DNase γ -dependent and -independent apoptotic DNA fragmentations in Ramos Burkitt's lymphoma cell line

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ABSTRACT

DNA fragmentation is a biochemical hallmark of apoptosis. Several endonucleases, including CAD/DFF40 and endonuclease G, are implicated in DNA fragmentation. DNase γ has also been considered to be one of the enzymes involved, but its role in relation to CAD/DFF40 in apoptosis has not been fully elucidated. Here, we distinguished between DNase γ -dependent and CAD/DFF40-dependent DNA fragmentations. We found that DNase γ activities appeared in the late apoptotic phase and accelerated DNA fragmentation. Thus, even if the apoptotic DNA fragmentation is initiated by CAD/DFF40, DNase γ is required for the more complete digestion of the genomic DNA in dying cells.

Apoptosis, or programmed cell death, eliminates unwanted cells during development, homeostasis, and immune responses (11, 20). It is characterized by changes in cell morphology, including the formation of membrane-associated apoptotic bodies, membrane blebbing, nuclear breakdown, and chromatin condensation. At the molecular level, these changes are accompanied by the cleavage of several proteins and DNA fragmentation. DNA fragmentation is the final step in the disposal of the genomic DNA in cells undergoing apoptosis. The fragmentation of the genomic DNA in apoptotic cells occurs in two steps: the initial macrofragmentation (fragments ≥ 50 kb, a size consistent with that of a chromatin loop domain) and the subsequent internucleosomal DNA fragmentation, which is often observed as DNA laddering (12).

The DNA fragmentation during apoptosis is not a passive process but rather an active process that participates in the enhancement of the apoptotic cas-

cade in response to proapoptotic stimulations (2). However, the mechanism of the DNA fragmentation in apoptotic cells remains to be clarified. Although several endonucleases have been implicated in this process, their specific roles are still under debate.

The DNA fragmentation factor (DFF) is a well-characterized apoptotic endonuclease (9). DFF is a heterodimer composed of a 40-kDa molecule and a 45-kDa molecule (DFF40 and DFF45, respectively). DFF40 is also termed caspase-activated DNase (CAD) or caspase-activated nuclease (CPAN), and DFF45, the inhibitor of CAD (ICAD) (4, 5). DFF40/CAD exists in living cell nuclei as an inactive complex with its inhibitor DFF45/ICAD (9, 4, 15). After apoptosis induction, DFF40/CAD is released from the complex by the caspase-3-mediated cleavage of DFF45/ICAD and catalyzes internucleosomal DNA fragmentation. DNase γ , a nuclear Mg^{2+}/Ca^{2+} -dependent endonuclease, also termed DNAS1L3, LS-DNase, or DNaseY, has been reported to be an apoptotic endonuclease (1, 8, 14, 16, 21). DNase γ is located in the nuclear envelope in living cells and translocates into the nucleus in dying cells, where it induces DNA fragmentation (17).

Although some of the catalytic properties of DNase γ have been reported (17, 10), the role of

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DNase γ in relation to those of other endonucleases, particularly CAD/DFF40, has not yet been elucidated. In this study, we focused on the roles of DNase γ and CAD/DFF40 in the apoptosis-associated DNA fragmentation using Burkitt's lymphoma cell line Ramos. We found that each endonuclease plays a distinct role in specific situations.

MATERIAL AND METHODS

Cells. A stably DNase γ -expressing clone, γ Ramos-25, was generated previously by introducing the DNase γ expression vector to Ramos cells (13). The expression vector encoding the caspase-resistant form of ICAD (ICAD-CR) was stably introduced into Ramos and γ Ramos-25 cells as reported previously (18).

Isolation of nuclei and induction of DNA fragmentation. Cells were suspended and lysed in a hypotonic buffer (10 mM Hepes, 10 mM KCl, 3 mM MgCl_2 , 0.5 mM spermidine, and 0.1% NP40) for 5 min on ice and nuclei were harvested by centrifugation. Then, the isolated nuclei (5×10^5) were suspended in 60 μL of a buffer containing 25 mM Tris-HCl (pH 7.4), 150 mM KCl, and 5 mM MgCl_2 , with or without 5 mM CaCl_2 . The reaction was performed at 37°C for 1 h with or without 1 ng of the recombinant DNase γ (10, 19). DNA was purified from the nuclei with Wizard DNA purification resin (Promega, Madison, WI, USA) as described previously (21) and DNA fragmentation was analyzed by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.1 $\mu\text{g/mL}$).

Fig. 1 DNase γ -dependent DNA fragmentation. **(A)** DNA fragmentation in isolated nuclei was induced by DNase γ . Nuclei isolated from Ramos (lanes 1–3) and γ Ramos-25 cells (lanes 4–6) were incubated in a buffer containing 25 mM Tris-HCl (pH 7.4), 150 mM KCl, 5 mM MgCl_2 and with 5 mM CaCl_2 (+, lanes 2, 3, 5, and 6) or recombinant DNase γ (+, lanes 3 and 6). **(B–D)** Cell death was induced by etoposide (70 μM). Cultured cells were harvested at each incubation time (0–48 h) and cell viability was determined by trypan blue dye exclusion **(B)**, DNA fragmentation by agarose gel electrophoresis **(C)** and DNA content by flow cytometric analysis of PI-stained cells **(D)**, top panels; Ramos cells, bottom panels; γ Ramos-25 cells). Values in **(B)** represent the mean $\pm$ SD. DNA fragmentation **(C)** and a clear sub-G1 peak **(D)**, arrow) were detected in γ Ramos-25 cells after 48 h of incubation.

Apoptosis induction. Cells were cultured in RPMI medium supplemented with 10% fetal bovine serum and 100 U/mL penicillin-streptomycin. Apoptosis was induced with the addition of two chemotherapeutic drugs (2, 3), etoposide (70 μ M); a DNA topoisomerase II inhibitor, or staurosporine (1 μ M); a potent protein kinase inhibitor. The cultured cells were harvested at indicated incubation times (0–48 h) and cell viability was determined by microscopic examination by trypan blue dye exclusion (6). DNA content was determined by propidium iodide (PI) staining followed by flow cytometry (6). DNA was prepared from the harvested cells with Wizard DNA purification resin as mentioned above.

Western blot analysis. Western blot analysis was performed using rabbit anti-mouse ICAD (eBioscience Inc., San Diego, CA, USA) and goat anti- β -actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) antibodies as described previously (17). Blots were visualized using ECL Plus Western blotting detection reagent (GE Healthcare UK Ltd., Amersham Place, England).

RESULTS AND DISCUSSION

DNase γ -dependent DNA fragmentation

Ramos expresses a low level of DNase γ mRNA (13). In our previous study, a stably DNase γ -expressing cell line, γ Ramos-25, was established (13). We assessed DNA fragmentation in isolated nuclei of these cell lines (Fig. 1A). Nuclei from Ramos and γ Ramos-25 cells were incubated with Mg^{2+} in the presence or absence of Ca^{2+} or the recombinant DNase γ . Nuclei from Ramos cells showed no DNA fragmentation in the presence of both Mg^{2+} and Ca^{2+} (lane 2), but showed DNA fragmentation after the addition of the recombinant DNase γ (lane 3). On the other hand, nuclei from γ Ramos-25 cells clearly showed DNA fragmentation in the presence of Mg^{2+} and Ca^{2+} regardless of the addition of the recombinant DNase γ (compare lanes 5 and 6). Thus, exogenous DNase γ and endogenous DNase γ can cause the Ca^{2+} -dependent DNA fragmentation in isolated nuclei. Next, etoposide-mediated apoptosis was induced in these cells (Figs. 1B–D). Etoposide, a DNA topoisomerase II inhibitor, is

Fig. 2 DNase γ -independent DNA fragmentation. Cell death was induced by staurosporine (1 μ M). Cultured cells were harvested at each incubation time (0–48 h) and cell viability was determined by trypan blue dye exclusion (**A**), DNA fragmentation by agarose gel electrophoresis (**B**) and DNA content by flow cytometric analysis of PI-stained cells (**C**, top panels; Ramos cells, bottom panels; γ Ramos-25 cells). Values in (**A**) represent the mean $\pm$ SD. Although DNA fragmentation was detected in both Ramos and γ Ramos-25 cells to similar extents (**B**), a clear sub-G1 peak was detected only in γ Ramos-25 cells after 48 h of incubation (**C**, arrow).

a chemotherapeutic drug (2). Most of Ramos and γ Ramos-25 cells died within 48 h, as determined by trypan blue dye exclusion (Fig. 1B). DNA fragmentation was detected in γ Ramos-25 cells but not in Ramos cells at 48 h (Fig. 1C). Flow cytometric analysis of PI-stained cells showed a prominent sub-G1 peak (arrow) for γ Ramos-25 cells after a 48-h incubation (Fig. 1D), suggesting that DNA fragmentation is promoted by DNase γ . These data clearly indicate that DNase γ is responsible for the DNA fragmentation in the etoposide-induced apoptosis of γ Ramos-25 cells.

DNase γ -independent DNA fragmentation

We next tested the DNA fragmentation of Ramos and γ Ramos-25 cells when apoptosis was induced by staurosporine, a potent protein kinase inhibitor that is widely used as an apoptosis-inducing drug (3) (Fig. 2). Most of the cells died within 24 h, as determined by trypan blue dye exclusion (Fig. 2A). DNA fragmentation was detected in both Ramos and γ Ramos-25 cells to similar extents, starting at 3 h of incubation and peaking at 6 h (Fig. 2B). Flow cytometric analysis of PI-stained cells, however, showed

a clear sub-G1 peak for γ Ramos-25 cells after a 48-h incubation (Fig. 2C, arrow), suggesting that DNA fragmentation is enhanced in the stably DNase γ -expressing cells in the late apoptotic phase. These data indicate that the DNA fragmentation in the early apoptotic phase induced by staurosporine is independent of the DNase γ expression, although this expression enhances DNA fragmentation (Fig. 2C) in the late apoptotic phase as observed in etoposide-induced apoptosis (Fig. 1D).

DNase γ -independent and CAD-dependent DNA fragmentation

We next determined whether this DNase γ -independent DNA fragmentation detected in the early apoptotic phase after staurosporine treatment is mediated by CAD/DFF40. Using Ramos and γ Ramos-25 cells, we generated derivatives of each cell line expressing ICAD-CR, a caspase-resistant ICAD mutant that inhibits CAD activation (18). The expression level of ICAD-CR in both Ramos and γ Ramos-25 cells was determined by Western blot analysis (Fig. 3A). Although almost all the cells died within 48 h of the addition of staurosporine and there were no differ-

Fig. 3 DNase γ -independent/CAD-dependent DNA fragmentation. **(A)** Confirmation of the ICAD-CR expression in the stable transformants (+) of the ICAD-CR-expression vector by Western blot analysis. **(B)** Cell death was induced by staurosporine (1 μ M). Cultured cells were harvested at each incubation time (0, 6, and 48 h) and cell viability was determined by trypan blue dye exclusion. White bar; Ramos cells, hatched bar; ICAD-CR-expressing Ramos cells, gray bar; γ Ramos-25 cells, black bar; ICAD-CR-expressing γ Ramos-25 cells. **(C)** ICAD-CR expression inhibited the DNA fragmentation induced in staurosporine-treated Ramos and γ Ramos-25 cells. Cells with (+) or without ICAD-CR expression were induced into apoptosis with 6 h of staurosporine treatment and the genomic DNA prepared from the cells was examined by agarose gel electrophoresis. DNA contents in the cells were also determined by flow cytometry after PI staining **(D)**. The sub-G1 shoulder (arrows in top panels), a sign of DNA fragmentation, was not detected in ICAD-CR expressing cells (bottom panels).

ences between these cells in the apoptosis efficiency (Fig. 3B), the DNA fragmentation at 6 h of incubation was inhibited in the clones expressing ICAD-CR (Fig. 3C, +). By flow cytometry, PI-stained Ramos and γ Ramos-25 cells showed 'sub-G1 shoulders', which indicate the initiation of the DNA fragmentation at 6 h of incubation (arrows in Fig. 3D, top panels). No such shoulders were detected in stably ICAD-CR-expressing clones (Fig. 3D, bottom panels), suggesting that DNA fragmentation is inhibited by ICAD-CR. Thus, the DNA fragmentation in the early apoptotic phase induced by staurosporine is mediated by CAD/DFF40.

Here, we demonstrated that two apoptotic endonucleases, namely, CAD/DFF40 and DNase γ , have different roles in inducing DNA fragmentation in Ramos cells in response to different proapoptotic stimuli. The staurosporine-induced DNA fragmentation is mediated by CAD/DFF40, whereas the etoposide-mediated DNA fragmentation by DNase γ . We also showed that DNase γ is activated in isolated nuclei of unstimulated cells in the presence of Mg^{2+}/Ca^{2+} (Fig. 1A), indicating that no specific apoptotic signal or protein modification is necessary for DNase γ activation. The CAD/DFF40-dependent DNA fragmentation was detected within 3 h of incubation (Fig. 2B). In contrast, the DNase γ -dependent DNA fragmentation was detected in the late apoptotic phase, such as after 48 h of incubation (Figs. 1C and D, and Fig. 2C). Therefore, CAD/DFF40 may be involved in the DNA fragmentation that occurs in the early phase of apoptosis, as in the case of the staurosporine-induced DNA fragmentation. On the other hand, DNase γ may be involved in the DNA fragmentation that occurs in the late phase of apoptosis as in the case of the etoposide-induced DNA fragmentation (Figs. 1C). CAD/DFF40 activation is initiated by the caspase-3-dependent ICAD degradation, and the signal pathway to caspase-3 activation has been well defined (11, 20). The mechanism of DNase γ activation, however, remains to be clarified. DNase γ is located in the nuclear envelope (17) and remains separated from chromatin. Although DNase γ has a nuclear localization signal (16, 17), its translocation mechanism is also unclear. DNase γ may leak through damaged membranes of the nuclear envelope in the late phase of apoptosis and degrade chromatin. Because the late apoptotic phase, sometimes called secondary necrosis, is accompanied by membrane disruption (7), DNase γ may be also involved in necrosis. Therefore, DNase γ may be the final caretaker responsible for the disposal of the genomic DNA in dying cells, although the

mechanism of DNase γ activation and the actual function of DNase γ *in vivo* remain to be elucidated.

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