

Review

Degradation of chromosomal DNA during apoptosis

S Nagata^{*1,2,3}, H Nagase², K Kawane², N Mukae² and H Fukuyama^{1,2}¹ Integrated Biology Laboratories, Graduate School of Frontier Science, Japan Science and Technology Corporation, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan² Department of Genetics, Osaka University Medical School, Japan Science and Technology Corporation, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan³ Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan^{*} Corresponding author: S Nagata, Department of Genetics, Osaka University Medical School, B-3, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan.

Tel: +81 6 6879 3310; Fax: +81 6 6879 3319;

E-mail: nagata@genetic.med.osaka-u.ac.jp

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Abstract

Apoptosis is often accompanied by degradation of chromosomal DNA. CAD, caspase-activated DNase, was identified in 1998 as a DNase that is responsible for this process. In the last several years, mice deficient in the CAD system have been generated. Studies with these mice indicated that apoptotic DNA degradation occurs in two different systems. In one, the DNA fragmentation is carried out by CAD in the dying cells and in the other, by lysosomal DNase II after the dying cells are phagocytosed. Several other endonucleases have also been suggested as candidate effectors for the apoptotic degradation of chromosomal DNA. In this review, we will discuss the mechanism and role of DNA degradation during apoptosis.

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Keywords: apoptosis; DNase; DNA fragmentation; CAD; DNase II

Abbreviations: CAD, caspase-activated DNase; ICAD, inhibitor of CAD; DFF, DNA fragmentation factor

Introduction

Programmed cell death or apoptosis is a process that removes toxic or useless cells that are produced during animal development.¹ During tissue turnover, senescent cells die via apoptosis to maintain homeostasis. Apoptosis can be induced by a variety of external stimuli, including death factors such as Fas ligand, γ -irradiation, anticancer drugs, and factor deprivation.^{2–5} Failure of apoptosis causes an accumulation of abnormal cells leading to cancer and autoimmune diseases, whereas its exacerbation causes tissue destruction

such as that seen in hepatitis, AIDS, and neurodegenerative diseases.

Apoptosis is defined by morphological changes of cells and nuclei, such as cell shrinkage, condensation and fragmentation of nuclei, and blebbing of the plasma membranes.⁶ This process is accompanied by the degradation of chromosomal DNA in most cases. In contrast to the necrotic cell death that accompanies the rupture of plasma membranes, the cellular contents are thought not to be released during the apoptotic process. However, if dying cells are not properly disposed of, they rupture and undergo secondary necrosis. Thus, apoptotic cells must be swiftly recognized and engulfed by professional phagocytes (macrophages and dendritic cells) or neighboring cells.^{7,8} Extensive studies of the signal transduction of apoptosis identified caspases (cysteine-protease) as the effectors.⁹ Whatever the apoptotic stimuli are, a set of caspases seems to be activated to cleave cellular substrates to kill the cells¹⁰ and to present one or more 'eat me' signals to phagocytes.⁷

Identification of the DNase responsible for the apoptotic DNA degradation

In 1980, Wyllie reported¹¹ that when mouse thymocytes were treated with glucocorticoid, a substantial amount of chromatin complex was released from the nucleus. The DNA in the released complex was double stranded, and consisted of multimers of about 180 bp, which is the size of the DNA comprising a nucleosome. Since the protein composition in the complex was not different from that in intact nucleosomes, it was concluded that a double-strand specific endonuclease(s) is activated during apoptosis and cleaves the nucleosomal spacer regions to yield a DNA ladder. Subsequently, the application of pulsed-field gel electrophoresis showed that prior to or in the absence of internucleosomal cleavage, chromosomal DNA is cleaved into large fragments of 50–300 kb.^{12,13} The sequence of the cleavage sites is A/T-rich, as found in the nuclear scaffold region,¹⁴ suggesting that chromatins are cleaved by a nuclease at the nuclear scaffold attachment sites. A different requirement for metal ions (Mg^{2+} and Ca^{2+}) initially suggested that the DNase(s) for internucleosomal cleavage and large-scale degradation might differ.¹⁵

Many groups since then have searched for the nuclease(s) responsible for this process. However, the DNase was labile and scanty, making purification of the enzyme extremely difficult.¹⁶ Nonetheless, several enzymes were proposed as candidates, including DNase I,¹⁷ DNase II,¹⁸ cyclophilins,¹⁹ and DNase γ .²⁰ None of them, however, appeared to fulfill the criteria for the apoptotic DNase. For example, DNase I is a secreted protein with a signal sequence, expressed only in a limited set of tissues, such as the kidney, pancreas, and seminal plasma. It is not, however, expressed in lymphocytes,

which efficiently undergo apoptotic DNA fragmentation. DNase I-mutant mice were recently established by gene targeting, and the analysis of these mice showed that DNase I is responsible for the removal of nuclear debris at sites of high cell turnover,²¹ but not for the degradation of DNA in apoptotic cells (personal communication from Dr. Hans G Mannherz, Ruhr-Universität Bochum, Germany). DNase II is a lysosomal enzyme, and apoptotic DNA degradation occurs at the early stage of apoptosis when lysosomes have not yet been destroyed, suggesting that DNase II might not function to fragment DNA in apoptotic cells. However, as discussed below, DNase II seems to work in phagocytes to cleave the DNA of the apoptotic cells after phagocytes engulf them.

Meanwhile, caspases have been identified as effectors of the apoptotic process,²² and DNA fragmentation as well as nuclear morphological changes have been placed downstream of caspase activity.^{23–28} When cytosolic extracts from proliferating cells are treated with caspases, they induce DNA fragmentation in isolated nuclei.^{29–31} The factor responsible, designated as DNA fragmentation factor (DFF), has been purified from HeLa cells to homogeneity as a heterodimer of 45 and 40-kDa subunits.³⁰ When DFF was treated with caspase 3, the 45-kDa subunit (DFF45) was specifically

cleaved, and DFF then caused DNA fragmentation in nuclei. However, since the DNase activity was not detected in this preparation, Liu *et al.*³⁰ initially concluded that the DNase responsible for the DNA fragmentation resided in the nuclei, and that DFF activated the DNase(s). A similar approach by us³² and Williams' group³³ has led purification of a DNase of 40 kDa from mouse lymphoma WR19L and human Jurkat cells, respectively. This molecule was designated as caspase-activated DNase (CAD) by us or caspase-activated nuclease (CPAN) by Williams' group. The DNase activity of CAD/CPAN could be inhibited by ICAD (inhibitor of CAD, or DFF45), and the inhibitory activity of ICAD was destroyed by caspase 3. In addition to action on naked DNA, CAD/CPAN caused the fragmentation of chromosomal DNA in nuclei. These similarities between DFF and CAD eventually led us to conclude that DFF is a complex of CAD and ICAD.^{34,35} The initial failure to detect the DNase activity of human DFF40 was apparently because of its extreme instability.^{33,34}

Caspase-activated DNase (CAD, DFF40)

CAD has so far been identified in six species (human, mouse, rat, Zebrafish, *Drosophila*, and chicken) (Figure 1). Except for

	fly (1)	MISYIRDAIMSPTRSSSNKSNQPTGEVLKDVSSSE	
fly (40)	SRGTAGAPMRGYKVTDNERTKRYGIGANSLEMLIAKAKSKFPLEL	--HLYLASDGFVSDDEYLKSLPAQTLFIVSGPDAVITTDAD	
mouse (1)	MCAVLRQPKCKVLRLALHSACKFVGAARSCQELLRKGCVRFLPMPGSRCLYEDGTEVTDDCFGLPNDAE	LLLLLTAGETWHGYVSD	
rat (1)	MCAVLPQPKCKVLRLALHSSCKFVGAARSCQELLRKGCIRFQLPVPGSRCLMYEDGTEVTDDCFGLPNDSE	LLLLLTAGETWHGYVSD	
human (1)	MLQPKPSVKLRLALRSRKFVGAARSCQELLRKGCIRFQLPVPGSRCLMYEDGTELTEDYFPPSPVDNAELVLLTLGQAWQGVSD		
Zebrafish (1)	MHKTTPKPKLVKIRSANQARKYGIAAVNLKELIKKGCQLLVSSSGVLVCLYEDGTVVTEEFQNLDPDNTDLVLLPHGQSWNGFADE		
Chicken (1)	MAAPLRGFRLLRRPGSAQKFGAAAGSLRGLLRKGCRLQLPLAGSRCLYEDGTELSAFAFFRTLPPQTELVLLRPGESWPGCCGD		
fly (125)	FEFEKMRQSQPLLVK--ADIFYDFIEQHPEKFRMRMITEYEHQKQRRVLDNSKTHLSLKAHEVWFTGGEERFHSKEEAMATRAQTRVR		
mouse (88)	ITRFLSVFNPHAGVIAAARQLLSDEQAPLRQK----	LLADLLHHVSNQITAE TRE--QDPSWFEGLESFRFNK-----S	
rat (88)	ITRLLSVFNPHAGVIAAARQLLSDEQAPLRQK----	LLADLLHHVSNQITAE TRE--QDPSWFEGLESFRFNK-----S	
human (85)	IRRFLSAFHEPQVGLIQAAQQLLCDEQAQRQR----	LLADLLHNVSNQIAAE TRA--EDPPWFEGLESFRFQSK-----S	
Zebrafish (87)	INRVGLDRNTEELLVSAAGQLLSDESPKRRR----	ILGDLNLRDRSELENRL--QDHDWFEGIDTRFKTK-----S	
Chicken (85)	VERFLAALCSRTDAVVEAARRLLEDERAPRRQR----	LLADLIHNLSGDSAAERRD--EDGKWFEGLESFRFNK-----S	
fly (211)	GYYYKAKEELTRNPLRYQNAKARQVINSVLEKFRYLLIGCDFSSMMFDRNCKQKHEFLKQHLGDEETDAGRIPSKRLRQVIREYTKEN		
mouse (157)	GYLRYSCESRIRGYLREVSAYTSMVDEAAQEEYLRVLGSMCQKLK-----	SVQYNGSYF	
rat (157)	GYLRYSCESRIRGYLREVSAYISMVDAAREEYLRVLSSMCHKLK-----	SVQYNGSYF	
human (154)	GYLRYSCESRIRSYLREVSYPSTVGAEAEQEEFLRVLGSMCQRLK-----	SMQYNGSYF	
zebrafish (156)	AYMKYNCESRIRGYLKEVDGYTQTIKNAKTKSEYKKVAEALAEKLLK-----	AARYNGTYF	
chicken (154)	SYMRYSCESRIRSYMKEVSNFISNVHPTARDAYKRIIDLMSDKLK-----	SVKYNGSYF	
fly (299)	CILDEWS-TSLCSDLGDFYCQGSYSENGNSCSKQHTINPYASRENILFQVWNLDHQIELCRTILPALVANVEELVSHPTKCSIHKK		
mouse (211)	DRGAEAS-SRLCTPEGWFSCQG--PFDLESCLSKHSINPYGNRESRIIFSTWNLDHIEKKRTVVPTLAEAI-----		
rat (211)	DRGAEAS-SRLCTPEGWFSCQG--PFDLESCLSKHSINPYGNRESRIIFSTWNLDHIEKKRTVVPTLAEAI-----		
human (208)	DRGAKGG-SRLCTPEGWFSCQG--PFDMSCLSRHSINPYGNRESRIIFSTWNLDHIEKKRTIIPITLVEAIKE-----		
zebrafish (211)	DRSEKDA-NRLCTEEGWFSQCG--AFDENSCNLFHSINPYGNRESRIIFSTWNLDHIEKKRTVIIPALAKALEA-----		
chicken (208)	DRTEEAAMRLCTKEGWFSQCG--PFDRDDCPCKHSINPYTNRESRIIFSTWNLDHIEKKRSVVPPELAEAVKT		
fly (386)	QVVDISVLEYF-LEIFSLKNLKLHVHIVCHEKAQRSNRSNGRLLCSDCHEYRIVQELMEVQVDSLAT	450 aa, pI=7.2	
mouse (280)	QDGREVNWEYFYFSLFTAENLKLHVHIACHKK-----TTHKLECDRSRIYRPQTGSRRKQPARKKRPARKR	344 aa, pI=8.7	
rat (280)	QDGREVNWEYFYFSLFTAENLKLHVHIACHKK-----TTHKLQCDRSRIYRPQTGSRRKQPARKKRPARKRQ	349 aa, pI=9.1	
human (279)	QDGREVDWEYFYGLLFTSENLKLHVHIVCHKK-----TTHKLNCDSRIYKYPQTRLKRLKQPVRRKQ	338 aa, pI=9.1	
zebrafish (282)	NKNSDINVDYFYKLLFTRENLKLHVHIVCHKK-----GAHELSCDSRKIYKRVKKTIVK	333 aa, pI=9.5	
chicken (280)	RDGREVNWEYFYQLLFTMNNLKLHVHIACHKK-----TNHNLSCDKTKIYKRRKQTHEIS	333 aa, pI=9.3	

Figure 1 Alignment of the amino-acid sequences of CAD from six different organisms. The amino-acid sequences of CAD from *Drosophila melanogaster*,³⁶ mouse,³² rat,¹⁰³ human,^{34,35} zebrafish (GenBank Accession number: AF286179), and chicken⁷⁵ are aligned to give a maximum homology by introducing several gaps (—). The amino-acid residues conserved among six members are shown in bold. The caspase-recognition sequence in *Drosophila* CAD is boxed. Three lysine residues and one tyrosine residue essential for the CAD's DNase activity are shown in blue and brown, respectively. Four histidine residues required for the DNase activity are shown in red. Among these histidine residues, His-313 in mouse sequence is not conserved in *Drosophila*, but His-433 of *Drosophila* CAD may have a similar function. A putative nuclear localization signal is underlined. The total numbers of amino acids for each proteins, and their isoelectric points are listed at the end

Drosophila CAD, which is a 52-kDa protein with an isoelectric point of 7.2, the CADs from other species are basic proteins of about 40 kDa with a pI of 8.7–9.5. *Drosophila* CAD contains a caspase-recognition sequence (EETD) in the middle of the molecule, which is cleaved by caspases to become an active enzyme comprised of large and small subunits (p32 and p20).³⁶ In contrast, the CAD from other species does not undergo post-translational cleavage, and has no subunit structure. The enzymatic properties of CAD have been mainly characterized with the mouse and human proteins. CAD binds to DNA without Mg^{2+} ,³⁷ and has an endonuclease activity at neutral pH in the presence of Mg^{2+} .³⁸ It is specific for double-stranded DNA with a preference for A/T-rich region, and it does not cleave single-stranded DNA or RNA. CAD mainly generates DNA fragments with blunt ends carrying a 5'-phosphate and 3' hydroxyl group,³⁸ which can be substrates for the terminal transferase³⁹ used for the TUNEL (TdT-mediated dUTP-biotin nick end labeling) reaction.

CAD has a high specific activity. The homogeneous recombinant mouse CAD (0.1 pmol) generates about 30 pmol of 3' hydroxyl ends after 30 min at 30°C when it is assayed using optimal conditions.³⁹ Human and mouse CADs behave peculiarly in gel filtration: when analyzed using a low-salt buffer, they stick to the gel (H Sakahira and SN, unpublished result) or elute as aggregates,^{33,40} probably because of their extremely positive charge. On the other hand, active *Drosophila* CAD behaves like a protein of about 100 kDa consisting of (p32)₂(p19)₂.³⁶ Several histidine, lysine, and tyrosine residues in the C-terminal half of CAD are well conserved among different species (Figure 1).^{39,41} Chemical and site-directed mutagenesis showed that these conserved residues are essential for its DNase activity, indicating that CAD is a member of the histidine nuclease family that includes RNase T1, DNase I, and S1 nuclease.^{39,41,42} Histone H1, high mobility group protein (HMG)-1, HMG-2, and DNA topoisomerase II α were reported to bind CAD and stimulate its DNase or DNA fragmentation activity.^{34,40,43} On the other hand, when the recombinant CAD, free of the residual ICAD, was assayed under optimal conditions, histone H1 had no effect on CAD's DNase activity (H Sakahira and SN, unpublished result). Although we cannot rule out the possibility that these molecules regulate the CAD activity in cells, it is likely that the effect of these proteins is to stabilize CAD during the assay, or to remove caspase-cleaved ICAD from CAD.

Inhibitor of CAD (ICAD, DFF45)

ICAD has been identified in human, mouse, rat, and *Drosophila*. In human and mouse, there are two forms of ICAD that are encoded by alternatively spliced mRNAs; these are designated ICAD-L and ICAD-S for the long and short forms, respectively.^{32,44} ICAD-L and ICAD-S are acidic proteins of 45 and 30 kDa, and they inhibit CAD's DNase activity with the same efficiency.^{37,45} They inhibit CAD by blocking its binding to DNA.³⁹ In proliferating cells, ICAD-L but not ICAD-S exists as a heterodimer (CAD/ICAD-L) with CAD in mouse and human cells,^{32,40} or as a heterotetramer (CAD)₂(ICAD-L)₂ in *Drosophila* cells.³⁶ This is because

ICAD-L but not ICAD-S binds to the nascent polypeptide of CAD while it is on ribosomes.⁴⁶ During its synthesis, ICAD-L, together with general chaperones of hsp70 and hsp40, helps CAD to be folded correctly. It is released from ribosomes as a complex with CAD when the CAD synthesis is completed. Thus, the functionally folded CAD protein cannot be produced without ICAD-L,⁴⁷ and ICAD-L must be coexpressed in mammalian, insect, or *E. coli* cells to produce functional CAD.^{32,37,40} In addition to the regulation of CAD by ICAD-L, we recently found that the expression of ICAD-L but not ICAD-S is regulated by CAD at the post-translational level, suggesting that their expression is mutually regulated.¹⁰⁴ No specific function has been assigned to ICAD-S, and it is probably dispensable in most if not all species, as has been shown in *Drosophila*.⁴⁸ In this regard, results by Chen *et al.*⁴⁹ indicating that rat and mouse tissues express only ICAD-S are difficult to explain.

Human and mouse ICADs carry two caspase-recognition sites, at Asp-117 and 224,^{30,44} while there is a single caspase recognition site in *Drosophila* ICAD at Asp-119.⁴⁸ Among members of the mammalian caspase family, caspases 3 and 7 cleave ICAD.^{50,51} Mammalian ICAD must be cleaved at both sites to lose its CAD-inhibiting activity. That is, ICAD fragments carrying amino-acid residues from 1 to 224 or 118 to 331 still have a substantial affinity for CAD and can inhibit its DNase activity.^{44,52,53} The human breast carcinoma MCF-7 cell line carrying a deletion in the caspase 3 gene,²⁸ or mouse cells derived from caspase 3-null mice⁵⁴ do not undergo DNA fragmentation upon exposure to apoptotic stimuli. In these cells, ICAD is cleaved at Asp-117 but not Asp-224, suggesting an absolute requirement of caspase 3 to cleave ICAD at Asp-224 for full activation of CAD.⁵⁵ Differing from the initial report,⁵¹ two reports have claimed that granzyme B, a serine protease released from cytotoxic T cells (CTL), cleaves ICAD at Asp-117 to activate CAD.^{56,57} However, the fact that ICAD-L can still inhibit the CAD's DNase when cleaved only at Asp-117 may not support the idea that ICAD-L is cleaved in this way in CTL-induced apoptotic DNA fragmentation.

Interaction between CAD and ICAD

As described above, ICAD-L can be divided into three domains (amino acids 1–117, 118–224, and 225–331) that can be separated by cleavage with caspase 3. These three domains synergistically contribute to ICAD's ability to bind CAD and inhibit its DNase activity.^{52,53} Among these domains, the N-terminal domain of about 80 amino acids is significantly homologous to the N-terminal domain of CAD, and is called the CIDE or CAD (CD) domain.^{35,58} The ICAD-CD and CAD-CD specifically interact with each other, and this interaction is important for ICAD-L to recognize the nascent polypeptide of CAD on ribosomes.⁴⁶ The tertiary structures of the CAD-CD and its complex with the ICAD-CD have been resolved by the NMR analysis.^{59–61} Both CAD-CD and ICAD-CD are composed of one α -helix and five β -strands, and belong to the ubiquitin-superfold family. In the complex, they are aligned side by side through ionic interactions between basic residues in CAD-CD and acidic residues in ICAD-CD.⁵⁹

Thus, point mutations in the acidic residues of the ICAD-CD abolish the ability of ICAD-L to facilitate the correct folding of CAD during its synthesis, although the mutants can still inhibit the CAD's DNase.

Cellular Localization of CAD and ICAD

CAD must function in the nucleus to digest chromosomal DNA. CAD from human, mouse, rat, and zebrafish carries a typical nuclear localization signal (NLS) at the C terminus (Figure 1). An NLS-like sequence has also been identified in the C-terminal region of ICAD-L.^{62,63} However, there is contradictory evidence regarding which cellular compartments contain CAD and ICAD in proliferating cells. The CAD/ICAD (DFF) complex was purified from the cytosolic fraction of HeLa and mouse lymphoma cells.^{30,32} The fractionation of cellular organelles followed by Western blotting showed that ICAD-L is mainly localized in the cytosol.⁶⁴ From these results, we initially suggested that CAD/ICAD complex resides in the cytoplasm in proliferating cells and that CAD translocates into the nucleus after ICAD is cleaved by caspase 3.³² CAD but not the CAD/ICAD complex enters nuclei in a cell-free system, or in a semipermeabilized cell system, which seemed to agree with this proposal. On the other hand, immunostaining of endogenous ICAD showed it to be in the nucleus of human HeLa and epithelial cells,^{34,63} but in the cytoplasm of activated thymocytes and Hodgkin's lymphoma cells.^{65,66} The expression pattern of exogenous ICAD tagged with Flag or GFP also gave contradictory results. Two reports showed that the exogenously expressed ICAD is mainly localized in the nucleus,^{63,67} but one report showed a cytoplasmic localization.⁶⁵ Whether these contradictory results indicate that ICAD is localized in the nucleus or cytoplasm in a cell-specific manner, or whether they are because of technical problems in the cell fractionation and/or immunostaining procedure remains to be clarified. In this regard, it may be worth noting that *Drosophila* CAD and ICAD do not carry an NLS at the position corresponding to the NLS of their mammalian counterparts (Figure 1).^{36,48}

Contribution of CAD to DNA Degradation

During apoptosis, DNA is cleaved into blunt end double-stranded fragments carrying a 5' phosphate and 3' hydroxyl group.⁶⁸ Using this knowledge, two methods – TUNEL⁶⁹ and LM-PCR (linker-mediated PCR)⁷⁰ – were developed to label the cleaved DNA. The application of these methods indicated that DNA fragmentation takes place in most apoptotic processes in mammals, *Drosophila*, and *C. elegans*.^{69–71}

CAD and ICAD are expressed at the mRNA and protein levels in various tissues with abundant expression in lymphoid tissues such as the thymus, spleen, and lymph nodes.^{72,73} Thymocytes, lymphocytes, and lymphoid cell lines undergo massive DNA fragmentation upon exposure to apoptotic stimuli (Figure 2). In contrast, mouse embryonal fibroblasts (MEF) express a low level of CAD, and the presence of apoptotic DNA fragmentation is not clear.⁷⁴ The contribution of CAD to DNA fragmentation was examined by expressing a caspase-resistant ICAD, or by deleting the ICAD or CAD gene

by the gene-targeting method (KK, HF, and SN, unpublished results).^{47,50,75} ICAD that carries mutations (Asp to Glu mutation at P1 position) in the caspase-recognition sites binds CAD and inhibits its DNase, but since this molecule cannot be cleaved by caspases, CAD is not activated. Since ICAD-L is indispensable for the production of functional CAD protein, ICAD-null cells behave as if they lacked CAD. Thus, cells expressing the caspase-resistant ICAD, or that are deficient in the ICAD or CAD gene, show similar phenotypes. Splenocytes, thymocytes, and granulocytes from ICAD (DFF45)-null mice are resistant to DNA fragmentation upon exposure to various apoptotic stimuli such as staurosporine, etoposide, and actinomycin D.^{47,76} Human cell lines (Jurkat lymphoma and IL-3-dependent TF-1 myeloid lymphoma) and the *Drosophila* BG-2 neuronal cell line that were transformed with the caspase-resistant ICAD, or thymocytes from the transgenic mice carrying the caspase-resistant ICAD gene, do not undergo DNA fragmentation after treatment with Fas ligand, factor deprivation, anticancer drugs, or kinase inhibitors.^{48,50,73} Chicken DT40 cells that lack the CAD gene do not show fragmented DNA after treatment with etoposide.⁷⁵ Lymphocytes and hepatocytes from the CAD-null mice are also resistant to the apoptotic DNA fragmentation (Figure 2, data not shown). From these results, it is clear that CAD is responsible for the degradation of DNA into nucleosomal units during apoptosis.

There are, however, reports that challenge the idea that CAD is indispensable in apoptotic DNA fragmentation. During apoptosis induced by a set of stimuli, many proteins are released from the mitochondria.⁷⁷ One is endonuclease G, which is usually localized to the intermembrane space of the mitochondria. Since this nuclease caused DNA fragmentation in isolated nuclei, two groups suggested that endo-nuclease G works as an apoptotic endonuclease.^{78,79} However, this nuclease is (dG)_n/(dC)_n-specific,⁸⁰ preferentially generates single-stranded nicks, and cleaves not only DNA in inter-nucleosomal regions but also DNA within nucleosomes.⁸¹ As discussed by Wildlak *et al.*,⁸¹ it is unlikely that endonuclease G alone is involved in the apoptotic cleavage of chromosomal DNA. The observation that the number of TUNEL-positive cells increases in an endonuclease G-deficient mutant of *C. elegans*⁸² supports this notion.

As described above, the apoptotic fragmentation of DNA into nucleosomal units is preceded by the degradation of chromosomal DNA into a high-M_r DNA of 50–300 kb.^{12,13} Cells expressing the caspase-resistant ICAD,⁸³ or thymocytes and embryonal fibroblasts from ICAD (DFF45)-null mice, lack this type of chromosomal DNA degradation upon treatment with various apoptotic stimuli,^{74,84} suggesting that CAD is also responsible for the large-scale degradation of chromosomal DNA. On the other hand, the large-scale degradation of chromosomal DNA was observed in chicken DT40 cells lacking CAD, and Samejima *et al.*⁷⁵ proposed that an ICAD-inhibitable non-CAD DNase causes this type of degradation. No caspase-dependent DNase activity was detected when the extracts from the thymocytes from CAD-null mice were treated with caspase 3 (HN, and SN, unpublished results), and all the ICAD seems to be associated with CAD in *Drosophila* cells,⁴⁸ indicating that there are no additional ICAD-inhibitable DNases in mouse and *Drosophila*

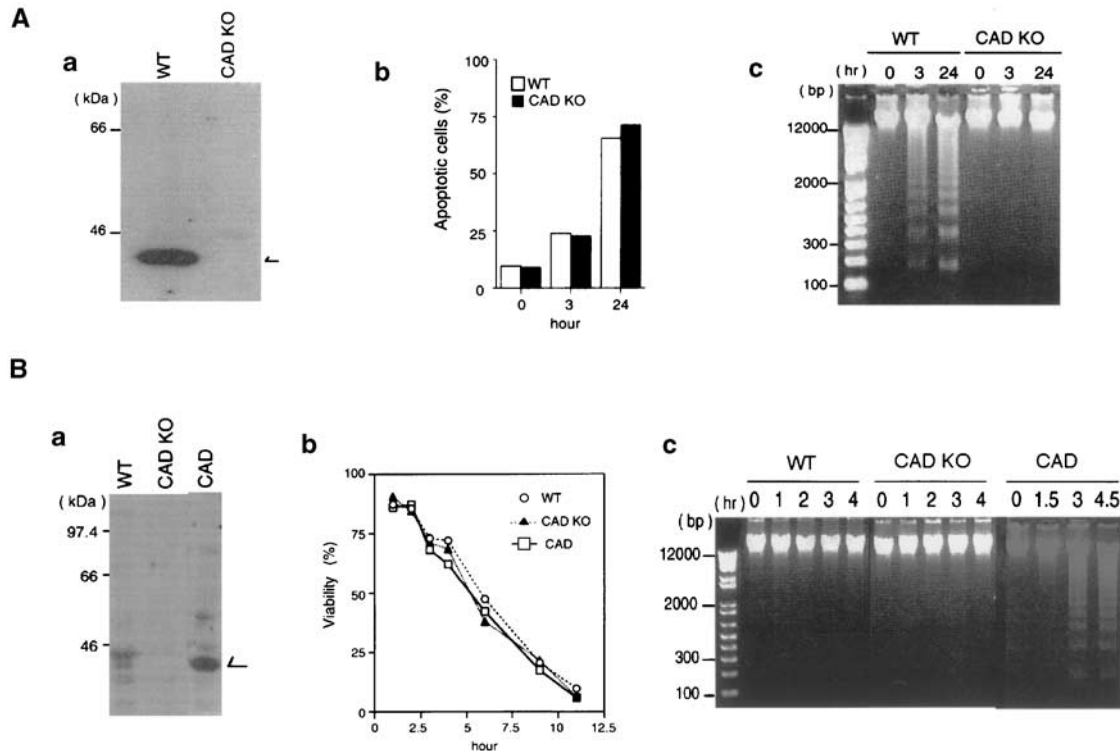


Figure 2 CAD-dependent apoptotic DNA fragmentation in mouse splenocytes and embryonal fibroblasts. (a) Apoptotic DNA fragmentation in mouse splenocytes. The S-30 fractions (20 μ g protein each) from splenocytes of the wild-type (WT) and CAD^{-/-} (CAD KO) mice were analyzed by Western blotting using the anti-CAD mAb (panel a). The relative molecular weights of the standard proteins are shown in kDa on the left. The band for CAD is indicated by an arrow on the right. Mouse splenocytes (1×10^6 cells/ml) from wild-type (\square) and CAD^{-/-} (\blacksquare) mice were incubated at 37°C with 500 U/ml of human Fas ligand. At the indicated time, cells were stained with FITC-labeled annexin V, and the percentage of annexin V-positive cells is shown in panel b. The chromosomal DNA was analyzed by electrophoresis on an agarose gel and is shown in panel c. The sizes of the marker DNAs are given in bp on the left. (b) Apoptotic DNA fragmentation in MEF. The S-30 fractions (40 μ g protein each) from the wild-type (WT), CAD^{-/-} (CAD-KO), and CAD-transformed (CAD) MEFs were analyzed by Western blotting using the anti-CAD mAb (panel a). The wild-type (\circ), CAD^{-/-} (\blacktriangle), and CAD-transformed MEFs (\square) (1×10^6 cells) were incubated in 96-well microtiter plates at 37°C for the indicated periods with 5000 U/ml Fas ligand in the presence of 1.5 μ g/ml cycloheximide. The cell viability was analyzed by staining the cells with crystal violet, and is shown as the percentage (panel b). The fragmentation of chromosomal DNA is shown in panel c. Similar results were obtained with treatment with staurosporine.

cells. Purification and characterization of the nuclease that causes the large-scale DNA degradation from chicken cells will be necessary to clarify this apparent controversy. In addition, apoptosis-inducing factor (AIF), which is also released from mitochondria during apoptosis, has been reported to cause the large-scale DNA degradation.⁸⁵ However, AIF has no nuclease activity, and the nuclease that is activated by AIF has not been identified. It is therefore unclear whether AIF has a role in the apoptotic DNA degradation.

Contribution of the DNA Degradation to Cell Death

When CAD is injected into cells, chromatin quickly condense and are fragmented.⁸⁶ We are certain that the degradation of chromosomal DNA by CAD is an efficient way to kill the cells. However, this system is apparently dispensable for cell death. That is, cells expressing caspase-resistant ICAD or cells deficient in ICAD or CAD are still efficiently killed by treatment with apoptotic stimuli (Figure 2). Caspases that are activated during the apoptotic process cleave more than 100 cellular

substrates such as actin and cyclin A.⁸⁷ Without the function of these molecules, cells will not survive. In some cases such as glucocorticoid-treated thymocytes, kainic acid-treated neuronal cells, or tumor necrosis factor (TNF)-treated MEF, CAD deficiency causes a delay in the cell death processes.^{74,76,88} Treatment of MEF cells with TNF does not cause DNA fragmentation, but induces the large-scale degradation of chromosomal DNA.⁷⁴ This is followed by activation of poly(ADP-ribose) polymerase (PARP), which leads to the depletion of NAD and ATP that kills the cells. Since the large-scale DNA degradation does not occur in DFF45 (ICAD)-null cells, NAD is not depleted and the cell death is delayed. On the other hand, staurosporine or Fas ligand, which induces apoptosis more strongly than TNF does, kills CAD-null, wild-type, or CAD-transformed MEF cells with the same efficiency (Figure 2). It is possible that strong apoptosis inducers such as staurosporine and Fas ligand activate caspases sufficiently to kill the cells directly. In contrast, if the caspases are not sufficiently activated by weak stimuli such as TNF, another pathway, such as CAD-dependent PARP activation, may contribute to the killing of the cells.

Mice deficient in CAD do not show adverse phenotypes, and do not accumulate undigested nuclei or DNA in their tissues. Although one report claims that spatial learning and memory are enhanced in DFF45 (ICAD)-null mice,⁸⁹ this was not confirmed by another group.⁹⁰ At a minimum, the longevity and fertility of CAD- or ICAD-null mice kept in specific pathogen-free conditions in our Medical School are not different from those of wild-type mice, indicating that the CAD-mediated apoptotic DNA degradation is not required for normal animal development.

DNA Degradation of Apoptotic Cells in Phagocytes

With a few exceptions,^{78,90} cells deficient in CAD do not undergo apoptotic DNA degradation *in vitro*, and do not become TUNEL-positive. However, very surprisingly, the thymus, ovary, and brain of CAD-deficient mice were found to contain TUNEL-positive cells to the same extent as in wild-type mice.⁷³ These TUNEL-positive cells were always inside macrophages, as revealed by electron microscopy. When wild-type mice were irradiated with γ -rays, massive TUNEL-positive cells were generated outside the macrophages in the thymus. The same treatment of the CAD-deficient mice killed many thymocytes, but they were not TUNEL-positive. This was explained as follows: During development, cells undergoing negative or positive selection in the thymus die in an orderly manner, and the dying cells, whether their DNA is fragmented or not, are immediately engulfed by phagocytes. The engulfed dead cells will enter lysosomes, and their DNA is degraded by a lysosomal enzyme(s). On the other hand, if massive cell death is induced by γ -rays, the capacity of phagocytes to engulf the cells is exceeded, and the corpses with their TUNEL-positive nuclei are left unengulfed. In any case, these results indicate that the DNA of apoptotic cells can be degraded in phagocytes after the cells are engulfed.^{73,91} In support of this idea, when the apoptotic CAD-deficient cells were cocultured with macrophages, their DNA was degraded in the lysosomes of macrophages.^{73,92}

Lysosomes carry a DNase called DNase II. It generates DNA fragments carrying 5' hydroxyl and 3' phosphate ends.⁹³ Thus, it is unlikely that the action of DNase II produces TUNEL-positive cells. However, lysosomes also carry acid phosphatases that may remove the 3' phosphate from the DNA fragments and allow them to become TUNEL-reactive at a certain time point. The DNase II-null mice accumulate many undigested nuclei in the macrophages of the fetal liver, and die as fetuses because of the failure of definitive erythropoiesis.⁹⁴ In addition, these embryos harbor a large amount of DNA that is left undigested in the macrophages of the tissues where apoptotic cell death occurs.⁹⁵ A similar deposit of DNA was previously reported in a *C. elegans* mutant lacking DNase II (Nuc-1).⁹⁶ Although there is a possibility that Nuc-1 may function in the dying cells in *C. elegans*,⁷¹ its localization in lysosomes strongly suggests that DNase II functions in phagocytes, at least in the mouse system.

Conclusion and perspective

The degradation of chromosomal DNA is a well-conserved apoptotic process. It can be seen in mammals, *Drosophila*, *C. elegans*, and plants. As discussed above, this process is mediated *in vivo* by two systems, one operating cell-autonomously in the dying cells, and the other in phagocytes after the dying cells are engulfed. In cell-autonomous DNA degradation, DNA is first cleaved into fragments of about 50 kb with intact nucleosomes, followed by degradation into nucleosomal units. Although we cannot rule out the possibility that other nucleases (endonuclease G, unidentified AIF-activated nuclease, and others) degrade DNA in certain circumstances, CAD is mainly responsible for this cell-autonomous DNA degradation. A simple explanation is, when CAD is activated, it first digests DNA at the nuclear scaffold region since this region is most exposed and A/T-rich. This unpacks the chromatin, and CAD then gains access to the spacer regions of nucleosomes to produce DNA fragments in nucleosomal units. There is a single gene for CAD in mammals and *Drosophila*. In *C. elegans*, Nuc-1 is known as a nuclease involved in DNA degradation,⁹⁶ but no CAD homolog has been identified. However, TUNEL-positive cells can be observed in *C. elegans*, in particular when Nuc-1 is mutated,⁷¹ suggesting that this animal also carries a CAD-like DNase.

One of the most surprising findings in our laboratory in recent years is that apoptotic DNA degradation or the generation of TUNEL-positive cells takes place *in vivo* in mice that are deficient in the CAD system. This and the identification of Nuc-1 as a DNase II-homolog by Wu *et al.*⁷¹ clarified the role for DNase II in digesting the DNA of apoptotic cells in macrophages. In *C. elegans*, Nuc-1 works in a Ced-3 (caspase)-dependent manner.⁷¹ Macrophages engulf only apoptotic cells by recognizing phosphatidylserine that is exposed on the cell surface in a caspase-dependent manner.⁷ In this regard, DNase II in macrophages also works in a caspase-dependent manner in the apoptotic process. In addition to DNase II, lysosomes contain abundant proteases that can digest the proteins of the phagocytosed apoptotic cells. Thus, the DNA of the engulfed apoptotic cells will be completely digested into nucleotides by DNase II in lysosomes.⁹⁷ We recently established *Drosophila* mutants lacking both CAD and DNase II.¹⁰⁵ In the CAD single mutant, there was no accumulation of DNA in embryonal tissues. In contrast, in the DNase II mutant, fragmented DNA accumulated in macrophages, and in the double mutant for the CAD and DNase II genes, large, probably intact, chromosomal DNA accumulated in macrophages. These results support the two-step model for apoptotic DNA degradation.

DNA is an immunogenic molecule, and activates immune responses.⁹⁸ If the DNA of apoptotic cells is left undigested, it may have adverse effects on the homeostasis of animals, as found in DNase I-null mice.²¹ The accumulation of undigested DNA in various tissues in DNase II-null mice but not CAD-deficient mice suggests that DNA degradation in macrophages may play a more vital role than CAD-mediated DNA degradation. If so, why have cells evolved an elaborate system to degrade chromosomal DNA by themselves? This

system is dispensable for cells to die, and it is not necessary for phagocytes to engulf dying cells. During development, apoptotic cells are recognized by phagocytes at a very early stage, when no apparent morphological change or DNA fragmentation has occurred.^{99,100} Once apoptotic cells present an 'eat me' signal, phagocytes can degrade the proteins and DNA of apoptotic cells, and may actively kill the cells.^{101,102} In this regard, the role of caspases may be just to present the 'eat me' signal to phagocytes. It is not clear to us, however, why caspases cleave so many cellular proteins, and CAD cleaves the chromosomal DNA if phagocytes can do the job. On the other hand, in pathological settings such as inflammation, ischemia, and acute infection, a large load of apoptotic cells may exceed the local phagocytic clearance capacity. In these situations, cell-autonomous degradation of chromosomal DNA by CAD, and the cleavage of cellular proteins by caspases may be more beneficial for organisms than releasing intact DNA or proteins into the circulation. We hope that in the near future, we will have a clearer picture of the role of DNA degradation in mammalian homeostasis and in various human diseases.

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