DNA Fragmentation Factor 45-deficient Cells Are More Resistant to Apoptosis and Exhibit Different Dying Morphology than Wild-type Control Cells*

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The DNA fragmentation factor 45 (DFF45) is a subunit of a heterodimeric DNase complex critical for the induction of DNA fragmentation in vivo. To understand the in vivo role of DFF45 in programmed cell death, we measured the expression of DFF45 during mouse development and compared DNA fragmentation and viability of DFF45-deficient cells with wild-type control cells after activation of apoptosis. We found that DFF45 is ubiquitously expressed throughout mouse development. Moreover, DFF45-deficient thymocytes are resistant to DNA fragmentation with in vivo dexamethasone treatment. Furthermore, primary thymocytes from DFF45 mutant mice are also more resistant to apoptosis than wild-type control cells on exposure to several apoptotic stimuli. Dying DFF45-deficient thymocytes exhibit different morphology than wild-type control cells in that they show reduced degree of chromatin condensation, absent nuclear fragmentation, intranuclear cytoplasmic invagination, and striking nuclear chromatin conglutination after release from disintegrating cells. These results indicate that DFF45 is essential during normal apoptosis.

Programmed cell death or apoptosis is a highly regulated physiological process critical in development and homeostasis (1–4). Alteration of apoptosis is implicated in many human diseases, including cancer, viral infections, autoimmune diseases, and AIDS (5). The hallmarks of apoptosis include regulation of apoptosis remain unclear.

DNA fragmentation in vivo may be an important step for disposal of large fragments of DNA from dying cells, which may be critical in maintaining normal tissue homeostasis (36, 37). DNA fragmentation and chromatin conglutination have been shown to depend critically on a heterodimeric protein complex composed of DNA fragmentation factors 45 and 40 (DFF45 or inhibitor of caspase-activated nuclease and DFF40 or caspase-activated nuclease, respectively; Refs. 38–41). Although DFF40 contains an intrinsic DNase activity, both DFF40 and DFF45 are required to generate this activity. DFF45 has been postulated to stabilize the synthesis of DFF40 (38, 40) or, alternatively, mediate correct folding and chromatin localization of DFF40 (40). On activation of apoptosis, DFF45 is cleaved by caspase-3 at amino acids 117 and 224 and dissociates from DFF40, resulting in the induction of DFF40 nuclease activity (38–41). Activated DFF40 oligomerizes to form a large functional complex that cleaves DNA by introducing double-strand breaks (42). Despite this knowledge, however, whether DFF45 is important for induction of DNA fragmentation in vivo and how DNA fragmentation plays a role in execution and regulation of apoptosis remain unclear.

We have used the previously generated DFF45 mutant mice to address these issues (43, 44). We found that DFF45 is ubiquitously expressed throughout mouse development. Moreover, DFF45-deficient thymocytes are resistant to DNA fragmentation after in vivo dexamethasone treatment. Furthermore, primary thymocytes from DFF45 mutant mice exhibit more resistance to apoptosis than wild-type control cells after exposure to several apoptotic stimuli. Dying DFF45-deficient thymocytes also show different dying morphology than wild-type control cells in that they show a reduced degree of chromatin condensation, absent nuclear fragmentation, intranuclear cytoplasmic invagination, and conglutination of liberated chromatin. These results indicate that DFF45 is essential during normal apoptosis, and its mutation could have adverse effects in vivo.

**Experimental Procedures**

**DFF45 Mutant Mice**—Mice homozygous for the DFF45 gene mutation were produced by crossing heterozygous mutant animals in our mouse colony and were identified by Southern blotting as described (43). DFF45 mutant and wild-type control littersmates 8–12 weeks of age were used for all subsequent analyses.

**Northern Hybridization**—Mouse poly(A) RNA blots representing embryonic and adult stages of development were purchased from CLONTECH. Northern hybridization was performed as described using a DFF45 gene-specific probe that contains DNA sequences from the first three exons (43, 45).

**DNA Fragmentation Assays**—For the in vivo DNA fragmentation

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§ The abbreviations used are: DFF40, 40-kDa subunit of DNA fragmentation factor; DFF45, 45-kDa subunit of DNA fragmentation factor; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate.
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Fig. 1. DFF45 is ubiquitously expressed throughout normal mouse development. Blots with mRNA isolated from embryonic (left) and adult (right) stages of mouse development were probed with a DFF45 gene-specific probe. Two species of DFF45 mRNA of indicated sizes are detectable. E, embryonic day; Br, brain; Ht, heart; Kd, kidney; Sp, spleen; Th, thymus; Li, liver; kb, kilobases.

Fig. 2. Resistance to DNA fragmentation in thymus from DFF45 mutant mice. Both wild-type control (+/+ ) and DFF45 mutant (-/-) mice (n = 3 each) were treated intraperitoneally with dexamethasone (12.5 mg/kg of body weight) for 2, 4, and 6 h. Untreated mice were included as 0-h controls (n = 3 each). Genomic DNA samples were extracted from thymi, separated on 1.5% agarose gels, and visualized by staining with ethidium bromide. 1-kilobase pair molecular weight standard is shown on the left.

DFF45 is ubiquitously expressed throughout normal mouse development, suggesting it may also function during the entire period.

DFF45-deficient Thymocytes Are Resistant to DNA Fragmentation When Treated with Dexamethasone in Vivo—We previously demonstrated that cultured primary DFF45-deficient thymocytes, splenocytes, and neutrophils exhibit resistance to DNA fragmentation in response to various apoptotic stimuli (43). To determine whether the lack of DFF40 nucleolytic activity results in changes in DNA fragmentation in vivo, we treated both DFF45 mutant and wild-type mice with dexamethasone for different periods. Genomic DNA samples were then isolated from thymi from all treated mice and resolved by gel electrophoresis. As shown in Fig. 2, wild-type thymocytes start to exhibit typical nucleosomal DNA fragmentation after 4 h of in vivo dexamethasone treatment. Moreover, the degree of DNA fragmentation in wild-type thymocytes increases with longer periods of treatment. In sharp contrast, DFF45-deficient thymocytes are resistant to DNA fragmentation in response to the same treatment. Thus, DFF45 is required for DNA fragmentation in vivo on apoptotic stimulation.

DFF45-deficient Thymocytes Exhibit More Resistance to Cell Death than Wild-type Thymocytes on Apoptotic Stimulation—It has been shown that cell nucleus and DNA fragmentation are not required for apoptosis under certain conditions (47). We also observed caspase 3 activation and degradation of caspase substrates poly(ADP-ribose) polymerase (48) and nuclear lamin B (48, 49) in DFF45-deficient cells with exposure to various apoptotic stimuli. These observations suggest that apoptosis in DFF45-deficient cells could occur despite the resistance to DNA fragmentation and chromatin condensation. However, it is not known whether apoptosis occurs at a reduced level in DFF45-deficient cells, which could have adverse effects in vivo. To address this issue, we compared the extent of cell death in DFF45-deficient and wild-type cells in response to several apoptotic stimuli. Specifically, we isolated primary thymocytes from DFF45 mutant and wild-type mice and exposed these cells to dexamethasone, etoposide, or staurosporine for different periods. We then measured the viability of these treated thymocytes using both the trypan blue exclusion and FITC-annexin V propidium iodide staining methods.

EXPERIMENTAL RESULTS

DFF45 Is Expressed Ubiquitously through Normal Mouse Development—To assess the function of DFF45 in vivo, we first performed a Northern blot analysis of poly(A) RNA isolated from different stages of mouse development using a DFF45 gene-specific probe (43). As shown in Fig. 1, two species of DFF45 mRNA—2.4 and 3.8 kilobases in sizes are detected, and they are detectable as early as embryonic days 7–17 (left) or as late as 18 months of age (right). Moreover, DFF45 is expressed in multiple tissues including the brain, spleen, thymus, and liver in adult mice. Thus, DFF45 is ubiquitously expressed throughout mouse development, suggesting it may also function during the entire period.

Analysis, thymocytes were prepared from dexamethasone-treated (12.5 mg/kg body weight) DFF45 mutant and wild-type mice at different time points as described (43). Untreated thymocytes from mutant and control mice were used as 0-h controls. For the in vitro DNA fragmentation analysis, primary thymocytes from both DFF45 mutant and wild-type mice were isolated and resuspended at 2 ×10⁶/ml in Dulbecco’s modified Eagle’s medium and 10% fetal calf serum and were cultured in the absence or presence of dexamethasone (1 μM), etoposide (50 μM), or staurosporine (2 μM), respectively, for different lengths of time at 37 °C (43). DNA samples were then extracted by overnight incubation at 56 °C in 0.1 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 150 mM NaCl, 50 μg/ml freshly made proteinase K). These DNA samples were recovered by isopropanol precipitation, resuspended in Tris-EDTA-RNase, analyzed on 1.5% agarose gels, and visualized by ethidium bromide staining as described (43).

Cell Death Analyses—Cell viability on activation of apoptosis was determined using two different methods. Thymocytes were collected, and viable cells were counted using the trypan blue exclusion method after dexamethasone or etoposide treatment at different time points. Viability of thymocytes after dexamethasone, etoposide, or staurosporine treatment was also determined by flow cytometric analysis counting 20,000 events using a FACS Calibur system (Becton Dickinson, San Jose, CA) at the Children’s Hospital Research Foundation. Viable cells are negative populations for both FITC-annexin V and propidium iodide staining (Immunotech; Refs. 43, 46).

Statistical Analyses of Data—Cell viability determined by the trypan blue exclusion assay was analyzed by Tukey’s multiple comparison for the pairwise comparison between wild-type and DFF45-deficient thymocytes at different time points. In addition, Bartlett’s test was also performed to ensure that the underlying assumption about the homogeneity of the variance for Tukey’s multiple comparison was appropriate. Cell viability determined by the FACS analysis was analyzed by pairwise t test between wild-type and DFF45 deficient thymocytes for different drug treatments.

Ultrastructural Studies—Primary DFF45-deficient and wild-type control thymocytes were isolated and were treated with 1 μM dexamethasone for 6 or 16 h, respectively. Treated thymocytes were harvested and centrifuged at 1,500 rpm. The cell pellets were fixed in cacodylate-buffered 2% glutaraldehyde and prepared for electron microscopy by standard methods (6, 46).

Thymocytes were classified as normal or degenerate using the following grading system: stage 1, early nuclear chromatin condensation with nuclear clefts in otherwise intact cells; stage 2, moderate nuclear chromatin condensation with intact cytoplasm and cell membrane; and stage 3, advanced nuclear chromatin condensation with disintegrating cytoplasm. Comparable stages of degeneration of DFF45-deficient thymocytes were judged by their cytoplasmic changes. Also recorded were cytoplasm. Comparable stages of degeneration of DFF45-deficient thymocytes were observed with increasing length of apoptotic exposure. However, viability of thymocytes from the DFF45 mutant mice is significantly higher than that of the wild-type mice after 24 h of dexamethasone (Fig. 3A; p < 0.01) or etoposide treatment (p < 0.01; data not shown). These results suggest that DFF45-deficient cells are more resistant to cell death than the wild-type cells after exposure to apoptotic stimulation. To confirm further
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**Fig. 3.** DFF45-deficient thymocytes are more resistant to cell death than wild-type control thymocytes on apoptotic stimulation. DFF45-deficient and wild-type thymocytes (n = 3 mice each) were cultured in the presence of dexamethasone (1 μM) for 0, 2, 4, 8, 16, and 24 h, stained with trypan blue, and counted (A). DFF45-deficient and wild-type thymocytes (n = 3 mice each) were treated with dexamethasone (1 μM) for 0, 2, 4, 8, 16, and 24 h (B) or etoposide (50 μM) or staurosporine (2 μM) for 24 h (C) and stained with FITC-annexin V/propidium iodide and analyzed by FACS. D, dexamethasone; E, etoposide; S, staurosporine. The percent of viable cells is presented as mean ± S.E.

As shown in [Fig. 3B](#), DFF45-deficient thymocytes exhibit a significantly higher percent viability than that of the wild-type control thymocytes after 16 and 24 h of dexamethasone treatment (p < 0.01). The difference in viability between DFF45-deficient and wild-type control thymocytes after 16 h of dexamethasone treatment may be attributable to a difference in detection methods. Further analyses showed also that viability of thymocytes from the DFF45 mutant mice is significantly higher than that from the wild-type mice after 24 h of exposure to etoposide (Fig. 3C; p < 0.01) or staurosporine (Fig. 3C; p < 0.05). Therefore, DFF45-deficient cells are more resistant to cell death than normal control cells on activation of apoptosis. Moreover, the appearance of FITC-annexin V+/propidium iodide− cells from both DFF45 mutant and wild-type mice indicates that 15 of 165 (9%) dying wild-type thymocytes showed nuclear fragmentation compared with 0 of 160 (0%) DFF45-deficient thymocytes. Finally, dying DFF45 deficient thymocytes exhibit a remarkable tendency to fusion of nuclear chromatin liberated from disintegrated cells, whereas dying wild-type thymocytes do not (Fig. 5, stage 2) after dexamethasone treatment. A quantitative survey of one electron microscopic field each for wild-type and DFF45 deficient thymocytes indicates that 15 of 165 (9%) dying wild-type thymocytes showed nuclear fragmentation compared with 0 of 160 (0%) DFF45-deficient thymocytes. This is consistent with our previous observation that DFF45-deficient cells are more resistant to apoptosis than wild-type control cells.

**Fig. 4.** Resistance to DNA fragmentation in thymocytes from DFF45 mutant mice. Genomic DNA samples were isolated from primary thymocytes from both wild-type (+/+ ) and DFF45 mutant (−/− ) mice (n = 3 each) with 0, 1, 2, 4, 8, 16, or 24 h of dexamethasone (1 μM) treatment. All DNA samples were extracted, separated on 1.5% agarose gels, and visualized by ethidium bromide staining. 1-kilobase pair molecular weight standard is shown on the left.

It has been shown that DFF45 are detected, and they presumably encode the long plasma membrane changes of apoptosis can still happen in the absence of DFF45.

To be certain that the exposure to various apoptotic stimuli were effective in inducing apoptosis, we isolated DNA samples from the above-treated thymocytes and analyzed them on agarose gels. As shown in [Fig. 4](#), the DNA samples from dexamethasone-treated thymocytes from DFF45 mutant mice were devoid of DNA fragmentation, similar to those from the untreated cells. In contrast, the DNA samples from wild-type cells exhibited increasing amount of DNA ladder after longer dexamethasone treatment. Similar results were obtained with etoposide- or staurosporine-treated cells (data not shown). Thus, treatments from these three drugs produced expected DNA fragmentation patterns in the wild-type thymocytes.

**DISCUSSION**

**DFF45 in Apoptosis—**We found that DFF45 is ubiquitously expressed during early embryonic development and in adult tissues, suggesting an important role of DFF45 throughout mouse development. Interestingly, two forms of mRNA for DFF45 were detected, and they presumably encode the long
and short forms of DFF45 (inhibitor of caspase-activated nuclease-L and -S, respectively; Ref. 50). Whereas the long form of DFF45 likely acts as both a chaperone and an inhibitor for DFF40 function, the role of the short form of DFF45 remains unknown (38–41, 43, 50).

Using the previously generated DFF45 mutant mice, we found that DFF45-deficient thymocytes exhibit more resistance to cell death than wild-type control thymocytes on activation of apoptosis. This is the first experimental demonstration that DNA fragmentation factor(s) could modulate the overall apoptotic process in response to various apoptotic stimuli. This may not be surprising, because double-stranded DNA breaks that are generated by DFF40 could themselves be apoptotic signals (42). Thus, the lack of DNA double-stranded breaks in DFF45-deficient thymocytes results in a deficiency of signaling, leading to more resistance to apoptosis than wild-type cells. Moreover, DNA fragmentation may be the true point of no return in cell death in some cases. It could be argued that degraded DNA to nucleosomal sizes in thymocytes is responsible for degrading DNA to nucleosomal sizes in thymocytes. Whether there are unlikely additional mechanisms, such as the existence of DFF45 homologs or other nuclease systems, remains to be determined. Regardless, the more resistance to apoptosis may have adverse functional consequences in vivo when timely apoptosis is needed. Future detailed analysis of physiological function in the DFF45 mutant mice and how DFF45 mutant mice respond to adverse conditions that demand appropriate apoptosis in wild-type mice will provide useful clues.

What might be the underlying mechanisms for the observed resistance to programmed cell death in DFF45-deficient cells? One possibility is that activation of DFF45 feedback regulates one or more steps in the apoptosis process. A thorough comparison of the time course of cytochrome c release, activation of various caspases, and cleavage of various caspase substrates, such as poly(ADP-ribose) polymerase, nuclear lamins, and various plasma membrane or cytosolic structural proteins that contribute to morphological changes of apoptosis process in response to apoptotic stimuli, will provide initial answers. Alternatively, the responses to double-stranded DNA breaks are absent in DFF45-deficient cells, thus contributing to the apparent resistance to apoptosis in these cells. We have made DFF45-deficient mouse embryonic fibroblasts, and these cells will permit us to address these and other mechanistic questions directly in a more homogenous cell population.

**DFF45 in DNA Fragmentation and Chromatin Condensation**—We found that DFF45-deficient thymocytes are resistant to DNA fragmentation, whereas wild-type thymocytes exhibit standard DNA laddering on exposure to dexamethasone. These in vivo results substantiate and extend our previous finding that DFF45 is essential for DNA fragmentation in vitro (43, 44). Together, these results establish that DFF45 plays a critical role in vivo in internucleosomal cleavage of DNA on exposure to apoptotic stimulation. Moreover, these findings argue that there are unlikely additional mechanisms, such as the existence of DFF45 homologs or other nuclease systems, responsible for degrading DNA to nucleosomal sizes in thymocytes in vivo. Whether the DFF45 mutation affects chromatin degradation into 30–50-kilobase pair fragments remains to be seen (52, 53).

Our electron microscopic studies demonstrate that DFF45-deficient thymocytes exhibit strikingly different dying morphology than wild-type cells on induction of apoptosis. Features observed only in DFF45-deficient cells include poor chromatin condensation, intranuclear cytoplasmic invagination, fusion of nuclear chromatin released from disintegrating cells, and the absence of normal nuclear fragmentation or apoptotic body formation. These findings support and extend previous reports that DFF45 is essential for chromatin condensation during apoptosis (40, 43). It is likely that because of conglutination of liberated chromatin and the absence of nuclear fragmentation, phagocytosis would be altered. The consequence of the possible abnormal phagocytosis is under investigation.

Nagata and colleagues (53) recently reported that chromatin condensation could still occur on activation of apoptosis when DFF45 activity is inhibited in a dominant negative transfection system. One possible explanation of this finding is that different cell systems may contain a different set of factors for inducing chromatin condensation. Sahara et al. (54) reported recently that in addition to DFF, other proteins could also serve as chromatin condensation factors on activation of apoptosis. Thus, DFF45 and DFF40 may be important in chromatin condensation in mouse thymocytes but may not be in human Jurkat cells. Another possible explanation is that the caspase-resistant DFF45 inhibits the nuclease activity of DFF40 but not the chromatin condensation activity of DFF40 because of conformational and stoichiometric differences between the caspase-resistant DFF45-DFF40 complex and the wild-type
DFF45-DFF40 complex. The continuous use of DFF45 mutant mice as a model system will help answer these and other questions regarding how DFF45 might function in vivo in the near future.

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