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Fas-Mediated Apoptosome Formation Is Dependent on Reactive Oxygen Species Derived from Mitochondrial Permeability Transition in Jurkat Cells

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Generation of reactive oxygen species (ROS) and activation of caspase cascade are both indispensable in Fas-mediated apoptotic signaling. Although ROS was presumed to affect the activity of the caspase cascade on the basis of findings that antioxidants inhibited the activation of caspases and that the stimulation of ROS by itself activated caspases, the mechanism by which these cellular events are integrated in Fas signaling is presently unclear. In this study, using human T cell leukemia Jurkat cells as well as an in vitro reconstitution system, we demonstrate that ROS are required for the formation of apoptosome. We first showed that ROS derived from mitochondrial permeability transition positively regulated the apoptotic events downstream of mitochondrial permeability transition. Then, we revealed that apoptosome formation in Fas-stimulated Jurkat cells was clearly inhibited by N-acetyl-L-cysteine and manganese superoxide dismutase by using both the immunoprecipitation and size-exclusion chromatography methods. To confirm these in vivo findings, we next used an in vitro reconstitution system in which in vitro-translated apoptotic protease-activating factor 1 (Apaf-1), procaspase-9, and cytochrome c purified from human placenta were activated by dATP to form apoptosome; the formation of apoptosome was markedly inhibited by reducing reagents such as DTT or reduced glutathione (GSH), whereas hydrogen peroxide prevented this inhibition. We also found that apoptosome formation was substantially impaired by GSH-pretreated Apaf-1, but not GSH-pretreated procaspase-9 or GSH-pretreated cytochrome c. Collectively, these results suggest that ROS plays an essential role in apoptosome formation by oxidizing Apaf-1 and the subsequent activation of caspase-9 and -3. *The Journal of Immunology*, 2004, 173: 285–296.

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3 Abbreviations used in this paper: MPT, mitochondrial permeability transition; Apaf-1, apoptotic protease-activating factor 1; ROS, reactive oxygen species; NAC, N-acetyl-L-cysteine; SOD, superoxide dismutase; MnSOD, manganese SOD; GSH, reduced glutathione; DiOC6(3), 3,3′-dihexyloxacarbocyanine iodide; CsA, cyclosporin A; NAME, Nω-nitro-L-arginine methyl ester; Atr, atracylloside; BA, bongkrekic acid.
N-benzoylcarbonyl-Val-Ala-Asp fluoromethyl ketone inhibits caspase-8 upstream of MPT as well as caspases downstream of MPT such as caspase-9 and -3.

More importantly, how ROS is integrated in the caspase cascade activation induced by Fas remains unresolved. The implication of ROS in caspase cascade has been suggested, because antioxidants such as ascorbic acid or reduced glutathione (GSH) inhibited the activation of caspase-3 in the apoptosis signal induced by a topoisomerase inhibitor (15) or hyperosmotic shock (16), and the stimulation of hydrogen peroxide resulted in the increment of caspase-3 activity (17). However, the mechanism by which ROS generation is involved in the cascade of caspase activation is unclear.

In this study, using Fas-stimulated Jurkat cells in which the involvement of both ROS and caspase cascade through MPT induction and a subsequent apoptosis formation has been previously elucidated, we have clarified the fact that the second-wave ROS is indeed derived from MPT and is integrated in caspase cascade by playing a key role in apoptosis formation.

**Materials and Methods**

**Reagents**

Agonistic anti-human Fas mouse mAb (CH-11) and FITC-conjugated anti-annexin V mouse mAb were purchased from Medical and Biological Laboratories (Nagoya, Japan). Cyclosporin A (CSA) was from Nakarai Tesque (Kyoto, Japan). Hydroethidine and 3,3’-dihexyloxycarbocyanine iodide (DiOC6) were from Molecular Probes (Eugene, OR). Bongkrekic acid (BA) was from Biomol (Plymouth Meeting, PA). Human cytochrome c purified from placenta was from R&D Systems (Minneapolis, MN). Atracylsidone (Atr), DTT, GSH, hydrogen peroxide, dATP, /H11032-GGC AGC ACT AGC AGC ATG TTG AGC C-3/5 were prepared as described above. The pellet of mitochondrial fraction was re-suspended and washed twice with PBS. The cytosolic fraction (S-100) was collected, washed once with PBS, and suspended in ice-cold isosmotic buffer (200 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM HEPES-KOH (pH 7.4), 1 μg/ml milk protein A, 1 μg/ml leupeptin, and 1 mM Pefabloc SC (Sigma-Aldrich, St. Louis, MO)). After incubation for 5 min, the cells were Dounce homogenized using a type B (loose) pestle and centrifuged at 100,000 g for 10 min to separate nuclei and unbroken cells. Then the supernatant was centrifuged at 8000 × g for 10 min to pellet heavy membranes (mitochondrial fraction). The supernatant was then centrifuged at 100,000 × g to obtain the cytosolic fraction (S-100). Procaspase-9 in the supernatant was immunoprecipitated with 1 μg of anti-caspase-9 rabbit polyclonal Ab, 0.1% Triton X-100, 1 mM EDTA, and incubated for 18 h. The beads were then washed four times and boiled in standard reducing sample buffer for 3 min. Aliquots were loaded for SDS-PAGE, followed by transfer to a nitrocellulose membrane (Bio-Rad, Hercules, CA) and hybridization with anti-caspase-9 mouse mAb (clone 5B4; Medical and Biological Laboratories), anti-Apaf-1 mouse mAb (clone 94408.11; R&D System), or anti-cytochrome c mouse mAb (clone 7H8.2C12; BD Pharmingen, San Diego, CA). Proteins were visualized with HRP-conjugated anti-mouse IgG Ab (Sigma-Aldrich) followed by use of the ECL plus chemiluminescence system (Amersham Pharmacia Biotech).

**Analysis of cytochrome c release**

After 1 × 10⁶ parental Jurkat and MnSOD clone 1 cells were stimulated by anti-Fas Ab with or without the pretreatment of NAC, the cells were harvested and washed twice with PBS. The cytosolic fraction (S-100) was prepared as described above. The pellet of mitochondrial fraction was re-suspended in 10 mM Tris-HCl (pH 7.4) containing 1% SDS. Western blot analysis was performed with the anti-cytochrome c mAb mentioned above.

**Western blot analysis for Apaf-1, cytochrome c, procaspase-9, and MnSOD**

A total of 1 × 10⁶ parental Jurkat cells or MnSOD transfectants were washed once with PBS and lysed in buffer containing 140 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1.0% Triton X-100, 10% glycerol, 1 mM EDTA, 1.5 mM MgCl₂, 1 mM sodium vanadate, 50 mM sodium fluoride, 1 μg/ml milk protein A, 1 μg/ml leupeptin, and 1 mM Pefabloc SC, and then centrifuged at 15,000 rpm for 30 min. Western blot analysis using anti-caspase-9, Apaf-1, or cytochrome c Ab was performed as mentioned above. For the detection of MnSOD, anti-MnSOD rabbit polyclonal Ab (StressGen Biotechnologies, Victoria, BC, Canada) was used.

**Caspase-3 and -9 activity assay**

The activities of caspases 3 and 9 were measured using the Colorimetric Protease Assay Kit for caspase-3 or -9 (Medical and Biological Laboratories). Briefly, 1 × 10⁶ parental Jurkat cells or MnSOD transfectants were collected, washed once with PBS, and suspended in 50 μl of provided cell lysis buffer. After incubation for 10 min on ice, the cells were centrifuged at 15,000 rpm for 3 min. The cytosolic fraction (S-100) was diluted with 2× reaction buffer, and 200 μM pNA-conjugated colorimetric substrate of caspase-3 or -9 was added. After incubation for 45 min at 37°C, released pNA was detected by a spectrophotometer ImmunoMini NJ-2300 (InteMed, Tokyo, Japan), and caspase activity was expressed as OD₄⁰₅ (×10⁻³).

For further confirmation, procaspase-9, procaspase-3, and their cleaved fragments (active forms) were detected by Western blot analysis using gating of forward and side scatter. ROS production was expressed as the percentage of fluorescent cells in contrast with the control cells, which had not been Fas stimulated.

**Measurement of MPT**

The induction of MPT was quantified fluorometrically using DiOC6, which is a mitochondrial membrane potential-indicating fluorophore. Following the stimulation of Fas, parental Jurkat cells or MnSOD transfectants were harvested, centrifuged, and suspended in 200 μl of PBS with 60 nM DiOC6. After 30 min of incubation, the cells were analyzed on a FACSScan flow cytometer (13). Dead cells and debris were excluded from the analysis by electronic gating of forward and side scatter. MPT induction was expressed as the percentage of nonfluorescent cells in contrast with the control cells, which had not been Fas stimulated.

**Immunodetection of apoptosis formation**

A total of 2 × 10⁷ parental Jurkat cells or MnSOD transfectants were washed once with PBS and suspended in ice-cold isosmotic buffer (200 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM HEPES-KOH (pH 7.4), 1 μg/ml milk protein A, 1 μg/ml leupeptin, and 1 mM Pefabloc SC (Sigma-Aldrich, St. Louis, MO)). After incubation for 5 min, the cells were Dounce homogenized using a type B (loose) pestle and centrifuged at 100,000 g for 10 min to separate nuclei and unbroken cells. Then the supernatant was centrifuged at 8000 × g to obtain the cytosolic fraction (S-100). Procaspase-9 in the supernatant was immunoprecipitated with 1 μg of anti-caspase-9 rabbit polyclonal Ab, H-170 (Santa Cruz Biotechnology, Santa Cruz, CA), and 30 μl of 50% protein G-Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ) by rotation at 4°C for 18 h. The beads were then washed four times and boiled in standard reducing sample buffer for 3 min. Aliquots were loaded for SDS-PAGE, followed by transfer to a nitrocellulose membrane (Bio-Rad, Hercules, CA) and hybridization with anti-caspase-9 mouse mAb (clone 5B4; Medical and Biological Laboratories), anti-Apaf-1 mouse mAb (clone 94408.11; R&D System), or anti-cytochrome c mouse mAb (clone 7H8.2C12; BD Pharmingen, San Diego, CA). Proteins were visualized with HRP-conjugated anti-mouse IgG Ab (Sigma-Aldrich) followed by use of the ECL plus chemiluminescence system (Amersham Pharmacia Biotech).
FIGURE 1. MPT induction and two waves of ROS generation induced by Fas stimulation. Jurkat cells were cultured with anti-Fas Ab (100 ng/ml) for the indicated periods of time. ROS generation (A) and MPT induction (B) were analyzed as described in Materials and Methods. The representative patterns of histogram are shown in A and B. C. MPT (○) and ROS production (●) were quantified as described in Materials and Methods. The data represent the means (bars, SD; n = 5).
FIGURE 2. Inhibitory effects of antioxidant or MnSOD overexpression on Fas-mediated apoptosis. Parental Jurkat cells (A and B) or MnSOD (□) and neo clones (■) (C and D) were cultured with anti-Fas Ab (100 ng/ml) for 6 h (A and C) or 12 h (B and D). NAC was added to culture medium at the indicated concentrations, 2 h before the stimulation of Fas (□) (A and B). The cells incubated with anti-Fas Ab without NAC are the control (□) (A and B). Fas-induced ROS production (A and C) and apoptosis (B and D) were estimated as described in Materials and Methods. The data represent the means (bars, SD; n = 5).
mouse anti-caspase-9 Ab (mentioned above) and mouse anti-CPP32 Ab (Medical and Biological Laboratories), which are known to detect both proenzymes and their activated forms of these caspases.

Chromatographic detection of apoptosome formation

HiPrep 16/60 S-300 Sephacryl high-resolution column (Amersham Pharmacia Biotech) was equilibrated with 5% (w/v) sucrose, 0.1% (w/v) CHAPS, and 20 mM HEPES/NaOH (pH 7.0), and calibrated with a gel filtration protein standards kit (Amersham Pharmacia Biotech). Then, the cytosolic fraction (S-100) of 2 x 10^6 parental Jurkat cells or MnSOD transfectants were applied to and eluted from the column at a flow rate of 0.2 ml/min and fractionated every 20 min. All procedures were conducted at 4°C. Each fraction was concentrated in Centricon YM-3 (Millipore, Bedford, MA) and analyzed by Western blot using the same Abs mentioned above.

In vitro apoptosome formation

Apaf-1 was translated in vitro from pcDNA3-Apaf-1XL-Myc plasmid with a Flexi Rabbit Reticulocyte Lysate System (Promega, Madison, WI) in which no reducing reagents were included. Similarly, procaspase-9 was translated from pcDNA3-caspase-9-FLAG plasmid. A volume of 40 μl of
FIGURE 4. Effects of antioxidant or MnSOD overexpression on Fas-induced MPT and cytochrome c release. A, Parental Jurkat or MnSOD clone 1 cells were cultured with 100 ng/ml anti-Fas Ab for 6 h ( ). The antioxidant NAC (20 mM) was added to the culture medium of parental Jurkat cells 2 h before the stimulation of Fas ( ). The cells incubated with no reagent are the control ( ). MPT were estimated as described in Materials and Methods. B, The same samples were prepared as described above. Both the released cytochrome c into cytosolic fraction and the retained one in mitochondrial fraction were examined as described in Materials and Methods. The data in A represent the means (bars, SD, n = 5), and representative results are shown in B.
FIGURE 5. Inhibitory effects of antioxidant or MnSOD overexpression on Fas-induced apoptosome formation as detected by the immunoprecipitation method. MnSOD and neo clones (A) or parental Jurkat cells (B and C) were cultured with 100 ng/ml anti-Fas Ab (A and B) or 50 μM MPT inducer Atr (C) for 6 h. Antioxidant NAC (20 mM) was added to the culture medium 2 h before the stimulation of Fas or Atr (B and C). The cells incubated with no reagent are the control (B and C). Immunodetection of apoptosome formation was performed as described in Materials and Methods. In D, the expression of procaspase-9, Apaf-1, and cytochrome c in parental Jurkat cells treated with NAC (20 mM) for 8 h, and in MnSOD or neo clones without any stimulation, was estimated by Western blot analysis as described in Materials and Methods. Parental Jurkat cells incubated with no reagent are the control.

FIGURE 6. Inhibitory effects of antioxidant or MnSOD overexpression on Fas-induced apoptosome formation as detected by size-exclusion chromatography. Neo clone 1 (A–C and E) and MnSOD clone 1 (D) cells were cultured with 100 ng/ml anti-Fas Ab for 6 h (B–E). A concentration of 20 mM antioxidant NAC (C) or 500 μM NO inhibitor L-NAME (E) was added to the culture medium 2 h before the stimulation of Fas. The cells incubated with no reagent are the control (A). Chromatographic detection of apoptosome formation was performed as described in Materials and Methods.
in vitro-translated procaspase-9 and the same volume of Apaf-1 were incubated with 1/25 pl of cytochrome c (250 ng/μl) in the presence of a reducing reagent, DTT (20 mM), GSH (25 mM), or the oxidizing reagent hydrogen peroxide (50 or 100 μM) in a final volume of 97.5 μl at 25°C for 30 min. A volume of 2.5 μl of dATP (100 mM) was added to the mixture, which was then incubated at 37°C for 3 h to facilitate apoptosome formation. When Apaf-1, procaspase-9, or cytochrome c was independently reduced with GSH (25 mM) at 25°C for 30 min, each protein was subsequently dialyzed to exclude residual GSH by Slide-A-Lyzer Mini Dialysis Units, 3500 MWCO (Pierce, Rockford, IL) in PBS, in which oxygen was

FIGURE 7. Inhibitory effects of antioxidant or MnSOD overexpression on Fas-induced activation of caspases 9 and 3. Parental Jurkat cells (A–D) were cultured with 100 ng/ml anti-Fas Ab (A and B) or 50 μM MPT inducer Atr (C and D) for 12 h. A concentration of 20 mM antioxidant NAC (A–D) or 100 μM MPT inhibitor BA (A and B) was added to the culture medium 2 h before the stimulation of Fas or Atr. In E and F, MnSOD and neo clone 1 cells were cultured with 100 ng/ml anti-Fas Ab for 12 h. The cells incubated with no reagent are the control. Activity of caspases 9 and 3 were estimated by both colorimetric assay (A, C, and E) and Western blot analysis (B, D, and F) as described in Materials and Methods. In the colorimetric assay, the activities of caspase-3 (□) and -9 (■) represent the means (bars; SD; n = 5). Representative results are shown for Western blot analysis.
removed by a continuous nitrogen flushing before use. For the control experiment, each protein was dialyzed without reducing. Then, apoptosome formation was induced by dATP at 37°C as described above. The cleavage of procaspase-9 was detected by Western blot analysis as described above. For the chromatographic detection of apoptosome formation, 2 ml of the reaction mixture was applied to the column. The fractions 1 and 2, in which the components of apoptosome were eluted, were three times more concentrated before Western blot analysis than other fractions, allowing them to detect apoptosome clearly.

**Results**

**Time course of ROS generation and MPT induction**

To study the time course of ROS generation, hydroethidine positivity of Fas-treated Jurkat cells was analyzed by flow cytometric analysis. Fig. 1A represents typical patterns of histogram. At 30 min, a small, transient peak, which diminished at 1 h, was observed. After 3 h, another ROS burst appeared and continued to increase until 6 h. The time course of MPT induction was also monitored by flow cytometry. Representative patterns of histogram are shown in Fig. 1B. MPT induction, as measured by negativity for DiOC₆, started at 1 h and continued to increase until 6 h. The results of five such experiments are summarized in Fig. 1C. A small initial peak of ROS at 30 min apparently preceded MPT, and from 1 h to 6 h thereafter, both ROS and MPT increased in parallel. Because the small peak at 30 min was surmised to correspond to the first-wave ROS in a previous report (13), which is irrelevant to apoptosis, in the following experiments, we analyzed ROS generation at 6 h, which appeared to correspond to the second wave of ROS in the previous report (13).

**Effects of ROS inhibition on apoptotic cell death**

To confirm the essentiality of ROS in Fas-mediated apoptosis (3–11), Jurkat cells were pretreated with antioxidant NAC, a GSH precursor, and were stimulated with the anti-Fas Ab for 6 h, at which point ROS generation and apoptotic cell death were quantified by flow cytometry as the positive fractions of hydroethidine and annexin V, respectively. As shown in Fig. 2, A and B, NAC suppressed both ROS and apoptosis in a dose-dependent manner, and almost complete inhibition was observed at 20 mM.

To verify the above observation, an expression vector of MnSOD, which should be more specific than the above-mentioned antioxidants in terms of ROS-scavenging function, was transfected into Jurkat cells. The three clones selected from MnSOD transfectants all showed a ~3-fold enhancement of the expression, quantified by Western blot analysis (data not shown). When these clones were stimulated by Fas, substantial suppression of both ROS production (Fig. 2C) and apoptosis (D) was observed.

**Effects of MPT inhibition or MPT induction on ROS generation and apoptotic cell death**

We then treated the cells with MPT inhibitors, CsA and BA, and found that these inhibitors suppressed Fas-induced ROS production (Fig. 3A) and apoptosis (B) at almost the same degree as the suppression rates on MPT (C). Furthermore, when we treated the cells with a MPT inducer Atr, induction of ROS (Fig. 3A) and apoptosis (B) was clearly observed at almost the same degree as that of MPT (C) without the stimulation of Fas. These results suggest that MPT induction is the cause of second-wave ROS production.

**Effects of ROS inhibition on MPT and cytochrome c release**

To negate the possibility that ROS generation is the causative factor for MPT induction resulting in cytochrome c release, we analyzed these events in the presence of NAC or under MnSOD overexpression. Neither Fas-induced MPT (Fig. 4A) nor cytochrome c release into cytosol with the reduction of retained cytochrome c in mitochondria (B) of Jurkat cells was affected by NAC treatment. Similarly, Fas-induced MPT (Fig. 4A) and cytochrome c release with the reduction of mitochondrial cytochrome c (B) were clearly observed in a MnSOD-overexpressed Jurkat clone.

**Effects of ROS inhibition on apoptosome formation**

We then examined the effects of ROS inhibition on apoptosome formation downstream of MPT by the immunoprecipitation method using the anti-caspase-9 Ab. The precipitated bands of Apaf-1 and cytochrome c indicated apoptosome formation. The precipitation of Apaf-1 and cytochrome c induced by the Fas stimulation was substantially inhibited by MnSOD overexpression (Fig. 5A) and almost completely by the antioxidant NAC (B); the precipitation induced by the MPT inducer Atr in the absence of the anti-Fas Ab was also completely blocked by NAC (C). We further assured that neither the antioxidant NAC nor MnSOD overexpression influenced the expression of any apoptosome component itself (Fig. 5D).

Effects of the antioxidant NAC and MnSOD overexpression on apoptosome formation were further substantiated by chromatographic fractionation. As shown in Fig. 6B, with the stimulation of Fas, all of the components of the apoptosome complex were detected at high molecular fractions (fractions 1 and 2) with the remaining unbound procaspase-9 at fractions 12–15. Apaf-1 at fractions 4–9, and cytochrome c at fractions 17–19. The components at the high molecular fractions were markedly diminished by the overexpression of MnSOD (Fig. 6D) or became almost completely undetectable with the treatment of NAC (C). However, 500 μM L-NAME, at which concentration this NO synthase inhibitor suppressed the first wave completely (13), merely inhibited apoptosome formation (Fig. 6E).

**Effects of ROS inhibition on activation of caspase-9 and -3**

In Fas-treated cells, activation of caspase-9, which is evoked by apoptosome formation and subsequent activation of caspase-3, was almost completely suppressed by NAC and appreciably by the MPT inhibitor BA as determined by enzyme activity assay (Fig. 7A) and Western blot analysis (B). Similar suppressive effects of NAC on caspase-9 and -3 were observed in the cells treated with the MPT inducer Atr in the absence of Fas (Fig. 7, C and D). MnSOD overexpression also resulted in significant suppression of the activity of these caspases in Fas-treated cells (Fig. 7, E and F).

**Effects of reducing agents and hydrogen peroxide on apoptosome formation within in vitro reconstitution system**

Because the effect of ROS on protein function is surmised to be based on oxidation of the protein (18), we then attempted to prove the direct effect of ROS on apoptosome proteins by using an in vitro reconstitution system in the presence of ROS and reducing agent. In vitro-translated Apaf-1 and procaspase-9 as well as cytochrome c purified from human placenta were mixed and activated with dATP to form an apoptosome complex. In the first series of experiments, the formation of apoptosome was examined by the cleavage of procaspase-9 (Fig. 8). As shown in Fig. 8A, a simple mixture of these four elements resulted in the formation of apoptosome, suggesting that, because in vitro translation was performed without any reducing reagents, the resultant Apaf-1 and procaspase-9 were in oxidized forms. When we used reducing reagents such as GSH or DTT, the apoptosome formation was clearly suppressed. This inhibitory effect of GSH on apoptosome formation was reversed by hydrogen peroxide, which was used because of its relative stability in vitro compared with other ROS (Fig. 8B). However, hydrogen peroxide by itself did not enhance
FIGURE 8. Inhibitory effects of reducing reagents on caspase-9 activation in the in vitro reconstitution system. In vitro-translated Apaf-1 and procaspase-9, as well as cytochrome c purified from placenta, were mixed and pretreated with DTT, GSH, or hydrogen peroxide for 30 min (A and C). In B, the mixture was first reduced with GSH for 30 min and then oxidized with hydrogen peroxide for 30 min. In D, Apaf-1, procaspase-9, and cytochrome c were independently reduced with GSH (25 mM) and dialyzed to remove residual GSH. For the control, they were dialyzed without pretreatment of GSH. Then, Apaf-1, procaspase-9, and cytochrome c with or without reduction were mixed as indicated in D. Thereafter, the mixture was activated in vitro with dATP (A–D), and Western blot analysis was performed as described in Materials and Methods.

FIGURE 9. Inhibitory effects of reducing reagent on apoptosome formation in the in vitro reconstitution system. In vitro-translated Apaf-1 and procaspase-9, as well as cytochrome c purified from placenta, were mixed and pretreated with 25 mM GSH for 30 min (B) and treated further with 100 mM hydrogen peroxide for another 30 min (C). For the control, the mixture was pretreated with no reagent (A). In D and E, Apaf-1 was independently reduced with 25 mM GSH and dialyzed to remove residual GSH. For the control, Apaf-1 was dialyzed without pretreatment of GSH. Then, Apaf-1 with (E) or without (D) reduction, procaspase-9, or cytochrome c were mixed. Thereafter, the mixture was activated in vitro with dATP (A–E), and chromatographic detection of apoptosome formation was performed as described in Materials and Methods.
the formation of apoptosome, indicating that oxidation of in-vitro-translated proteins was saturated (Fig. 8C). Then Apaf-1, procaspase-9, and cytochrome c were each reduced with GSH, and subsequently dialyzed to exclude residual GSH. As shown in Fig. 8D, when reduced Apaf-1, reduced procaspase-9, and reduced cytochrome c were activated together, apoptosome formation was largely impaired (lane 2). Similar results of impaired formation of apoptosome were obtained with the combination of reduced Apaf-1 with nonreduced procaspase-9 and nonreduced cytochrome c (lane 3). However, the combination of reduced procaspase-9 with nonreduced Apaf-1 and nonreduced cytochrome c (lane 4) or that of reduced cytochrome c with nonreduced Apaf-1 and nonreduced cytochrome c (lane 5) formed apoptosome.

In the second series of experiments, the chromatographic gel filtration method was used. With this method, the presence of procaspase-9, Apaf-1, and cytochrome c in high molecular fractions 1 and 2 indicated apoptosome formation, similarly as shown in Fig. 6. All of the components of apoptosome were detected in these two fractions when they were activated in vitro by dATP (Fig. 9A). The formation was apparently inhibited by the addition of GSH (Fig. 9B), and this inhibition was abrogated by hydrogen peroxide (C). When Apaf-1 reduced with GSH was combined with nonreduced procaspase-9 and nonreduced cytochrome c, no bands at fractions 1 or 2 were detected (Fig. 9D).

Discussion

Although ROS generation is evoked in apoptotic cell death induced by various kinds of stimulation, the relationship between ROS and MPT differs depending on the type of cells and apoptotic stimulation (19–24).

In activation-induced apoptosis of murine T lymphocytes, the implication of ROS production resulting in MPT and apoptotic cell death was demonstrated using the antioxidant manganese III tetrakis (21). Similarly, TGF-β reportedly induced cytochrome c release and apoptotic cell death, which were both inhibited by the radical scavenger pyrroliidine carbodithioic acid in combination with ascorbic acid in rat hepatocytes (20).

However, in human myeloid leukemia HL60 cells stimulated by staurosporine, ROS production and apoptotic cell death were both diminished by the overexpression of Bcl-2 (19), which protects mitochondria from MPT (22). In rat hepatocytes stimulated by hydrophobic bile acids, the MPT inhibitors CsA and BA were able to suppress ROS production and apoptotic cell death (23). Similar results have been obtained based on experiments using human lymphocytes and human myeloid leukemia U937 cells stimulated by glucocorticoid or TNF-α, respectively (24).

With regard to Fas-induced apoptosis, two waves of ROS generation, early and late onset, of which only the latter has been proven to be essential for apoptotic cell death, have been reportedly observed in Jurkat cells (13). However, although the second wave was surmised to have a close relationship with MPT induction, the question as to which precedes and is responsible for the other remained unresolved.

In the present study, we found that the MPT inhibitors CsA and BA abolished the second-wave ROS generation and apoptotic cell death, and that scavenging ROS by NAC or MnSOD overexpression inhibited neither MPT induction nor cytochrome c release in Fas-stimulated Jurkat cells. These results clearly indicate that the second-wave ROS derived from MPT is responsible for the apoptotic cell death. Furthermore, the possibility that ROS generated downstream of subordinate caspases such as caspase-3 functions only as a positive-feedback enhancer of MPT induction was ruled out by the latter results.

Our results also suggest that cell viability is maintained for a certain period of time (in the present study, 12 h), even though mitochondrial membrane potential is impaired, and cytochrome c is released. This is not a peculiar phenomenon, because it is well known that cells can survive at least for 15 wk after they are treated by ethidium bromide to block mitochondrial function by inhibiting its DNA synthesis (25).

Although we could not find a clear explanation as to why in previous reports some investigators detected only the first wave, but others could detect both, a plausible reason may be that they used different fluorogenic probes for ROS. We, as well as Beltran et al. (13), were able to detect both waves by the use of hydroethidine. In contrast, we could not distinguish these two waves by dihydrorhodamine 123 (data not shown), which was used by Banki et al. (12).

The most crucial aim of the present investigation was to clarify the interrelationship between ROS generation and caspase cascade, because both are indispensable cellular events for apoptotic death. Because ROS was proven to be derived from MPT, we postulated that ROS might interact with caspase cascade downstream of MPT. Therefore, we explored the effects of ROS on apoptosome formation, which occurs just after MPT. The results that the scavenging of ROS impaired formation of apoptosome as revealed by two independent methods, immunoprecipitation and chromatographic fractionation, clearly suggested that ROS facilitates apoptosome formation.

In contrast, the NO synthase inhibitor L-NAME, which is known to suppress the first wave of ROS (13), merely inhibited the apoptosome formation. These data suggest that the essential wave for the apoptosome formation is not the first but the second, being consistent with the reported fact that a suppression of the first wave of ROS by L-NAME did not result in a prevention of the second wave of ROS or apoptotic cell death (13).

Furthermore, experiments using an in vitro reconstitution system evidently substantiate the notion that ROS generation is requisite for apoptosome formation. In addition, using this system, we were able to disclose that, among three components of apoptosome, only Apaf-1 might be a target molecule of ROS (Figs. 8D and 9D). Although the molecular mechanism for activation of Apaf-1 by ROS is merely speculative, it is highly plausible that ROS oxidizes the thiol of Apaf-1, because the thiol oxidation is known to alter protein structure and function (18).

Recently, some investigators have shown that oxidative stress rather inhibits apoptotic signaling (26–28). However, the fact that exogenous stimulation of surplus oxidation inhibits apoptosis does not contradict our finding that endogenous ROS generated in Fas-mediated apoptosis positively regulates apoptosome formation, because the excessive stress of oxidation is known to lower intracellular ATP (28), which is also indispensable to apoptosome formation.

For the first part of the present investigation, we used Jurkat cells because they are well known to activate caspase cascade and generate ROS by Fas stimulation, and are therefore suitable for studying the integration of both cellular events.

Regarding the species of oxygen radical that is responsible for oxidation of Apaf-1, we surmise that hydroxyl radical is the most plausible candidate, because it is ultimately inhibited by both SOD (29) and NAC (30), which we used for in vivo experiments; hydrogen peroxide, which was used for the in vitro experiment, itself a rather inert oxidant (31), may be readily converted to hydroxyl radical in the presence of trace divalent metal ions such as Fe^{2+} in reaction reagents.

Whether or not the findings with this cell line are common to other cell types stimulated by Fas or other is an important issue.
Contrarily, a representative type I cell, SKW6.4, which is known to undergo apoptosis by Fas stimulation without MPT apoptosome formation (32), was much less sensitive to NAC than type II Jurkat cells when these cell lines were stimulated with the anti-Fas Ab (data not shown).

In summary, we have proven that ROS derived from MPT is integrated in the apoptotic signaling by the formation of apoptosome in Fas-stimulated Jurkat cells, and thus, we may be able to modulate the immune network or immunological responses that are relevant to apoptotic cell death by using ROS scavengers.

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References


