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Role of Macrophage Lysosomal Enzymes in the Degradation of Nucleosomes of Apoptotic Cells

Chikako Odaka¹ and Toshiaki Mizuochi

Although apoptotic cells are recognized and engulfed by macrophages via a number of membrane receptors, little is known about the fate of apoptotic cells after the engulfment. We observed in this study that nucleosomal DNA fragments of apoptotic cells disappeared when they were engulfed by the macrophage cell line J774.1 at 37°C. Pretreatment of J774.1 cells with chloroquine inhibited intensive DNA degradation, indicating that the cleavage of nucleosomal DNA fragments of apoptotic cells may take place in the lysosomes of J774.1. When apoptotic cells were exposed to a lysosome-rich fraction derived from J774.1 cells under an acidic condition, nucleosomal DNA fragments of apoptotic cells were no longer detectable by agarose gel electrophoresis. Additionally, we found that the lysosome-rich fraction of J774.1 cells contained an acid DNase that is similar to DNase II with respect to its m.w., optimal pH, and sensitivity to the inhibitors of DNase II. By exposure of apoptotic cells to the lysosomal-rich fraction, nucleosomal core histones of apoptotic cells were hydrolyzed along with degradation of nucleosomal DNA fragments. Addition of pepstatin A to the reaction buffer resulted in accumulation of ~180-bp DNA fragments and inhibition of hydrolysis of nucleosomal core histones. Leupeptin or CA-074 partially inhibited the degradation of nucleosomal DNA fragments and core histones. These findings suggest that lysosomal enzymes of macrophages, e.g., DNase II-like acid DNase and cathepsins, are responsible for the degradation of nucleosomes of apoptotic cells. The Journal of Immunology, 1999, 163: 5346–5352.

Programmed cell death is recognized as the physiological mechanism by which a large number of unwanted cells are deleted from the body. Once cells undergo programmed cell death, their corpses are swiftly engulfed by other cells and degraded. This engulfment process involves the recognition and subsequent phagocytosis of cell corpses by engulfing cells. The process is important for tissue remodeling and for the resolution of inflammatory responses (1).

The mechanisms by which apoptotic cells can be recognized and removed have been the subject of intense investigation for the last few years. An important consequence of the apoptotic process is cell surface alterations that lead to rapid recognition by phagocytes. A number of surface molecules are involved in the recognition of apoptotic cells by macrophages or dendritic cells, among which are an uncharacterized lectin inhibited by N-acetylgalactosamine (2), the vitronectin receptors (αvβ3, integrin) (3), which is thought to cooperate with CD36 in binding to thrombospondin on the surface of the apoptotic cells (4, 5), a phosphatidyl-l-serine receptor (6, 7), scavenger receptors (8 –10), and the macrophage Ag identified by the mAb 61D3 (11), which is identical to CD14 (12). The ABC1 transporter has also been suggested to be involved in phagocytosis (13). In contrast, little is known about the intracellular processing by which macrophages dispose of apoptotic cells after engulfment.

The biochemical hallmark of apoptosis is the appearance of a fragmentation pattern in chromatin, which is indicative of the DNA cleavage at the linker regions between nucleosomes. The DNA fragments yield discrete multiples of a 180-bp subunit that is detected as a “DNA ladder” on agarose gels after isolation of the DNA from apoptotic cells (14).

The thymus is the organ in which the repertoire of T cells is selected from a much larger number of immature thymocytes, and extensive apoptotic cell death occurs in immature thymocyte populations. Although numerous immature thymocytes undergo apoptosis, few dead cells are observed in situ due to rapid engulfment by phagocytic macrophages in the thymus (15). Recent studies, using the sensitive TUNEL technique to examine the distribution of apoptotic thymocytes, demonstrated an increase in the number of TUNEL-positive cells in the cortex of the thymus within a few hours after administration of glucocorticoid or anti-CD3 Ab (16–18). The clearance of TUNEL-positive apoptotic thymocytes was found to be conducted by macrophages in thymus. After apoptotic cells are engulfed by macrophages, the number of TUNEL-positive apoptotic thymocytes gradually reduces and finally becomes comparable with that of TUNEL-positive cells in untreated thymus (17, 18). These observations prompted us to predict that the nucleosomal DNA fragments of apoptotic cells might be further degraded when apoptotic cells are phagocytosed by macrophages.

In the present study, we investigated the fate of apoptotic cells after engulfment by macrophages. In particular, we traced the DNA fragments of apoptotic cells upon their engulfment by macrophages and observed a disappearance of nucleosomal DNA ladder formation in apoptotic cells. Furthermore, our study demonstrated that the lysosomal enzymes in macrophages were involved in the degradation of nucleosomes of engulfed apoptotic cells. These findings will be discussed with reference to the importance of apoptotic cell scavenger.

Materials and Methods

Cells and reagents
IL-2-dependent CTLL-2 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 50 μM 2-ME, 20 U/ml penicillin, 20 μg/ml streptomycin, and 100 U/ml recombinant mouse IL-2 (19). Mouse macrophage cell line J774.1 cells were maintained in RPMI 1640...
supplemented with 10% heat-inactivated FCS, 50 μM 2-ME, and the antibiotics. The RPMI 1640 and supplements were purchased from Life Technologies (Grand Island, NY), Pepstatin A, CA-074, and leupeptin were obtained from Peptide Institute (Osaka, Japan). All other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted.

Assay for phagocytosis of apoptotic cells

One day before assay for phagocytosis, J774.1 cells were seeded at a density of 1 × 10^6/ml in 100-mm^2 dishes. CTL-2 cells were cultured in the absence of IL-2 for 15 h, with the result that almost all cells showed apoptosis. These cells were used as apoptotic CTL-2 in following experiments.

Apoptotic CTL-2 cells (1 × 10^7) were added to macrophage monolayers and then cultured at 37°C for 1 h. After nonphagocytosed apoptotic cells were removed by extensive washing with RPMI 1640, J774.1 cells were incubated in RPMI 1640 containing 10% FCS at 37°C or at 4°C for the indicated period. In some experiments, J774.1 cells were treated with the indicated concentration of chloroquine. One hour later cells were extensively washed with RPMI 1640 three times and then cultured with apoptotic cells for 1 h. Then, J774.1 cells were washed to eliminate nonphagocytosed apoptotic CTL-2 cells and incubated in fresh medium for 3 h.

Detection of DNA cleavage in situ by TUNEL

For TUNEL staining, J774.1 cells were plated on 8-well chamber slides at 1 × 10^5 cells/well and incubated overnight. Apoptotic CTL-2 cells (1 × 10^5 cells/well) were added to the above J774.1 cells and incubated at 37°C for 1 h. Nonphagocytosed apoptotic cells were removed and then incubated at 4°C or at 37°C. At the indicated time, the cells were fixed in 4% buffered formaldehyde and permeabilized with 0.5% saponin/1% BSA in PBS. The cells were further incubated for 60 min at 37°C in a reaction buffer consisting of 100 mM sodium cacodylate (pH 7.2), 1 mM CoCl_2, 10 mM biotin-16-dUTP (Boehringer Mannheim, Mannheim, Germany), and 100 U/ml TdT (Takara Shuzo, Kyoto, Japan), followed by incubation with avidin-biotin-peroxidase complexes using the Vectastain-ABC kit, (Vector, Burlingame, CA) for 30 min. Cells were washed and then incubated with a mixture of 0.06% 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 0.03% H_2O_2 in 0.1 M Tris-HCl (pH 7.5). Counterstaining was performed with Meyer’s haematoxylin. Microscopic observations were conducted by using Microflex UVF-II (Nikon, Tokyo, Japan).

Isolation and detection of DNA fragments

Cells were resuspended in hypotonic lysis buffer (0.25% Triton X-100, 10 mM Tris-HCl, and 10 mM EDTA, pH 8.0) and centrifuged for 15 min at 20,000 × g. The supernatant, containing small DNA fragments, was treated with 1000 units proteinase K and 50 μg/ml RNase A. The DNA was extracted by phenol/chloroform and precipitated in isopropyl alcohol containing 0.5 M NaCl. After the sample was centrifuged, the pellet was washed with absolute ethanol and allowed to dry at room temperature. The pellets were suspended in 20 μl of solution containing 0.1 M glycine, 0.2% SDS, and 4 M urea (pH 10), and SDS-PAGE for analysis of histone hydrolysis was performed as described by Panyin and Chalkley (21) with a slight modification in a 17.5% polyacrylamide gel. The proteins in the gel were stained with Coomassie brilliant blue.

Results

Fate of DNA strand breaks in apoptotic cells engulfed by J774.1 cells

In the previous studies, deprivation of IL-2 from IL-2-dependent CTL-2 was shown to result in apoptotic cell death (23, 24). In our study, almost all CTL-2 cells were found to be dead when cultured in the absence of IL-2 for 15 h, as assessed by trypan blue dye exclusion (data not shown). When murine macrophage-like cells J774.1 were exposed to a 10-fold excess of apoptotic CTL-2 cells and incubated for 1 h at 37°C, ~70% of apoptotic cells were engulfed by J774.1 cells (data not shown). To examine the processing of apoptotic CTL-2 cells that were engulfed by J774.1 macrophages, we performed TUNEL staining, which allows detection of DNA strand breaks in the apoptotic CTL-2 cells (16). J774.1 cells were coincubated with dead CTL-2 cells for 1 h at 37°C, and they were extensively washed and fixed. DNA strand breaks of apoptotic cells in J774.1 macrophages were then evaluated by the TUNEL method. A significant number of DNA breaks of apoptotic cells was detected in J774.1 cells (Fig. 1a). After further incubation for 6 h at 37°C or at 4°C, the cells were fixed and processed for TUNEL staining. In an additional incubation for 6 h at 37°C, the number of TUNEL-positive cells was substantially decreased in the engulfing cells. (Fig. 1c). The process was inhibited when J774.1 cells were kept at 4°C over a 6-h period (Fig. 1b). Thus, when J774.1 cells that engulfed apoptotic cells were incubated for 6 h at 37°C, a disappearance of DNA breaks in nuclei of apoptotic cells was observed.

Degradation of apoptotic DNA fragments in J774.1 cells

DNA fragmentation of apoptotic CTL-2 cells can be visualized on a gel as a series of fragments that are multiples of 180 bp (14). When DNA isolated from apoptotic CTL-2 cells was subjected to agarose gel electrophoresis, DNA fragmentation, as demonstrated by a characteristic “DNA ladder” formation, was observed (data not shown). After J774.1 cells were coincubated with a 10-fold excess of apoptotic CTL-2 cells at 37°C for 1 h, they were extensively washed to eliminate nonphagocytosed apoptotic cells and incubated in a fresh medium at 37°C for up to 6 h. The cells were lysed with hypotonic lysis buffer, and the DNA fragments of apoptotic cells engulfed by J774.1 cells were isolated and then subjected to agarose gel electrophoresis.

After J774.1 cells were coincubated with dead CTL-2 cells for 1 h, nucleosomal DNA ladder formation of apoptotic cells engulfed by J774.1 cells was detectable on the agarose gel (Fig. 2, lane 2). An additional incubation at 37°C leaded to a disappearance of DNA ladder in a time-dependent manner. In a 2-h incubation, internucleosomal DNA fragments of apoptotic cells were still visible on the gel (Fig. 2, lane 4), whereas the DNA ladder was washed away in 40 mM Tris-HCl (pH 7.4), the gel was divided into three sections. Each gel was incubated in 40 mM Tris-HCl (pH 7.4) or 40 mM sodium acetate buffer (pH 5.2) with or without iodoacetic acid for 20 h at room temperature. The gels were stained with ethidium bromide (0.5 μg/ml) and illuminated under UV light.

Detection of histone hydrolysis

Cells were resuspended in 0.4 N H_2SO_4 and kept on ice for 30 min. After centrifugation at 20,000 × g for 15 min, the supernatant was collected and then four times the volume of absolute ethanol was added to the samples. After centrifugation, the pellet was washed with absolute ethanol and allowed to dry at room temperature. The pellets were suspended in 20 μl of solution containing 0.1 M glycine, 0.2% SDS, and 4 M urea (pH 10), and SDS-PAGE for analysis of histone hydrolysis was performed as described by Panyin and Chalkley (21) with a slight modification in a 17.5% polyacrylamide gel. The proteins in the gel were stained with Coomassie brilliant blue.
These results are representative of three independent experiments.

6 h at 4°C (lane 5). In a 6-h incubation at 37°C, internucleosomal DNA fragments of apoptotic cells engulfed by J774.1 cells was no longer detectable on the gel (Fig. 2, lane 6), which is concordant with the result obtained by the TUNEL method shown in Fig. 1. On the other hand, when apoptotic CTL-2 cells engulfed by J774.1 cells were incubated for 6 h at 4°C, the DNA ladder remained prominent (Fig. 2, lane 3). These findings suggest that the degradation of DNA fragments of apoptotic cells occurs in J774.1 cells and that macrophages may contain enzymes capable of digesting the DNA fragments of apoptotic cells. The above process was significantly inhibited when the experiment was performed at 4°C, i.e., at a temperature that does not permit phagocytosis.

Lysosomes are highly specialized organelles that have a low internal pH and contain hydrolytic enzymes that have an optimum acidic pH. Chloroquine is known to raise the pH in lysosomes/endosomes and to be concentrated inside them (25, 26). To investigate whether lysosomal enzymes of macrophages are involved in digesting the internucleosomal DNA fragments of apoptotic CTL-2 cells, J774.1 cells were pretreated with chloroquine before the exposure of apoptotic CTL-2 cells. Pretreatment of J774.1 cells with 50 or 100 μM chloroquine did not affect engulfment of apoptotic cells (data not shown). As shown in Fig. 3, when apoptotic cells engulfed by untreated J774.1 were incubated for 3 h at 37°C, nucleosomal DNA fragments were hardly detectable on the gel (Fig. 3, lane 2). Chloroquine at the concentration of 100 μM was able to inhibit DNA degradation in phagocytosis by macrophages (Fig. 3, lane 3). Chloroquine at 50 μM only slightly inhibited DNA degradation (Fig. 3, lane 4). Thus, blocking the acidification of lysosomes/endosomes by chloroquine caused the inhibitory effect on the processing of DNA cleavage of apoptotic cells in macrophages. Taken together, these results indicate that lysosomal enzymes including DNase(s) in macrophages are responsible for degradation of nucleosomal DNA fragments of apoptotic cells.

Acid DNase activity in the lysosome-rich fraction of macrophages

We next sought to identify the lysosomal enzymes involved in DNA degradation of apoptotic cells. To find out whether DNase(s)
results demonstrated that the lysosomal fraction derived from apoptotic cells was completely inhibited (Fig. 5, presence of each reagent, degradation of DNA fragments of apoptotic cells was included in the reaction buffer. When apoptotic cells were exposed to the lysosome-rich fraction at pH 5.2, the DNA ladder was no longer detectable on a 2% agarose gel (Fig. 5, lane 2). No detectable hydrolysis of the DNA ladder was observed when the incubation was performed at pH 7.4 (data not shown). As described above, DNase II is shown to be inactivated in the presence of sulfate ions, zinc ions, or iodoacetic acid (27, 28). Therefore, 2 mM ZnSO4 or 1 mM iodoacetic acid was included in the reaction buffer. When apoptotic cells were incubated with the lysosome-rich fraction of J774.1 cells in the presence of each reagent, degradation of DNA fragments of apoptotic cells was completely inhibited (Fig. 5, lanes 3 and 4). These results demonstrated that the lysosomal fraction derived from J774.1 cells was able to degrade nucleosomal DNA fragments of apoptotic cells into small random-sized fragments that were hardly visible on a 2% agarose gel. ZnSO4- or iodoacetic acid-sensitive acid DNase of the lysosome-rich fraction of J774.1 cells may be responsible for the DNA degradation.

Cathepsins B and D are major lysosomal proteinases, and each enzyme can contribute to up to 10% of the total lysosomal proteins. So far, a number of studies have discovered several other cathepsins. To determine whether cathepsins in lysosomes of J774.1 cells would be involved in the degradation of apoptotic cells, cathepsin inhibitors were introduced into the reaction buffer containing apoptotic CTL-L-2 cells and the lysosome-rich fraction of J774.1 cells. Leupeptin strongly inhibits trypsin, plasmin, papain, and cathepsins B and L, but it has little or no inhibitory activity on cathepsin A and D (29). It inhibits cathepsin L less effectively than cathepsin B (30). CA-074 exhibits greater inhibitory effects on cathepsin B than leupeptin (31). Pepstatin A is known as a strong inhibitor of acid proteases, including pepsin, renin, and cathepsin D (32) and cathepsin E (33). As shown in Fig. 5, lane 7, the addition of pepstatin A induced a significant increase in the accumulated ~180-bp DNA fragments, i.e., mono-nucleosomal DNA. Leupeptin or CA-074 showed a weak inhibitory effect on the degradation of nucleosomal DNA fragments of apoptotic cells (Fig. 5, lanes 5 and 6). Combination of pepstatin A and leupeptin were more effective than pepstatin A alone (Fig. 5, lane 8). The presence of 1% DMSO in the reaction buffer did not affect the lysosome-rich fraction-induced DNA degradation (data not shown). Thus, cathepsins in the lysosome-rich fraction of J774.1 appear to be necessary for the degradation of DNA fragments of apoptotic cells. Pepstatin A-sensitive cathepsins are likely to be involved in the degradation of nucleosomal DNA. Moreover, partial inhibition by leupeptin or CA-074 may suggest the involvement of several other lysosomal proteinases in the DNA cleavage.

Proteolysis of nucleosomal core histones in apoptotic cells exposed to the lysosome-rich fraction of macrophages

As shown in Fig. 5, when apoptotic cells were incubated with the lysosome-rich fraction of J774.1 cells in the presence of cathepsin...
were extracted from 8\times 10^4 cells. A total of 8 \times 10^4 apoptotic CTL-2 cells were incubated at 37°C for 2 h in the presence of the lysosome-rich fraction of J774.1 cells with the following reagents: 0.5 mM leupeptin and 0.5 mM pepstatin A (lane 4), 0.5 mM CA-074 (lane 5), 0.5 mM leupeptin (lane 6), and 0.5 mM pepstatin A (lane 7). Acid proteins were extracted from these cells and subjected to SDS-PAGE. The proteins in the gel were stained with Coomassie brilliant blue. The molecular masses at left indicate the migration of standard proteins. Each core histone was run in the gel and these migrations are shown at right. The result is representative of three independent experiments.

inhibitors, the degradation of mono-nucleosomal DNA fragment was largely protected. During apoptosis, double-stranded DNA is cleaved at most accessible internucleosomal linker region, resulting in the generation of mono- and oligo-nucleosomal DNA. DNA of the nucleosomes is tightly complexed with the core histones H2A, H2B, H3, and H4 and therefore protected from the cleavage by endonuclease (34). With respect to these findings, when apoptotic cells are exposed to macrophage lysosomes, proteolytic degradation of core histones may take place simultaneously with the digestion of nucleosomal DNA fragments into smaller DNA fragments.

A number of studies have been reported on histone-hydrolyzing proteinase activities in chromatin isolated from calf thymus (35–38) or from rat liver (39, 40). On the other hand, it has been demonstrated that lysosomal cathepsins are capable of hydrolyzing all types of core histones (41–43). Therefore, it was hypothesized that core histones of apoptotic cells might be degraded by lysosomal cathepsins in macrophages. To confirm this, we examined by in vitro studies using cathepsin inhibitors whether the lysosome-rich fraction of J774.1 cells contains histone-hydrolyzing proteinases. After CTL-2 cells were cultured in the presence or absence of IL-2 for 16 h, histones of apoptotic CTL-2 cells were extracted and thereafter subjected to SDS-PAGE for the detection of histone subtypes and their hydrolysis. The amount of core histones of apoptotic cells was relatively smaller than that of CTL-2 cells cultured in the presence of IL-2 (Fig. 6, lane 1 vs lane 2). It may be due to the release of cellular contents including nucleosomes into culture medium during apoptosis. However, nucleosomal core histones of apoptotic cells including H2A, H2B, H3, and H4 remained intact during apoptosis (Fig. 6, lane 2). By exposure of apoptotic CTL-2 cells to the lysosome-rich fraction of J774.1 cells for 2 h, histones H2A, H2B, H3, and H4, which are bound to DNA, were degraded almost completely (Fig. 6, lane 3). The proteolytic fragments of these histones were no longer detected because they might be run away from the polyacrylamide gel during the experimental procedure. Pepstatin A effectively inhibited core histones’ degradation, indicating that nucleosomal core histones of apoptotic cells may be degraded mainly by pepstatin A-sensitive lysosomal cathepsins of J774.1 cells (Fig. 6, lane 7). Leupeptin showed an inhibitory effect (Fig. 6, lane 6), whereas CA-074 had a weak inhibitory effect on the histone degradation (Fig. 6, lane 5). Addition of both pepstatin A and leupeptin to the reaction buffer induced the inhibition of proteolysis of core histones most effectively (Fig. 6, lane 4).

From these results, we concluded that lysosomal proteinases of J774.1 cells were capable of hydrolyzing nucleosomal core histones. Proteolytic degradation of core histones may be a necessary step in the cleavage of nucleosomal DNA fragments into small DNA fragments.

Discussion

In the present study, we observed a rapid disappearance of DNA strand breaks in apoptotic cells that were phagocytosed by macrophage J774.1 cells, as assessed by TUNEL staining. Furthermore, internucleosomal DNA fragments in apoptotic cells engulfed by J774.1 cells became undetectable when the DNA of apoptotic cells was analyzed by electrophoresis on an agarose gel. When the fate of DNA fragments of apoptotic cells after engulfment by resident peritoneal macrophages of BALB/c or C3H/HeJ mice was examined, we also observed the disappearance of nucleosomal DNA fragments of apoptotic cells (data not shown). Pretreatment of J774.1 cells with chloroquine inhibited the degradation of DNA fragments in apoptotic cells. This finding implies that DNA hydrolytic activity of macropahges is mainly localized in the lysosomes. Furthermore, our study using a cell-free system indicated that an acid DNase in the lysosomes of J774.1 cells may be responsible for the degradation of internucleosomal DNA fragments of apoptotic cells.

DNase II hydrolyzes DNA to 3'-phosphoryl oligonucleotides under acidic conditions and therefore has been designated as an “acid DNase” (44). de Duke et al. (20), from the results of differential centrifugation of homogenate of rat liver, demonstrated that DNase II is a lysosomal enzyme. Furthermore, DNase II was directly isolated from lysosomes in the rat liver (27) and in porcine spleen (45, 46), which provided additional evidence that DNase II is lysosomal. DNase II activity can be detected in various mammalian tissues and species (47). The enzymatic properties of DNase II from different tissues and animals are found to be very similar, but their structures and the estimated molecular weights are significantly diverse. Porcine spleen DNase II is a heterodimeric protein, consisting of a 1:1 complex of an α and β subunit with molecular masses of 35 kDa and 10 kDa, respectively, whereas DNase II from other sources consists of a single polypeptide chain with the following molecular masses: 36–38 kDa from rat liver (27), 26.5 kDa from bovine liver (48), 45 kDa from human lymphoblasts (49), and 32 kDa from human urine (50). Although the reasons for the variability remain unknown, recent studies of molecular cloning of porcine, human, and murine DNase II have explained the previously reported discrepancies among the molecular weights of DNase II (51, 52). These sequence analyses indicate that mature human or murine DNase II is a 344 aa protein, which contains four potential N-linked glycosylation sites, and that its predicted size is 42–44 kDa. The reported α and β subunits of porcine DNase II are encoded by one cDNA, indicating that the porcine 10-kDa subunit results from cleavage of a larger precursor protein. In addition, Yasuda et al. (53) indicated that structural organization of the cDNA encoding human DNase II is similar to those of lysosomal cathepsin families. Thus, our results suggest that a DNase in the lysosome-rich fraction of J774.1 cells is similar to DNase II.
with respect to its m.w., optimal pH, and sensitivity to DNase II inhibitors.

Endonuclease has been proposed to be responsible for the internucleosomal cleavage of the nuclear DNA during apoptosis and has received much attention for the past few years. Barry and Eastmann (54) implicated DNase II as the enzyme that degrades DNA in apoptosis associated with intracellular acidification. Torriglia et al. (55, 56) showed the involvement of L-DNase II in nuclear degradation in lens fiber cells. de Duve et al. (57) proposed that lysosomal DNase II serves a scavenging function in phagosomes. Our observation may support their hypothesis. In Caenorhabditis elegans, programmed cell death occurs normally in the mutants that lack the activity of nuc-1-encoded acid nuclease, although the nucleus remains intact after phagocytosis (58–60). It is proposed that the acid nuclease functions in late event after phagocytosis of dead cells (59, 61). Although a gene coding nuc-1 has not been isolated yet, the sequence of nuc-1 protein may be similar to that of mammalian Dnase II. In fact, C. elegans is shown to possess three different proteins (CEC07B5-5, YMV6-CAEEL, and YLS2-CAEEL), which share a homology with human and murine Dnase II (52). The porcine Dnase II cDNA sequence is also similar to that of a cDNA of C. elegans (GenBank accession number L11247) (51). Although the functions of these cDNA sequence-encoding proteins are unknown at present, it is likely that one of these proteins is identical to nuc-1 protein.

DNA of the nucleosomes is tightly complexed with the core histones H2A, H2B, H3, and H4 and is therefore believed to be protected from cleavage by the endonuclease during apoptosis. Kutsyi et al. (62) observed that histones H2A, H2B, and H1 were degraded in rat thymus treated with gamma-irradiation or hydrocortisone. In contrast, core histones of IL-2-depleted apoptotic CTLL-2 cells appeared to be intact in our study. When apoptotic CTLL-2 cells were exposed to the lysosome-rich fraction of J774.1 cells, hydrolysis of nucleosomal core histones, i.e., H2A, H2B, H3, and H4, was observed along with degradation of nucleosomal DNA fragments. Pepstatin A was the most potent inhibitor of degradation of core histones that we tested. It efficiently inhibited the cleavage of mono-nucleosomal DNA fragments into smaller DNA fragments. These results suggest that the proteolysis of core histones may be a necessary step in the degradation of mono-nucleosomal DNA fragments. Pepstatin A is known to inhibit cathepsin D (32) and cathepsin E (33). Because cathepsin E is reported to be nonlysosomal (63, 64), cathepsin D seems to play a major role in hydrolysis of core histones. A mixture of pepstatin A and leupeptin exhibited a stronger inhibitory effect than pepstatin A alone. Since leupeptin or CA-074 showed an inhibitory effect on the degradation of histones, cathepsin B or L may be responsible for hydrolysis of core histones of apoptotic cells. These results indicate that cathepsins in lysosomes of J774.1 cells, such as cathepsins D, B, and L, are responsible for degradation of nucleosomal core histones. In particular, cathepsin D appears to be most effective in hydrolyzing all types of histones compared with cathepsins B and L. This result is consistent with previously reported findings that used various proteolytic enzymes including cathepsins (43). Therefore, we suggest that DNase II-like acid nuclease and cathepsins in macrophage lysosomes are involved in the degradation of nucleosomes in apoptotic cells. Because cathepsin H is shown to hydrolyze all types of histones (43), it would be of interest to ask whether cathepsin H in macrophage lysosomes is involved in histone degradation.

Recently, it was reported that the processing of apoptotic cells engulfed by macrophages yields peptide epitopes that may be presented to T lymphocytes (65). However, Albert et al. (66) showed that dendritic cells, but not macrophages, efficiently present Ags derived from apoptotic cells, although macrophages are shown to phagocytose apoptotic cells efficiently. Thus, the capacity of dendritic cells and macrophages to phagocytose apoptotic cells is still a matter of debate and the difference between the process of engulfment and phagocytosis in these two cell types remains to be investigated.

Rapid engulfment of apoptotic cells is beneficial for the host because it prevents the release of potentially toxic and immunogenic intracellular contents from the apoptotic cells into the surrounding tissue (1). Nucleosomes have been found to circulate at high levels in patients with systemic lupus erythematosus (SLE) (67). Interestingly, increased rates of apoptosis in lymphoid cells have been detected both in human and murine lupus (68, 69). It has been demonstrated that nucleosomes serve as a major immunogen for pathogenic autoantigen- inducing T cells in both mouse and human with SLE (70). Therefore, mono- and oligo-nucleosomes that may be released from poorly engulfed apoptotic cells might act as an autoantigen in SLE. A reduced phagocytic activity of SLE patients’ polymorphonuclear leukocytes, monocytes, and macrophages has been reported (71–73). Moreover, Hermann et al. (74) recently found that phagocytosis of apoptotic cells is indeed decreased in SLE patients. Alternatively, it is possible that the impaired proteolytic hydrolysis of apoptotic cells in macrophages leads to an decrease of nucleosome degradation, as has been demonstrated by Zurier (75) that sera from SLE patients interfere with phagocytosis and lysosomal enzyme release from leukocytes. Consequently, a reduction of lysosomal enzymes may cause release of nucleosomes and serve as an immunogen for the induction of autoreactive lymphocytes.

Additional experiments will define the mechanisms by which macrophages in both mouse and human with SLE recognize and engulf apoptotic cells and the intracellular processing by which their macrophages dispose of apoptotic cells after engulfment.

References


