, arrow) allowed us to confirm the precise association of deiminated histone H3 with chromatin fibers. Ionophore A23187 treatment of neutrophils resulted in a rapid deimination of histone H3 and its association with DNA (Fig. 5E).

To explore the relation between protein translation and histone deimination, we pretreated cells with 20 μM cycloheximide, to inhibit protein synthesis (Fig. 5F). In the presence of cycloheximide, the deimination of histone H3 was greatly reduced, as assessed by binding of the anti-deiminated H3 Abs. Despite the reduced H3 deimination, cells were able to release NETs, demonstrating that deimination is not required for the release of extracellular chromatin. These data support the notion that deimination of histone H3 is tightly linked to translation and that, in the absence of active protein synthesis, histone deimination is greatly reduced. As expected, apoptotic cells showed no evidence of histone deimination (Fig. 5G). Anti-citrullinated H3 Abs and stimulation of neutrophils were required for red immunofluorescence, as induced (or uninduced) cells incubated with a nonspecific rabbit IgG or with secondary reagents alone failed to generate detectable signals (Fig. 5H).

In summary, our microscopic analyses demonstrate that neutrophils deiminate histones in response to a wide range of substances arising in infections. The response is rapid and sustained. Moreover, it overlaps in time and may be coordinated with the extrusion of chromatin from the nucleus and with its release from the cell. Thus, stimulation of neutrophils in the course of an infection may result in the extrusion of a modified self-Ag from the cells.
Discussion

Neutrophils undergo vastly different forms of cell death depending on whether they encounter an infection while circulating in the blood. In the absence of infection, neutrophils senesce within 6–10 h of entering the circulation, when they return to the bone marrow and undergo TRAIL-mediated apoptosis (34). In contrast, neutrophils that encounter an infection experience a drastic change in cellular activity (22). At the site of infection, neutrophils exit the vasculature and migrate toward the pathogens in response to a gradient of chemotactic substances such as IL-8, C5a, and leukotrienes. Bacterial products and cytokines activate the neutrophils and delay the induction of apoptosis (35). Activated neutrophils perform a set of functions that kill the pathogen, amplify the inflammatory response, and modify local and distal immune responses (28). Neutrophils accomplish these functions by engulfing microbial pathogens, mobilizing granule contents that mediate microbial killing and inflammation, and producing reactive oxygen and nitrogen. In addition, neutrophils deposit a meshwork of DNA and histones called NETs that physically immobilizes a pathogen (32). Clearly, phagocytosis, degranulation, and release of nuclear contents result in a type of cell death that is very different from the classic apoptosis of senescent neutrophils (29, 33, 36, 37).

Both genetic and structural analyses provide insight into the differences between apoptosis and activation-induced cell death in neutrophils. Microarray analysis indicates the induction of two different, yet overlapping sets of genes in neutrophils stimulated to undergo apoptosis vs those activated by the presence of complement and Ab-coated particles (38). Microscopy demonstrates that the exposure of neutrophils to bacteria induces the release of extracellular chromatin incorporating bactericidal granule components (33). The extracellular chromatin kills bacteria even if phagocytosis is prevented. The morphology of neutrophils after the release of extracellular chromatin is clearly distinguishable from the classical outward signs of apoptosis (33). The expression of PAD4 during the terminal differentiation of neutrophils (39) raised the possibility that the reaction conducted by this enzyme, the deimination of core histones, plays a unique role in the neutrophil response to infections. We report that deiminated histone H3, the major product of PAD4 activation in neutrophils, provides a very clear distinction between neutrophil activation by inflammatory stimuli and the apoptotic death of neutrophils.

We found that conventional treatments for inducing apoptosis, such as incubation of HL-60 cells with camptothecin or staurosporine, failed to stimulate histone H3 deimination (Fig. 2). In contrast, physiological stimuli that induce neutrophil degranulation resulted in histone H3 deimination (Fig. 3). In general, freshly isolated human blood neutrophils also responded with histone deimination to stimuli that signal the presence of bacterial pathogens (Fig. 4). Neutrophils responded more vigorously than HL-60 cells at lower concentrations of LPS, TNF, fMLP, and H₂O₂ (Fig. 3 vs 4). Because histone deimination in neutrophils represented a rapid and robust reaction to signals arising in a microbial infection, we propose that histone deimination forms an integral part of the neutrophil response to infections. In responses that induced both deimination and caspase 3 activation, caspase activation was secondary to deimination, and the inhibition of caspase activity by z-VAD did not reduce the extent of deimination (Figs. 1 and 3).

At present, very little is known about the regulation of histone deimination, except that the enzyme responsible for this modification, PAD4, requires calcium. The results presented in this study suggest that a variety of signals, converging on a common pathway, regulate the activity of PAD4 and that induction of apoptosis prevents PAD4 activity. Because the NF-κB family of transcription factors is of fundamental importance in the regulation of genes that respond to inflammatory stimuli, as well as in the regulation of neutrophil apoptosis, we suspect that NF-κB intersects or regulates the signaling pathways that lead to histone deimination.

What are some of the possible roles for citrullinated histones in an infection? Our observation that deiminated histone H3 is associated with NETs suggests that deiminated histones may have a role in the killing of trapped microbes. Support for this idea comes from the observations that histones are among the most potent bactericidal proteins in eukaryotic cells (40) and that peptides derived from histones are produced and secreted in epithelia that act as barriers to microbial invasion in frogs, fish, and mammals (41–43). The association with DNA in NETs may allow deiminated histones to gain closer proximity to bacteria and help them in disrupting their structural integrity. The loss of positive charge in the terminus of H3 may affect the interactions with the bacterial cell wall (44) or plasma membrane, in parallel to observations with other amphiphilic peptides (45).

Alternatively, deimination of histones may be required for constructing the relaxed structure of chromatin in NETs. It is notable that histone deimination affects the N terminus of H3, a histone domain that mediates interactions between adjacent nucleosomes (46). Perhaps, nuclear chromatin must be loosened and nucleosomes rearranged, to generate suitable extended structures that are characteristic of NETs. In addition, a relationship may exist between the formation of NETs and the need to terminate RNA transcription. Deiminated histones are considered to function as transcriptional repressors, because they antagonize histone arginine methylation at certain inducible promoters (6). Cessation of transcription may be necessary to alter the structure of chromatin because RNA-DNA heteroduplexes provide structural stability to large chromatin regions (47).

In addition, deiminated histones may assume an important signaling function upon their release from neutrophils. Such is the case with HMGB-1, a non-histone chromosomal protein that is released from damaged cells and functions as an inflammatory signal in the extracellular space (48). Release of HMGB-1 from cells appears to depend on the type of cell death, such that unscheduled death caused by infection may lead to immune activation (49, 50). Because the formation of deiminated histones depends on inflammatory signals received by neutrophils, it is tempting to consider that release of citrullinated H3 to the extracellular space may convey an important message regarding the cause of cell death to the immune system.

In parallel with the possible beneficial functions of deiminated histones in combating microbial pathogens, the expression of a modified form of a major human autoantigen in the context of an inflammatory response may have deleterious consequences for the host. It is conceivable that the combination of covalently altered histones and bacterial structures that signal via TLR and other pattern recognition receptors may break tolerance and initiate adaptive immune responses that, over time, may extend to the more common unmodified form of the autoantigen. If so, the production of deiminated histones in the course of an infection, or due to persistent neutrophil activation, may provide the initial stimulus for autoimmunity. In that case, histone deimination may provide a molecular link between infections and the induction of systemic autoimmunity.

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Disclosures

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