

Macrophages are involved in DNA degradation of apoptotic cells in murine thymus after administration of hydrocortisone

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Abstract

In the present study, we undertook kinetic analyses of DNA degradation and acid DNase activity in murine thymus after administration of hydrocortisone. Hydrocortisone induced apoptosis in thymocytes, and a large number of cortical thymocytes became TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP biotin nick end labelling)-positive (TUNEL⁺). F4/80⁺ macrophages infiltrated through the cortico-medullary junction into the cortical region, and thereafter engulfed apoptotic cells in the cortex of thymus. The distribution of acid DNase-active cells appeared to be similar to that of F4/80⁺ macrophages. Eighteen hours after the injection, although the foci of apoptotic cells were situated within massively distended F4/80⁺ macrophages, oligonucleosomal DNA fragments on an agarose gel were undetectable. Our results showed that macrophages were involved in the disappearance of oligonucleosomal DNA fragments in apoptotic thymocytes. Taken together, macrophages play a role in the hydrolysis of DNA in apoptotic cells upon their phagocytosis of the dead cells.

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Keywords: apoptosis; thymus; macrophages; DNA degradation

Abbreviations: CAD, caspase-activated DNase; ICAD, inhibitor of CAD; DFF, DNA fragmentation factor; DFF45, 45-kDa subunit of DFF; DFF40, 40-kDa subunit of DFF; CPAN, caspase-activated nuclease; DN, double negative; DP, double positive; SP, single positive; TdT, terminal deoxynucleotidyl transferase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP biotin nick end labelling.

Introduction

Apoptosis is a cell death process that removes unwanted cells during mammalian development.¹ One of the hallmarks of apoptosis is the degradation of nuclear DNA into nucleosomal

units, which is demonstrated as a 'DNA ladder' by gel electrophoresis.² These oligonucleosomal fragments of DNA in some apoptotic cells are derived from DNA fragments of 300 and/or 50 kb in length.^{3,4}

The endonuclease responsible for the cleavage of chromosomal DNA during apoptosis has been intensively investigated. From the results of early studies, a endonuclease that is activated in the presence of Ca²⁺ and Mg²⁺, has been proposed to be responsible for the internucleosomal cleavage of nuclear DNA during apoptosis.⁵ The Ca²⁺/Mg²⁺-dependent endonuclease is constitutively expressed in the nucleus isolated from various cells, and its optimum pH has been considered to be around 7–8. In contrast, some myeloid leukemia cells are shown to possess a Ca²⁺-independent and Mg²⁺-dependent endonuclease in their nuclei.⁶ Thus, the ion dependence of the nuclear DNase differs among various studies. Furthermore, several enzymes such as DNase I,^{7,8} DNase II,^{9,10} DNase γ ,¹¹ NUC18¹² and cyclophilin¹³ have been proposed as candidates for the nuclease responsible for DNA cleavage during apoptosis, but the involvement of DNase I in the apoptotic DNA fragmentation was ruled out by characterization of it deficient cells.¹⁴

Recently, a DNase, designated as caspase-activated DNase (CAD), was found to be responsible for oligonucleosomal DNA degradation during apoptosis. It exists as a complex with its inhibitor (ICAD) in growing cells. Caspase-3, which is activated during the apoptotic process, cleaves ICAD, and CAD released from ICAD digests the chromosomal DNA.^{15,16} Human homologues of CAD and ICAD have been identified as DFF40 or CPAN, and DFF45, respectively.^{17–19} CAD is also shown to be responsible for the large-scale degradation of chromosomal DNA.²⁰ Although ICAD and CAD have previously been identified as cytoplasmic factors,^{15,16} recent study demonstrated that these molecules are nuclear proteins.²¹ In addition, it remains unclear whether the Ca²⁺/Mg²⁺-dependent or Mg²⁺-dependent endonuclease might be identical to CAD/DFF40/CPAN. Recently identified mitochondrial apoptosis-inducing factor (AIF) is shown to be able to cleave the chromatin of isolated nuclei into the fragments of ~50 kb,²² and it is likely that at least a couple of DNases may be involved in the appearance of DNA fragmentation during apoptosis.

The preferential apoptosis of DP thymocyte subset by glucocorticoids, irradiation, or anti-CD3 antibody has been well documented in *in vivo* as well as *in vitro* systems.^{23–30} Recent studies, using the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) method to examine the distribution of apoptotic thymocytes after administration of glucocorticoids or anti-CD3 antibody,^{31–33} have shown that the majority of immature thymocytes die in the cortical region of the thymus and

that most of detectable apoptotic cells are localized within thymic macrophages. These studies indicate that thymic macrophages are the principal stromal cells responsible for phagocytosis of dying thymocytes and that the number of TUNEL⁺ thymocytes declines to the original basal level after apoptotic cells are phagocytosed by macrophages. We have previously investigated the fate of apoptotic cells after the engulfment by macrophage cell lines and found a rapid disappearance of DNA strand breaks in apoptotic cells, as assessed by TUNEL staining. Moreover, when the DNA of apoptotic cells engulfed by macrophages was analyzed by electrophoresis on an agarose gel, oligonucleosomal DNA fragments in apoptotic cells became undetectable. Based on these observations, we have proposed that DNase II-like acid nuclease in the lysosomes of macrophages is responsible for degradation of nucleosomal DNA fragments of apoptotic cells.³⁴ McIlroy *et al.*³⁵ also demonstrated DNA cleavage of apoptotic cells by macrophages *in vivo* and *in vitro*.

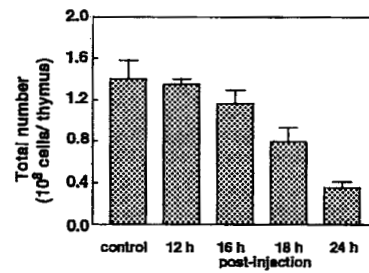
In this study, to extend our previous findings to an *in vivo* system, we undertook kinetic studies of DNA degradation in murine thymus after administration of hydrocortisone and found that macrophages were involved in the disappearance of oligonucleosomal DNA fragmentation. Furthermore, we examined histochemically the activity of acid DNase in the thymic sections, and the distribution of acid DNase-active cells in the thymus appeared to be similar to that of F4/80⁺ macrophages. These data of the experiments using murine thymus after administration of hydrocortisone support our previous proposal³⁴ that acid DNase in macrophages plays a role in the hydrolysis of DNA in apoptotic cells upon their phagocytosis of the dead cells.

Results

Injection of hydrocortisone into normal mice induces depletion of cortical CD4⁺ CD8⁺ thymocytes

It is well established that the exposure of thymocytes to glucocorticoids leads to depletion of CD4⁺ CD8⁺ (DP) thymocytes.^{25,26} To confirm this, we injected hydrocortisone into 6-week-old C57BL/6 mice and examined total number of thymocytes and thymocyte subpopulations using fluorescent anti-CD4 and anti-CD8 antibodies. As shown in Figure 1A, a decrease in the total number of thymocytes was detectable after 12 h of hydrocortisone-injection. As expected from the previous report,²⁶ at 24 h after the injection, the decrease was apparent and ranged from 75–83% of the values obtained for control mice. The cell surface marker analysis showed a decrease in the number of DP thymocytes following the administration of hydrocortisone (Figure 1B). We observed a significant decrease in the percentage of DP thymocytes (from 81 to 68% for 16 h post-injection and to 30% for 18 h post-injection). By 24 h, hydrocortisone induced the depletion of nearly all DP thymocytes, resulting in a proportional increase in the percentage of SP thymocytes and of DN thymocytes, and most of residual thymocytes were SP and DN thymocyte populations.

A



B

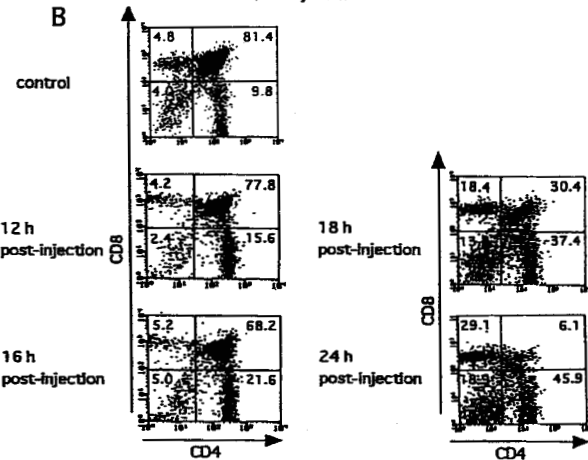


Figure 1 Injection of hydrocortisone into normal mice induces the depletion of CD4⁺CD8⁺ thymocytes. Six-week-old C57BL/6 mice were injected i.p. with 125 mg/kg hydrocortisone sodium phosphate. (A) Reduction of thymocyte cell numbers after administration of hydrocortisone. After the indicated period, each thymus was removed and the cell number of thymocytes was counted. The data represents the mean \pm S.D. of total thymocyte numbers of four mice in each experiment. (B) Thymocyte subpopulations after administration of hydrocortisone. After each thymus was removed at the indicated period, thymocytes were stained with FITC-anti-CD4 and PE-anti-CD8 and analyzed using a FACScan. Numbers represent percentages in each quadrant. The data are representative of four mice in each experiment

In situ detection of apoptotic cells and macrophages in the thymus after administration of hydrocortisone

Apoptotic cells with DNA strand breaks in the thymus after *in vivo* injection of glucocorticoid can be visualised by TUNEL method.^{32,33} Next, we also used the method for detecting the cells that exhibit DNA cleavage in the thymus after administration of hydrocortisone. Additionally, we examined the distribution of macrophages in the thymus by staining with F4/80 antibody.³⁶ In the thymus of normal mice, the cells with nuclear DNA breaks were rare but scattered in the cortex and to a lesser degree in the medulla. F4/80⁺ macrophages were dispersed throughout normal thymus (Figure 2a). Although the shrinkage of the cortex was not manifest until 12 h after the injection, cortical thymocytes showed extensive signs of apoptosis, as judged by DNA strand breaks as early as 12 h post-injection (Figure 2b). At this time, F4/80⁺ macrophages appeared in the thymus, principally in the medulla and the cortico-medullary region and in a lesser number in the cortex. These cells appeared to form a gradient, which was peaked in

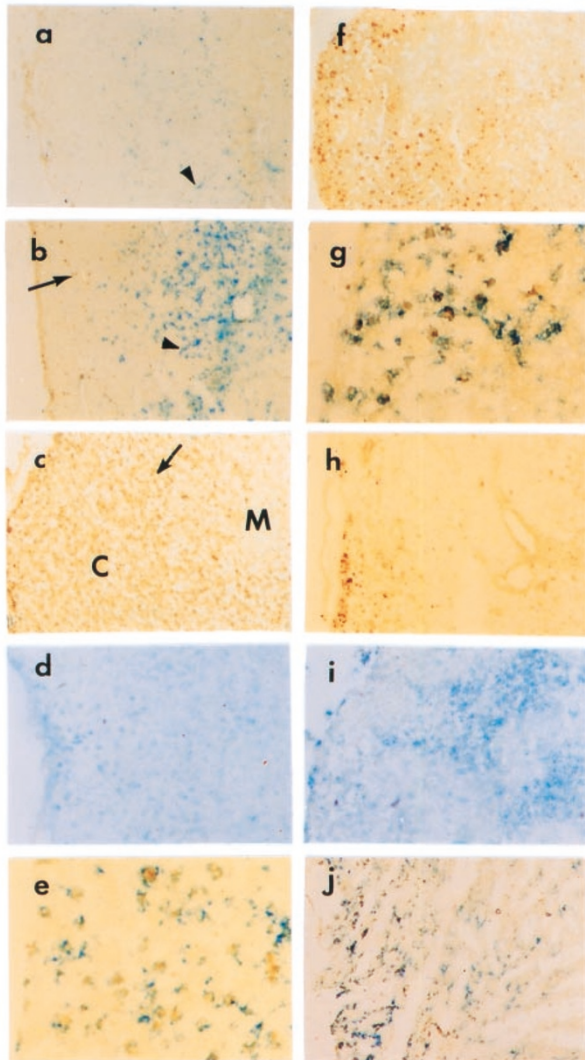


Figure 2 *In situ* detection of apoptotic cells and macrophages in murine thymus after administration of hydrocortisone. C57BL/6 mice were injected i.p. with 125 mg/kg hydrocortisone, and each thymus was removed at the indicated time. Cryostat sections of thymus were stained by TUNEL method (c, f, h) or with F4/80 antibody (d, i). Sections were double-labelled with TUNEL and F4/80 antibody (a, b, e, g, j). Counterstaining was performed with Meyer's haematoxylin. (a) Thymus from control untreated mouse; (b) thymus from mouse at 12 h post-injection; (c–e) thymus from mouse at 16 h post-injection; (f, g) thymus from mouse at 18 h post-injection; (h–j) thymus from mouse at 24 h post-injection. Thymic cortex (C) and medulla (M) are indicated; Arrows and arrow heads indicate thymocytes expressing TUNEL⁺ cells (brown) and F4/80⁺ macrophages (blue), respectively; Magnifications; (a–d, f, h) $\times 40$, (j) $\times 100$, (e, g) $\times 250$. These figures are representative of four mice for each experiment

the cortico-medullary junction (Figure 2b). Injection of hydrocortisone into mice induced a massive increase in the number of apoptotic cells in the thymus within 16 h (Figure 2c). Consistent with the previous study,³² a great number of TUNEL⁺ cells were scattered throughout cortical area, and some of the TUNEL⁺ thymocytes were aggregated to form clusters. The staining with F4/80 antibody showed a number of enlarged macrophages dispersed throughout the cortical thymus (Figure 2d). Moreover, double stainings for nuclear

DNA breaks and macrophages revealed that most of the apoptotic cells in the cortex were situated within F4/80⁺ cells, suggesting that TUNEL⁺ thymocytes were engulfed by macrophages (Figure 2e). This finding agrees with the previous study which reported that apoptotic cells are rapidly engulfed by macrophages and that most of the macrophages in the thymus are found to contain apoptotic bodies.^{32,33} At 18 h after the injection, the number of the TUNEL⁺ clusters further increased (Figure 2f). The foci of 5–10 TUNEL⁺ thymocytes were observed within F4/80⁺ macrophages, and nearly all TUNEL⁺ thymocytes appeared to be engulfed by F4/80⁺ macrophages (Figure 2g). The number of TUNEL⁺ thymocytes decreased drastically between 18 and 24 h, and by 24 h TUNEL⁺ cells were localised only in the subcapsular area of the thymus (Figure 2h). A large number of enlarged F4/80⁺ macrophages could still be observed (Figure 2i). F4/80⁺ macrophages that were localised in the subcapsular area engulfed TUNEL⁺ cells, whereas TUNEL⁺ thymocytes could be no longer detected within F4/80⁺ cells that were in the medullary area of the thymus (Figure 2j).

***In situ* detection of acid DNase activity in the thymus after administration of hydrocortisone**

We have shown that DNase II-like acid nuclease in the lysosomes of macrophages is responsible for the degradation of DNA fragments in apoptotic cells.³⁴ Since the histochemical lead nitrate method has been used to demonstrate an acid DNase activity in tissue sections,^{37,38} we considered it a useful method for identification of the morphological localisation of acid DNase activity and therefore used this method for the detection of acid DNase activity in the thymus after administration of hydrocortisone. The site of enzymatic activity has an appearance of dense brown precipitates. As shown in Figure 3Aa, in the thymus of normal mice, acid DNase activity was detectable in rare stellate cells. Injection of hydrocortisone induced a massive increase of acid DNase-active cells in the thymus. At 12 h after hydrocortisone injection, an increase in the number of cells showing acid DNase activity was found and the enzyme was active mainly in the cells localised in the cortico-medullary junction of the thymus (Figure 3Ab). Acid DNase-active cells were scattered throughout the thymus within 16 h (Figure 3Ac). An intense acid DNase activity was detected throughout the thymus of 18 h post-injection (data not shown), and it could still be detected 24 h after the injection in the cells localised in the subcapsular area or in a number of cells of the medulla (Figure 3A,d).

Macrophages and polymorphonuclear cells contain acid DNase(s) in their lysosomes.^{39,40} We have also observed that DNA hydrolytic enzyme of macrophages is mainly localised in the lysosomes and that the acid DNase is sensitive to DNase II inhibitors such as iodoacetic acid or zinc ion.^{34,41,42} To determine whether acid DNase activity is associated with macrophages, serial sections of the thymus at 24 h after administration of hydrocortisone were stained with TUNEL followed by antibody F4/80 or histochemically for acid DNase activity. As observed in Figure 2i, a number of F4/80⁺ macrophages were detected in the subcapsular area or in the medullary area (Figure 3Ba). On the other

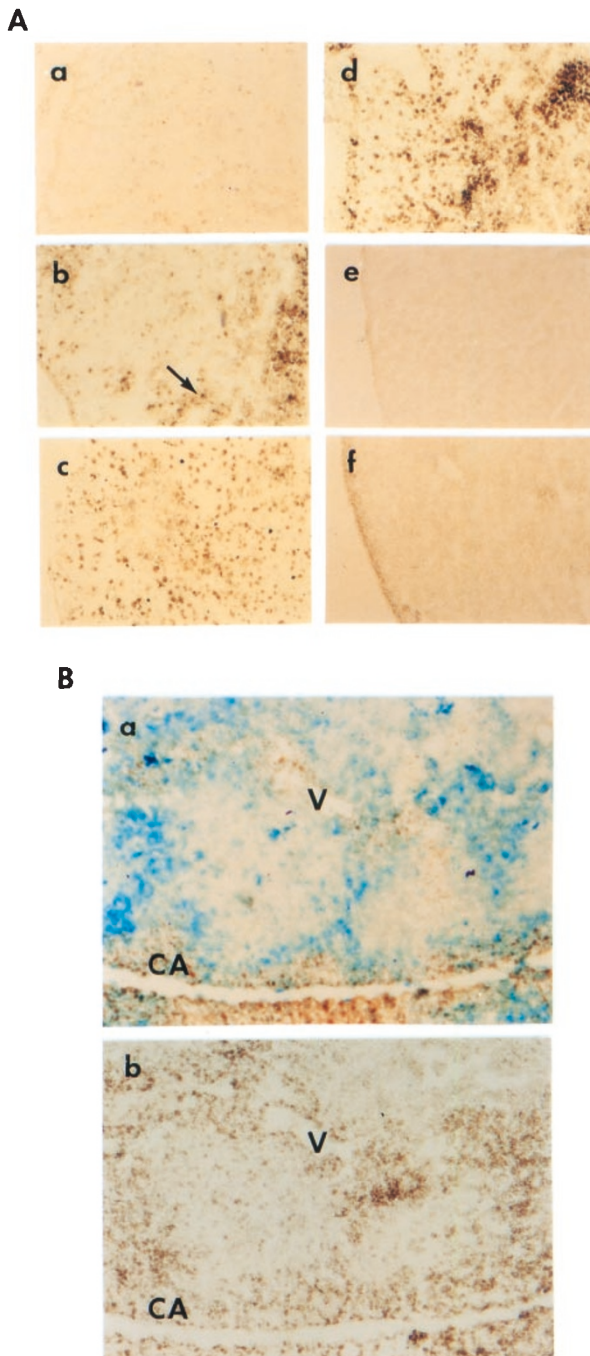


Figure 3 *In situ* detection of acid DNase in murine thymus after administration of hydrocortisone. (A) Acid DNase activity in the thymus after administration of hydrocortisone. C57BL/6 mice were injected i.p. with 125 mg/kg hydrocortisone, and each thymus was removed at the indicated time. The detection of acid DNase was performed as described in Materials and Methods. (a) Thymus from control untreated mouse; (b) thymus from mouse at 12 h post-injection; (c) thymus from mouse at 16 h post-injection; (d–f) thymus from mouse at 24 h post-injection; (e) zinc ion was added into the reaction buffer; (f) iodoacetic acid was added into the reaction buffer. An arrow indicates the cell expressing acid nuclease activity (dark brown). Magnification, $\times 40$. (B) Serial frozen sections of the thymus at 24 h post-injection of hydrocortisone. C57BL/6 mice were injected i.p. with 125 mg/kg hydrocortisone, and each thymus was removed at 24 h post-injection. (a) Serial section was double-stained for apoptotic cells (brown) and for mAb F4/80 (blue), and then counterstained with Meyer's haematoxylin. (B) Serial section was stained

hand, an intense acid DNase activity was mainly visible in the cells localised in the subcapsular area or in the cells of the medulla, some of which formed clusters in the site close to blood vessels (Figure 3Bb). Thus, the distribution pattern of the acid DNase-active cells was similar to that of F4/80⁺ macrophages, and most of macrophages appeared to display an intense acid DNase activity. In addition, we added iodoacetic acid or ZnSO₄ into the incubation buffer and examined their effects on acid DNase activity. The addition of iodoacetic acid or zinc ion into the incubation buffer resulted in the complete inhibition of acid DNase activity (Figure 3Ae and f).

Macrophages are involved in the disappearance of oligonucleosomal DNA fragments in the thymus after administration of hydrocortisone

Internucleosomal DNA cleavage is considered as a biological hallmark of apoptosis.² We next examined DNA fragmentation on an agarose gel after the isolation of DNA from each thymus of mice 16 or 18 h after hydrocortisone-injection. As shown (Figure 2c), a significant increase in the number of TUNEL⁺ cells was detected at 16 h post-injection, and oligonucleosomal DNA fragmentation was detectable but not clearly (Figure 4, lanes 2,3). However, in one mouse from the three in this group, DNA fragmentation in the thymus was hardly detectable (Figure 4, lane 4). We examined DNA fragmentation at the earlier time point, and oligonucleosomal DNA fragmentation was hardly detectable (data not shown). Moreover, DNA fragmentation was no longer detectable in each thymus of three mice at 18 h after the injection (Figure 4, lanes 5–7), at which time almost TUNEL⁺ thymocytes were phagocytosed by F4/80⁺ macrophages as shown in Figure 2g. Since the number of TUNEL⁺ cells rapidly decreased within 16–24 h post-injection, it is likely that the DNA fragments of apoptotic cells might be digested within macrophages, resulting in the disappearance of oligonucleosomal DNA fragments when DNA was isolated from the thymuses of hydrocortisone-treated mice and analyzed by agarose gel electrophoresis.

To determine whether macrophages are involved in the disappearance of DNA fragmentation in agarose gel electrophoresis, each thymus taken from the mice 12 h post-injection of hydrocortisone was used for the following experiments. At this time point, although many F4/80⁺ macrophages infiltrated into thymus, macrophages rarely engulfed apoptotic cells in the cortex, as observed in Figure 2b. When the cell suspension of one large lobe from each thymus was prepared and incubated at 37°C for 3 h, the engulfment of apoptotic thymocytes into macrophages was observed (Figure 5Ac), but DNA fragmentation was clearly detected in the cells (Figure 5B, lanes 3–5). As for another large lobe from each thymus, each cell suspension was prepared and passed through a nylon wool column to

histochemically for acid DNase activity (dark brown). CA; thymic capsule, V; blood vessel in medulla. Magnification, $\times 150$. The data are representative of four mice in each experiment

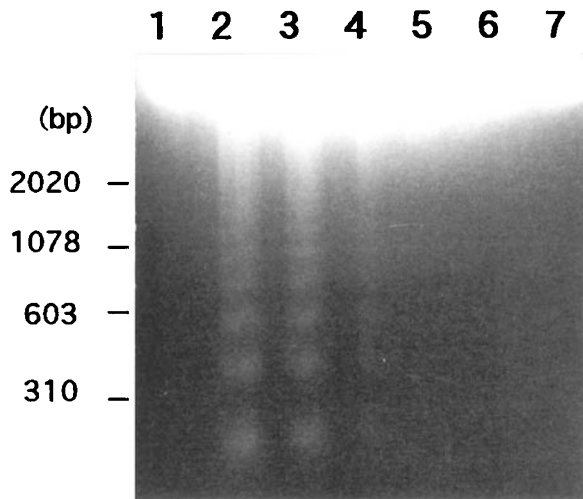


Figure 4 Detection of oligonucleosomal DNA fragments in the thymus after administration of hydrocortisone. C57BL/6 mice were injected i.p. with 125 mg/kg hydrocortisone, and each thymus was removed at 16 or 18 h post-injection. DNA was isolated from the thymus as described as Materials and Methods and then subjected to 1.8% agarose gel electrophoresis. Lane 1, thymus from control untreated mouse; lanes 2–4, thymuses from mice of 16-h post injection; lanes 5–7, thymuses from mice of 18-h post injection. Each lane represents an individual mouse. The size of fragments shown in the left serves as molecular size markers (*Hind*III digest of λ -phage DNA+*Hae*III digest of ϕ X174)

remove macrophages, and F4/80⁺ cells were hardly detectable in the cell suspension passed through a nylon wool column, as determined by FACS analysis (data not shown). When these macrophage-depleted thymocytes were incubated at 37°C for 3 h (Figure 5Ad), DNA fragmentation was detected more obviously than in thymocytes together with a number of infiltrated macrophages (Figure 5B, lanes 8–10).

Discussion

We herein confirmed and extended earlier studies, in which the lethal effect of glucocorticoids on DP thymocytes is shown to be caused by induction of apoptosis.^{2,25,26} Injection of hydrocortisone into mice induced a massive increase in the number of TUNEL⁺ cells in the thymus up to 16 h. Double stainings by TUNEL method and with F4/80 antibody revealed that TUNEL⁺ cells were rapidly engulfed by F4/80⁺ macrophages. After ingestion of apoptotic cells by macrophages, the number of TUNEL⁺ thymocytes rapidly decreased between 16 and 24 h. These findings are in agreement with previous studies.^{32,33}

Noteworthy is the reproducible observation that as early as 12 h after administration of hydrocortisone, a number of F4/80⁺ macrophages appeared in the thymus, principally in the medulla and the cortico-medullary region. Although the shrinkage of the cortex was hardly detected until 12 h post-injection, cortical thymocytes began to become TUNEL-positive. At 16 h of the injection, the number of F4/80⁺ cells in the cortex increased, suggesting the migration of macrophages from the medullary and cortico-medullary

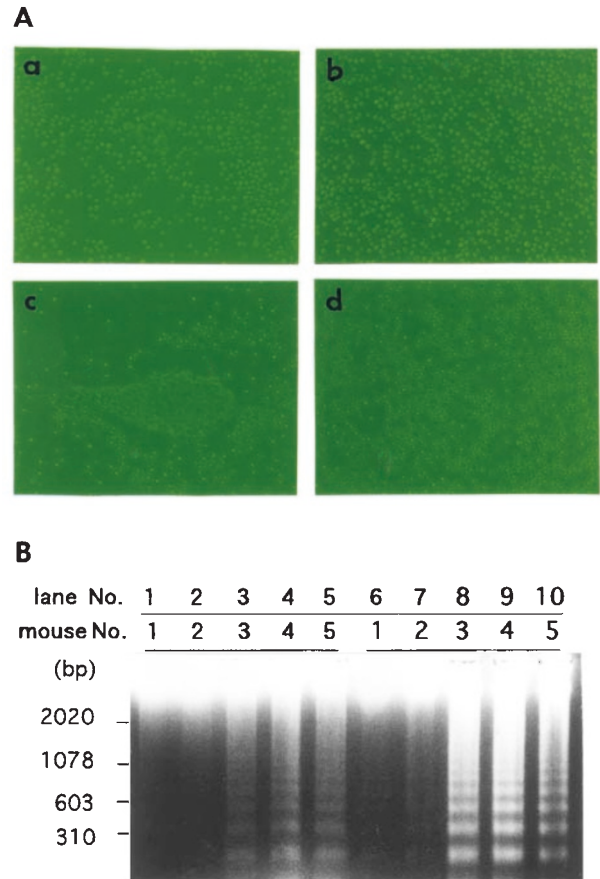


Figure 5 Appearance of DNA fragments in macrophage-depleted cell suspension from the thymus after administration of hydrocortisone. C57BL/6 mice were injected i. p. with saline (mice 1,2) or 125 mg/kg hydrocortisone (mice 3–5). At 12 h post-injection, each large lobe from thymus of mice was excised, and the cell suspension was prepared. Macrophage-depleted cell suspension from one large lobe of each thymus in individual mouse was prepared as nylon-nonadherent cells and then incubated at 37°C for 3 h. Unfractionated cell suspension from another large lobe was also incubated at the same temperature as the macrophage-depleted cell suspension during the procedure of nylon wool columns, and then incubated at 37°C for 3 h. (A) Engulfment of apoptotic thymocytes into macrophages; (a), unfractonated cell suspension from thymus (mouse 1); (b), macrophage-depleted cell suspension from thymus (mouse 1); (c), unfractonated cell suspension from thymus (mouse 3); (d), macrophage-depleted cell suspension from thymus (mouse 3). Magnification, $\times 100$. (B) DNA was isolated from the cells and then subjected to 1.8% agarose gel electrophoresis. Lanes 1,2, cell suspension from each thymus with administration of saline (mice 1,2); lanes 3–5, cell suspension from each thymus with administration of hydrocortisone (mice 3–5); lanes 6,7, macrophage-depleted cell suspension from each thymus with administration of saline (mice 1,2); lanes 8–10, macrophage-depleted cell suspension from each thymus with administration of hydrocortisone (mice 3–5). The size of fragments shown in the left serves as molecular size markers (*Hind*III digest of λ -phage DNA+*Hae*III digest of ϕ X174)

region to the cortical region. The estimated number of macrophages in murine thymus has been controversial due to the different methods used for collecting them. According to Zepp *et al.*,⁴³ who used bovine serum albumin (BSA) gradients, 1.5–3.5% of all thymic cells are low-density adherent cells including macrophages and dendritic cells.⁴³ In the previous studies on the ontogeny of rat thymus, the cortico-medullary region has been regarded as the

entrance site of blood monocytes.^{44,45} This increase of F4/80⁺ cells in the thymus is presumably due to the arrival of migrated macrophages through vessels, rather than *in situ* proliferation of pre-existent macrophages. These findings imply that cortical thymocytes undergoing apoptosis may release a macrophage-chemoattractive factor within 12 h post-injection. It would be of interest to elucidate the molecular mechanism(s) by which circulating macrophages are attracted to the cortical region of the thymus.

In this study, we examined histochemically the activity of acid DNase in the thymic sections, since the histochemical method is convenient for detecting the site of the nuclease activity. In the thymus of normal mice, a weak acid DNase activity was observed in some of the scattered cells of normal thymus. Injection of hydrocortisone into mice induced a massive increase in the number of acid DNase-active cells in the thymus, and our results confirmed four decades old observations by two groups.^{46–49} Douglas' group has reported that total body x-irradiation causes an increase in the activity of acid DNase in rat spleen.^{46,47} Additionally, they found that the activity is peaked 24–48 h post-irradiation at which time all the lymphocytes of spleen were already destroyed. Hempelmann's group also demonstrated that the activity of acid DNase increases markedly in spleen and thymus during the first 3 days after exposure of ionizing radiation or cortisone.^{48,49} They suggested that the radiation-sensitive lymphocytes contain little acid DNase activity and that radiation-resistant cells in the spleen or the thymus contribute to the increase of acid DNase activity.⁴⁹ In our observations, at 24 h after the injection, although the number of TUNEL⁺ apoptotic thymocytes drastically decreased and the thymus was almost entirely composed of medullary tissue, the duration of intense acid DNase activity could still be detected. In addition, the histological distribution of acid DNase activity was similar to that of F4/80⁺ macrophages in the thymus of hydrocortisone-treated mice. These results suggest that acid DNase is not involved in thymocyte apoptosis itself. If the enzyme had been involved in the execution of apoptosis, we could have hardly detected acid DNase activity at 24 h post-injection. We have reported that an acid DNase in lysosomes of macrophages which is responsible for DNA degradation of apoptotic cells is similar to DNase II with respect to its molecular weight, optimal pH and sensitivity to DNase II inhibitors.³⁴ The *in situ* activity of acid DNase was inhibited by the presence of iodoacetic acid or zinc ion, suggesting that the acid DNase possesses the character similar to that of DNase II in terms of the sensitivity to DNase II inhibitors. Although it remains unclear whether the acid DNase detected in the thymuses of hydrocortisone-injected mice is identical to DNase II, this issue, for instance, could be explored by an inhibition assay of acid DNase activity by anti-DNase II antibody in the histochemical method.

Glucocorticoid-induced apoptosis in thymocytes has been exclusively studied in cell culture systems and the oligonucleosomal fragmentation of genomic DNA is apparently detectable during the apoptosis.² In our study, a large number of apoptotic cells were found with a maximum at 16 h post-injection as assessed by TUNEL staining.

However, although DNA fragmentation was detected when DNA was isolated from each thymus at this time point, the amounts of DNA fragments on an agarose gel were smaller than we expected. At 18 h after the injection, nucleosomal DNA fragments were no longer detectable in each thymus of hydrocortisone-treated mice. Nakamura *et al.*⁵⁰ have reported that apoptosis in the thymuses of hydrocortisone-injected mice occurs without DNA fragmentation. However, they also observed TUNEL⁺ thymocytes, all of which are phagocytosed by F4/80⁺ macrophages. They therefore proposed that apoptotic thymocytes become TUNEL⁺ only after being engulfed by macrophages. This seems to conflict, in part, with our present results, and two differences in the experimental system might account for this discrepancy: (1) we injected with 125 mg/kg of hydrocortisone into each mouse, whereas the dose of the drug by Nakamura *et al.*⁵⁰ was 250 mg/kg and (2) we traced up to 24 h, whereas they removed thymus at 2 or 4 h after the injection. Rather, we favour a hypothesis that a disappearance of oligonucleosomal DNA ladder formation in apoptotic thymocytes occurs upon the engulfment by macrophages. As early as 12 h after administration of hydrocortisone, nearly all apoptotic cells were not yet engulfed by F4/80⁺ macrophages. When each thymus was removed at 12 h post-injection and each cell suspension was incubated at 37°C for ~4 h, DNA fragmentation on an agarose gel was detectable. Furthermore, when the cell suspension was depleted of the infiltrated macrophages, DNA fragmentation was detected more apparently than in cell suspension containing a number of macrophages. This finding implies that macrophages may be involved in the disappearance of DNA fragmentation. Since we found that a number of acid DNase-active cells were still distributed in the thymus even at 24 h post-injection and that the histological localisation of acid DNase-active cells appeared to be similar to that of F4/80⁺ macrophages, it is plausible that acid DNase, which is presumably resides in macrophages, may be responsible for the degradation of oligonucleosomal DNA fragments in apoptotic cells. Therefore, the oligonucleosomal fragments of genomic DNA may be undetectable in glucocorticoid-induced thymocyte cell death *in vivo*.

In *Caenorhabditis elegans*, programmed cell death normally occurs in the mutants that lack the activity of *nuc-1*-encoded acid nuclease although the nucleus remains intact after phagocytosis.^{51,52} This indicates that the acid nuclease functions in late event after phagocytosis of dead cells. We have speculated that *nuc-1* may encode a nuclease similar to DNase II.³⁴ Indeed, Wu *et al.*⁵³ demonstrated that the gene *nuc-1* is a homologue of mammalian DNase II. Furthermore, they indicated that mutations of *nuc-1* block the conversions of TUNEL-reactive DNA to a subsequent TUNEL-unreactive state. On the basis of our previous³⁴ and present results, we propose a model of DNA degradation of mammalian apoptotic cells (Figure 6). Upon induction of apoptosis, ICAD (DFF45) is cleaved by caspase-3 and dissociated from CAD (DFF40/CPAN), resulting in the induction of CAD (DFF40/CPAN) nuclease activity. Since the DNA of nucleosomes is tightly complexed with the core histones and therefore protected from the cleavage by the nucle-

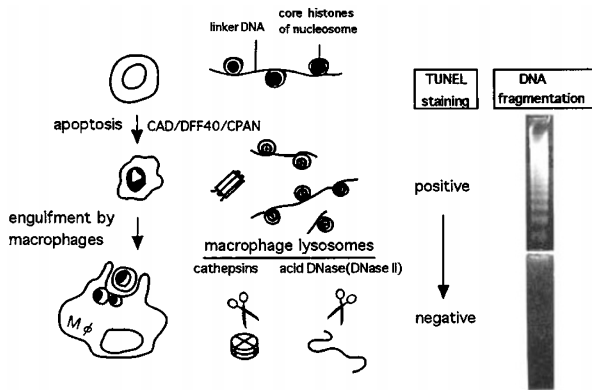


Figure 6 A model of DNA degradation of mammalian apoptotic cells

ase,⁵⁴ genomic DNA is cleaved at most accessible internucleosomal linker region, resulting that the DNA fragments yield discrete multiples of a 180 bp subunit which is detected as a 'DNA ladder' on a gel. Once cells undergo apoptosis, they are recognised and engulfed by macrophages via a number of membrane receptors. After rapid engulfment by phagocytic macrophages, DNase II-like acid nuclease and cathepsins in macrophage lysosomes are responsible for the degradation of nucleosomes in apoptotic cells. Consequently, DNA strand breaks in apoptotic cells are disappeared, as assessed by TUNEL staining. In addition, nucleosomal DNA fragments in apoptotic cells are degraded into smaller random-sized fragments, thereby DNA ladder is no longer detectable as analyzed by electrophoresis on a gel.

Material and Methods

Mice

Six-week-old C57BL/6 female mice were obtained from Clea Japan Inc (Tokyo, Japan). Mice were injected i.p. with 125 mg/kg hydrocortisone sodium phosphate (Sigma, St. Louis, MO, USA) or with saline. At the indicated times, mice were sacrificed, and freshly isolated thymuses were immersed in OCT compound (Miles, Naperville, IL, USA). They were then rapidly frozen on dry ice and sectioned at 5 μ m. Alternatively, thymus lobes were excised and a cell suspension was prepared from each lobe of individual mouse.

Fluorescent marker analysis

Thymocytes from each thymus were counted, and then stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (GK1.5; PharMingen, San Diego, CA, USA) and phycoerythrin (PE)-conjugated anti-CD8 (53-6.72; PharMingen). The staining was assessed by flow cytometry using a FACScan (Becton Dickinson, Mountain View, CA, USA). Dead cells were gated using propidium iodide (Sigma), and viable cells were analyzed for each thymus.

In situ detection of DNA cleavage by TUNEL

Frozen sections were air-dried and fixed in acetone. After incubation in 0.3% H₂O₂ for 30 min, the sections were incubated

for 60 min at 37°C in a reaction buffer consisting of 100 mM sodium cacodylate (pH 7.2), 1 mM CoCl₂, 10 nM biotin-16-dUTP (Boehringer Mannheim Biochemicals, Mannheim, Germany), and 100 units/ml terminal deoxynucleotidyl transferase (TdT) (Takara Biochemicals, Shiga, Japan), followed by incubation with avidin-biotin-peroxidase complexes using the Vecstain-ABC kit (Vector, Burlingame, CA, USA) for 30 min. The sections were washed and then incubated with a mixture of 0.06% 3-3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) and 0.03% H₂O₂ in 0.05 M Tris-HCl (pH 7.5). Counterstaining was performed with Meyer's haematoxylin. Microscopic observations were carried out by using Microflex UFX-II (Nikon Co. Ltd., Tokyo, Japan).

Immunohistochemistry

Frozen thymus sections were air-dried, fixed in acetone, and dehydrated in PBS. The sections were pre-incubated with PBS containing 5% normal goat serum, and incubated sequentially with an anti-macrophage antibody, F4/80³⁶ (rat IgG_{2b} mAb, Biomedicals AG., Augst, Switzerland) followed by the incubation with goat anti-rat IgG antibody conjugated with alkaline phosphate (Cappel, West Chester, PA, USA), and then incubated for 20 min at room temperature using the Alkaline Phosphate Substrate kit (Vector) to colour the reaction product blue. In some experiments, after the staining by TUNEL labelling, the sections were incubated first with F4/80, and second with goat anti-rat IgG antibody conjugated with alkaline phosphate. The sections were then developed with alkaline phosphate substrate. Counterstaining was performed with Meyer's haematoxylin.

In situ detection of acid DNase activity

Histochemical detection of acid DNase activity was performed according to the procedure described by Aronson *et al.*³⁷ with slight modifications by Vorbrodts.³⁸ Frozen thymus sections were air-dried and then incubated for 8 h at 37°C in a reaction buffer containing 0.05 M sodium acetate (pH 5.2), 1 mM Pb(NO₃)₂, 0.4 mg/ml herring sperm powder DNA (Sigma), and 0.1 mg/ml wheat germ acid phosphate (Sigma). Where indicated, iodoacetic acid or ZnSO₄ was added into the reaction buffer at a concentration of 1 mM or 2 mM, respectively. At the end of the incubation, the sections were rinsed with distilled water, and developed with 0.1% yellow aqueous solution of (NH₄)₂S. After the sections were washed with distilled water, they were fixed for 2 min in a solution of 3.7% formaldehyde and mounted.

Cell preparations and cell culture

Each lobe from thymus was excised, and the cell suspension was prepared in RPMI 1640 medium (Grand Island, NY, USA) supplemented with 10% FCS, 5 \times 10⁻⁵ M 2-ME, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 units/ml penicillin and 100 μ g/ml streptomycin. The cell suspension of one large lobe from each thymus was loaded in a nylon wool column and incubated in a 37°C, 5% CO₂ humidified incubator for 45 min. The macrophage-depleted cells were collected and resuspended in the above medium. Each cell suspension prepared from another large lobe was incubated at the same temperature as the macrophage-depleted cell suspension during the procedure of nylon wool columns. These cells were seeded into 24-well culture plates at a density of 5 \times 10⁶ cells/well and then incubated at 37°C for 3 h.

Isolation of DNA and detection of DNA fragmentation

The cleavage of DNA into nucleosomal fragments was assessed by fractionating DNA on an agarose gel as described previously.²⁹ In brief, thymocytes were lysed in the buffer containing 10 mM Tris-HCl (pH 8.0), 0.5% N-lauroylsarcosine and 0.1 M EDTA, and treated with 100 µg/ml 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 70% ethanol and allowed to dry at room temperature. The DNA was resuspended in TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA), and then DNA equivalent to 2.5×10^5 cells was fractionated by electrophoresis on a 1.8% agarose gel containing ethidium bromide. The gels were photographed using UV transilluminator.

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