We have previously shown that inhibition of catalase and glutathione peroxidase activities by 3-amino-1,2,4-triazole (ATZ) and mercaptosuccinic acid (MS), respectively, in rat primary hepatocytes caused sustained endogenous oxidative stress and apoptotic cell death without caspase-3 activation. In this study, we investigated the mechanism of this apoptotic cell death in terms of nucleosomal DNA fragmentation. Treatment with ATZ+MS time-dependently increased the number of deoxyribonucleotidyl transferase-mediated nick end-labeling (TUNEL)-positive nuclei from 12 h, resulting in clear DNA laddering at 24 h. The deoxyribonuclease (DNase) inhibitor, aurintricarboxylic acid (ATA), completely inhibited nucleosomal DNA fragmentation but the pan-caspase inhibitor, z-VAD-fmk was without effects; furthermore, the cleavage of inhibitor of caspase-activated DNase was not detected, indicating the involvement of DNase(s) other than caspase-activated DNase. Considering that endonuclease G (EndoG) protein was decreased to approximately half the level of untreated cells. Under these conditions, decreases in TUNEL-positive nuclei were significantly suppressed. These results indicate that EndoG is responsible, at least in part, for nucleosomal DNA fragmentation under endogenous oxidative stress conditions induced by ATZ+MS.

The generation and elimination of reactive oxygen species (ROS) are well balanced in normal cells. However, increased ROS generation or decreased antioxidative capacity inside cells shifts this balance toward excess ROS production, leading to oxidative stress (1–3). Cells exhibit diverse responses against oxidative stress, including proliferation (4, 5), differentiation (6, 7), and cell demise (apoptosis and/or necrosis) (8–13), depending on the cell types or levels of oxidative stress. Apoptosis is a major cellular response against oxidative stress. In fact, there have been numerous reports on apoptosis induced by oxidative stress, and its mechanisms have been extensively discussed (14–19).

The caspase family, which comprises a series of cysteine proteases, plays an important role in apoptosis and many groups have reported the involvement of caspases in apoptosis (20–22). Nucleosomal DNA fragmentation (DNA ladder formation) is one of the hallmarks of apoptosis and is executed by deoxyribonuclease (DNase) activated by apopotic stimuli. Of the DNases that are activated by apoptotic stimuli, caspase-activated DNase (CAD), which is induced in a caspase-dependent manner, is mainly involved in apoptotic DNA fragmentation. In normal cells, CAD exists as a heterodimer complex with an inhibitor subunit termed inhibitor of CAD (ICAD), and the DNase activity of CAD is masked by the state of the complex. ICAD has two sites cleaved by caspases. Once the caspases are activated and ICAD is cleaved, CAD is released from the complex and subsequently degrades chromosomal DNA to nucleosomal fragments (23, 24).

Whereas cell death under conditions in which the caspase family cannot function has been reported to resemble necrosis rather than apoptosis in several studies (25–28), a growing body of evidence that apoptotic cell death induced by oxidative stress occurs independently of caspase activation has been accumulated (29–32). In fact, cells have shown apoptosis-like morphological changes, chromatin condensation and/or DNA fragmentation without caspase activation in these papers, suggesting that these distinctive characteristics of apoptosis take place in a caspase-independent manner. An argument about the definition of apoptosis or necrosis has emerged in recent years (33–35). However, it is agreed that nucleosomal DNA fragmentation is a hallmark of apoptosis at this time. Thus, it is generally accepted that the presence or absence of nucleosomal DNA fragmentation distinguishes apoptosis from necrosis.

We have previously shown that sustained oxidative stress is evoked and, subsequently, chromatin condensation and nucleosomal DNA fragmentation (which are typical features of apoptosis) are observed in rat primary hepatocytes when catalase and glutathione peroxidase (GPx) activities are inhibited for 24 h by 3-amino-1,2,4-triazole (ATZ) and mercaptosuccinic acid (MS), respectively (36, 37). In addition, caspase-3 is not activated during this apoptotic cell death, indicating that the apoptotic morphological changes are induced in a caspase-3-independent manner (37). It should be noted that nucleosomal DNA fragmentation has been clearly observed in this apoptotic cell death without caspase-3 activation. However, considering that ICAD is
reportedly processed by caspases other than caspase-3, we cannot exclude the possibility of the participation of CAD in nucleosomal DNA fragmentation (38, 39). Thus, here we examined whether CAD is involved in the nucleosomal DNA fragmentation induced by ATZ + MS by investigating ICAD processing and, if not so, this nucleosomal DNA fragmentation can be considered to take place caspase-independently. We focused on endonuclease G (EndoG) as an executor of DNA fragmentation, which has been shown to be caspase-independently translocated from mitochondria to nuclei in response to apoptotic stimuli, followed by the induction of nucleosomal DNA fragmentation (40, 41).

EXPERIMENTAL PROCEDURES

Materials—WILLIAMS E medium, ATZ, MS, aurantricarboxylic acid (ATA), SKF-525A (SKF), tumor necrosis factor α (TNFα), actinomycin D (ActD), and Hoechst 33342 were obtained from Sigma-Aldrich. 1-Ascorbic acid sodium salt (vitamin C) was obtained from Wako Pure Industries, Ltd. (Osaka, Japan). z-VAD-fmk was obtained from BIOMOL (Plymouth Meeting, PA). Antibodies were purchased from Pure Industries, Ltd. (Osaka, Japan). Clontech) was used according to the manufacturer's instructions.

Materials—WILLIAMS E medium, ATZ, MS, aurantricarboxylic acid (ATA), SKF-525A (SKF), tumor necrosis factor α (TNFα), actinomycin D (ActD), and Hoechst 33342 were obtained from Sigma-Aldrich. 1-Ascorbic acid sodium salt (vitamin C) was obtained from Wako Pure Industries, Ltd. (Osaka, Japan). z-VAD-fmk was obtained from BIOMOL (Plymouth Meeting, PA). Antibodies were purchased from Pure Industries, Ltd. (Osaka, Japan). Clontech) was used according to the manufacturer's instructions.

Cell Fractionation—To separate the mitochondrial fraction from the cytosolic fraction, an ApoAlert® Cell Fractionation kit (BD Biosciences Clontech) was used according to the manufacturer’s instructions. Hepatocytes were homogenized in fractionation buffer, and the homogenates were centrifuged at 700 × g for 10 min at 4 °C. Supernatants were incubated with 200 nM DiOC6 (3) for 15 min under the above-mentioned culture conditions. The cells were then washed with ice-cold phosphate-buffered saline (PBS) and fixed with 4% formaldehyde in PBS. The cells were then stained with Hoechst 33342 (1 μg/ml) for 5 min at room temperature. The nuclei were visualized using a fluorescence microscope (Nikon, Tokyo, Japan) at an excitation wavelength of 365 nm. Data were captured by a digital CCD camera (Hamamatsu C4742-95-10, Hamamatsu, Hamamatsu, Japan). For the detection of DNA fragmentation, an Apoptosis DNA Ladder kit (Wako) was used. The extracted DNA was separated by 1.5% agarose gel, and then visualized under ultraviolet light. Fluorographs of the DNA ladder were stored on a computer-assisted image analyzer (Fluor-S Multimager, Bio-Rad).

Immunoblotting Analysis—Equal amounts of proteins were loaded and separated by SDS-PAGE using a 7.5–15% (w/v) gradient polyacrylamide gel, and then transferred onto a polyvinylidene difluoride membrane. The blocked membranes were incubated overnight at 4 °C with the following antibodies at the stated dilutions (depending on the specific experiment): anti-CAD (1:200), anti-ICAD (1:200), anti-cytochrome c (1:500), anti-EndoG (1:500), anti-COX IV (1:1000), anti-histone H1 (1:200), or anti-β-actin (1:200). The membranes were then incubated with alkaline phosphatase-conjugated secondary antibody (Bio-Rad) and visualized with alkaline phosphatase substrates. Band intensity was measured by Multianalyst (Bio-Rad).

Cloning of Rat EndoG—Rat liver QuickClone cDNA (BD Biosciences Clontech, Mountain View, CA) was amplified with Platinum TaqDNA Polymerase (Invitrogen) and PCR × Enhancer system (Invitrogen) using the EndoG-specific primers ESP1 (TACCCCGCTGGTCTCTCCGA, forward primer) and ESP2 (GCTTGAAGTGCCTCTAAGCTCCTC, reverse primer). The reaction conditions recommended by the manufacturer were used with minor modifications. Briefly, 30 cycles of amplification were carried out at 95 °C for 30 s, at 55 °C for 30 s and at 68 °C for 1 min. One-tenth of the amplification product from the first 30 cycles was reamplified under the same conditions. The products of the second amplification were analyzed by agarose gel electrophoresis and cloned using the TOPO TA Cloning kit (Invitrogen) in pCR 2.1-TOPO vectors. Positive clones were selected, followed by purification and sequencing of the cDNA.

Expression and Purification of Recombinant Rat EndoG—Rat EndoG cDNA with 6× His tag was subcloned into the pET 30 vector (Novagen, Madison, WI), and the construct was transformed into Escherichia coli BL21 (DE3). The transformed cells were cultured and then stimulated in recombinant EndoG by the addition of isoprropyl-β-D-thiogalactopyranoside. The collected cells were resuspended in 5 volumes of lysis buffer (50 mM NaHPO4, pH 8.0, 300 mM NaCl, and 0.05% Tween 20) containing 10 mM imidazole. All subsequent procedures were conducted at 4 °C. Lysozyme was added at a final concentration of 1 mg/ml. After 30 min incubation on ice, the cell suspension was sonicated and centrifuged at 10,000 × g for 30 min at 4 °C to remove the cellular debris. The supernatant was loaded onto a Ni-NTA agarose (Qiagen, Hilden, Germany) column. After the column was washed with lysis buffer containing 50 mM imidazole, the protein was eluted with lysis buffer containing 100 mM imidazole. The eluted protein was dialyzed against buffer A (20 mM HEPES, pH 7.0, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, and 1 mM dithiothreitol) containing 10 mM KCl and then loaded onto SP-500C (TOSOH Corp., Tokyo, Japan) column. The column was washed with buffer A containing 50 mM KCl and EndoG was eluted with a linear gradient from 50 to 700 mM KCl in buffer A.

DNase Assay—Recombinant rat EndoG activity was determined by the method of Wieldak et al. (42). Recombinant rat EndoG (100 ng) was mixed with pUC18 plasmids (1 mg) or nuclei isolated from rat primary hepatocytes (2 × 105 nuclei) and the mixture was incubated in the presence or absence of ATA or hydrogen peroxide for 20 min at 37 °C followed by agarose gel electrophoresis. DNA fragmentation was analyzed by electrophoresis using 1.5% agarose gel.

Measurement of Mitochondrial Membrane Potential—Hepatocytes were incubated with 200 nM DiOC6 (3) for 15 min under the above-mentioned culture conditions. The cells were then washed with ice-cold phosphate-buffered saline and detached from the plates by trypsinization. Fluorescence was determined using FACSCalibur™ (BD Bioscience, San Jose, CA) with an excitation wavelength at 488 nm and an emission wavelength at 530 nm. Generally, 10,000 events were monitored and data analysis was performed by Cell Quest software (BD Bioscience).

Cell Fractionation—To separate the mitochondrial fraction from the cytosolic fraction, an ApoAlert® Cell Fractionation kit (BD Biosciences Clontech) was used according to the manufacturer’s instructions. Hepatocytes were homogenized in fractionation buffer, and the homogenates were centrifuged at 700 × g for 10 min at 4 °C. Supernatants...
were further centrifuged at 10,000 x g for 25 min at 4°C. Supernatants from the second centrifugation were designated as cytosolic fractions and pellets resuspended in fractionation buffer were used as mitochondrial fractions. Isolation of nuclei was carried out by the method described by Staal et al. (43) with slight modifications. Cells were suspended in buffer B (10 mM HEPES, pH 7.8, 10 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 0.5 mM dithiothreitol, 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 20 µM APMSF) and incubated on ice for 15 min. Nonident-40 at a final concentration of 0.6% was added to the cell suspension, which was immediately vortexed followed by centrifugation. A white pellet was washed with buffer B and used as a nuclear fraction.

**RNA Interference (RNAi)—Silencing of EndoG gene expression in primary rat hepatocytes was achieved by the siRNA technique. Duplexes of 21 nucleotides with two 3’-overhanging TT against rat EndoG were designed and synthesized by Prologi (Kyoto, Japan). The sense strand of the siRNA against EndoG was GUA CAG CGG CAG CUU GAC UTT. Scrambled nonspecific siRNA was used as a negative control (the sense strand was CGU GCA GUC GUC UCG CAA ATT). Transfection of rat primary hepatocytes with synthesized siRNA was carried out by electroporation using the Nucleofector® system (Amaxa, Kolin, Germany), according to the protocols provided by the manufacturer. Briefly, 1.3 x 10⁶ cells were resuspended in 100 µl of nuclofactor solution (Human T Cell Nucleofector kit) containing 150 pmol of double-stranded siRNAs. After electroporation, transfected cell suspensions were transferred into 6-well collagen type I-coated plates in 1.2 ml of prewarmed cultured medium. After 2 h of incubation, the medium was changed, and the cells were further incubated for 12 h. Subsequently, the medium was changed, and the cells were further incubated with ATZ+MS in the presence or absence of inhibitors for 24 h.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis—**Total RNA extraction from hepatocytes was performed using an RNeasy Mini kit (Qiagen) according to the manufacturer’s instructions. First-strand cDNA was synthesized from 1 µg of total RNA using a TaKaRa RNA PCR kit (AMV) Ver. 2.1 (TaKaRa, Otsu, Japan). For PCR analysis, cDNA was amplified by Platinum TaqDNA polymerase (Invitrogen). β-Actin was used as the endogenous expression standard. Each PCR program consisted of a 2-min initial denaturation step at 94°C, followed by 25 cycles (for EndoG) or 20 cycles (for β-actin) at 94°C for 30 s, at 55°C for 30 s and at 72°C for 1 min, on a PCR Thermal Cycler Personal (TaKaRa). The primer sequences were as follows: EndoG forward primer, CCG CGA GTC CTA CGT GCT GA; EndoG reverse primer, ATC ACA TAG GAA CGC AGC TCG AC; β-actin forward primer, TTC AAC ACC CCA GCC ATG TA; and β-actin reverse primer, TGA TCC ACA TCT GCT GGA AG. The amplified products were separated by electrophoresis on agarose gels. The band intensity was measured using a Multianalyst (Bio-Rad).

**Terminal Deoxynucleotidyl Transferase-mediated Nick End-labeling (TUNEL) Assay—**The TUNEL assay was performed using the DeadEnd™ Fluorometric TUNEL System (Promega) according to the manufacturer’s instructions. After the hepatocytes were fixed, fluorescein-12-dUTP was catalytically incorporated into the fragmented DNA at the 3’-OH by terminal deoxynucleotidyl transferase. The fluorescein-12-dUTP-labeled DNA was visualized directly by fluorescence microscopy using an excitation wavelength at 488 nm. TUNEL-positive cells were determined from fluorographs for at least 300 nuclei.

**Determination of Protein Content—**The protein content was determined according to the method of Bradford (44) using bovine serum albumin as a standard.

**RESULTS**

**Time Course for Increases in TUNEL-positive Cells Induced by ATZ+MS Treatments—**TUNEL-positive nuclei were not observed for up to 24 h in untreated hepatocytes. Treatment with ATZ+MS for 9 h had no effect on DNA fragmentation. However, treatment periods longer than 12 h induced a time-dependent increase in the number of TUNEL-positive nuclei and almost all nuclei were TUNEL-positive after 24 h of incubation (Fig. 1A). The inset in Fig. 1A showed morphological features of hepatocytes treated with ATZ+MS at 12 h. By phase contrast microscopy (Eclipse TE300, Nikon), two hepatocytes in the figure were observed to be apart from interconnected hepatocytes and rounded up. However, they adhered to the surface of the culture dish. The sizes of ATZ+MS treated hepatocytes including rounded ones were smaller than those of untreated hepatocytes, showing that cellular shrinkage occurred. Nuclear condensation was noted in the rounded hepatocytes. In addition, electrophoretic analysis with extracted DNA at 24 h showed clear DNA laddering when cells were treated with ATZ+MS, confirming our previous reports (Fig. 1B) (36, 37). Pretreatment with SKF (a cytochrome P450 inhibitor) or vitamin C (an antioxidant) almost completely inhibited both the increase in the number of TUNEL-positive nuclei and the appearance of DNA laddering induced by ATZ+MS (Fig. 1, A and B). The onset and time course for the increase in TUNEL-positive nuclei were similar to those for the increase in chromatin condensation (37).

**Involvement of DNases Other Than CAD in ATZ+MS-induced DNA Fragmentation—**As described above, clear nucleosomal DNA fragmentation was observed when hepatocytes were treated with ATZ+MS. Thus, to ensure whether DNase(s) were responsible for this fragmentation, A, which is a DNase inhibitor, was used (45). Pretreatment with 30 µM ATA had no effect on DNA laddering induced by ATZ+MS. However, pretreatment with 100 µM ATA completely suppressed DNA laddering, suggesting that DNase(s) were involved in DNA fragmenta-
Cells were collected and lysed. CAD, ICAD, and its cleaved products in cell lysates were detected by immunoblotting.

FIGURE 2. Involvement of DNases other than CAD in ATZ+MS-induced DNA fragmentation. Primary rat hepatocytes were pretreated with ATA (30 or 100 μM) or z-VAD-fmk (10, 50, or 250 μM) for 20 min and then incubated for 24 h in the presence or absence of ATZ (20 μM) and MS (7 μM). A and C, cells were collected, and cellular DNA was extracted, followed by electrophoresis. B, TUNEL assay was performed, and then the percentage of TUNEL-positive nuclei was determined. Values are given as the mean ± S.E. of five separate experiments. D, primary rat hepatocytes were treated with ATZ (20 μM) and MS (7 μM), or TNF-α (50 ng/ml), and ActD (50 ng/ml), and then incubated for 24 h. Cells were collected and lysed. CAD, ICAD, and its cleaved products in cell lysates were detected by immunoblotting.

Primary rat hepatocytes were pretreated with ATA (30 or 100 μM) and MS (7 μM) neither affected TUNEL-positive nuclei (Fig. 2B) nor nucleosomal DNA fragmentation (Fig. 2C) induced by ATZ+MS. Secondly, we examined whether ICAD was cleaved in hepatocytes treated with ATZ+MS because CAD is released and activated by the processing of ICAD. After 24 h of incubation, few ICAD-cleaved products were observed in untreated hepatocytes. Treatment with ATZ+MS showed similar quantities of ICAD-cleaved products to those in untreated cells (Fig. 2D). Stimulation by TNF-α+ActD is well known to induce caspase-dependent apoptosis in many types of cell, including hepatocytes (46–48). In fact, treatment with TNF-α+ActD induced cell death in rat primary hepatocytes accompanied by caspase activation, chromatin condensation, and nucleosomal DNA fragmentation (data not shown). When cells were stimulated by TNF-α+ActD, the processing of ICAD occurred after 24 h of incubation, as evidenced by the increases in ICAD-cleaved products (Fig. 2D). These results indicate that DNAse(s) other than CAD are involved in nucleosomal DNA fragmentation induced by ATZ+MS.

Cloning, Expression, and Purification of Rat EndoG—The related sequences and the published reports regarding EndoG were scanned against the GenBank™ database. Human and mouse EndoG are reported to be apoptotic DNases that execute DNA fragmentation without caspase activation (40, 41). However, there have been no reports on the cloning of rat EndoG cDNA to date. Thus, we cloned rat EndoG cDNA to evaluate its enzymatic properties. Full-length rat EndoG cDNA was cloned from a rat liver cDNA library and then registered in the DNA Data Bank of Japan (DDBJ)/European Molecular Biology Laboratory (EMBL)/GenBank™ data base (DDBJ/EMBL/GenBank™ accession number: AB221075). Recombinant rat EndoG expressed in E. coli was purified to a single band on SDS-PAGE, as shown by Coomassie Blue staining by two-step chromatography on Ni-NTA agarose and Sp550C (Fig. 3). The purified His tag fusion proteins showed a molecular weight of 36 kDa on SDS-PAGE and were detected by anti-His tag or anti-EndoG antibodies (Fig. 3). These results indicate that we succeeded in cloning rat EndoG cDNA and obtaining purified recombinant rat EndoG.

Evidence for DNase Activities (Digestion and Nucleosomal Fragmentation) of Recombinant Rat EndoG—The enzymatic properties of recombinant rat EndoG were examined using the pUC18 plasmid (Fig. 4A) or isolated nuclei from rat primary hepatocytes (Fig. 4B).

Recombinant rat EndoG completely digested the pUC18 plasmid, indicating that recombinant EndoG has DNase activity (Fig. 4A). The addition of ATA inhibited the DNase activity of recombinant EndoG in a dose-dependent manner, and the DNase activity was almost completely inhibited by treatment with 100 μM ATA (Fig. 4A). Surprisingly, recombinant EndoG fully retained DNase activities in the presence of 10 mM hydrogen peroxide, which is considered to represent severe oxidative stress conditions (Fig. 4A).

When isolated hepatocyte nuclei and recombinant EndoG were mixed and incubated, clear nucleosomal DNA fragmentation was observed (Fig. 4B). The addition of 100 μM ATA suppressed this nucleosomal DNA fragmentation, whereas treatment with hydrogen peroxide, even at 10 mM, had no effect on nucleosomal DNA fragmentation (Fig. 4B). These results strongly indicate that EndoG is an ATA-inhibitable DNase that can induce nucleosomal DNA fragmentation under severe oxidative stress conditions.

Decreases in Mitochondrial Membrane Potential and EndoG Translocation from Mitochondria to Nuclei—To evaluate the changes in mitochondrial membrane potential during the apoptotic process, a strong cationic dye, DiOC6(3), was used. The mitochondrial membrane potential in untreated hepatocytes showed no changes up to 9 h of incubation (Fig. 5). Treatment with ATZ+MS for 6 h had no effect on the mitochondrial membrane potential (Fig. 5). However, after 9 h of incubation, the mitochondrial membrane potential in ATZ+MS-treated cells showed a 40% decrease compared with that of untreated cells (Fig. 5). Pretreatment with SKF or vitamin C significantly sup-
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EndoG localized predominantly in mitochondria and to a lesser extent in nuclei at 12 h in untreated cells according to immunoblotting (Fig. 6, A and B). Cytochrome c localized only in mitochondria (Fig. 6A). When cells were treated with ATZ+MS for 12 h, EndoG and cytochrome c were detected in the cytosol (Fig. 6A), suggesting that these molecules were released from mitochondria. Interestingly, EndoG levels in nuclei at ATZ+MS-treated cells were markedly increased compared with those in untreated cells (Fig. 6B). Pretreatment with SKF or vitamin C inhibited the release of both EndoG and cytochrome c from mitochondria (Fig. 6A), namely, both reagents suppressed the nuclear translocation of EndoG (Fig. 6B). At 9 h of incubation, neither EndoG nor cytochrome c was detected in the cytosolic fractions obtained from ATZ+MS-treated cells (data not shown), despite decreases in the mitochondrial membrane potential, suggesting that the mitochondrial membrane potential decreased before the release of these molecules from mitochondria to the cytosol occurred. These results suggest that treatment with ATZ+MS evokes oxidative stress, which causes the decreases in mitochondrial membrane potential, followed by the translocation of EndoG from mitochondria to nuclei.

EndoG Knockdown by RNAi—When EndoG siRNA was transfected into hepatocytes by electroporation, and the cells were incubated for another 14 h, EndoG mRNA was rarely detectable (Fig. 7A), and the EndoG protein levels were decreased (Fig. 7B). Densitometric analysis revealed that EndoG mRNA levels fell to 16% of those of untreated cells, and protein levels fell to 54% of those of untreated cells (Fig. 7, A and B). By contrast, scrambled nonspecific siRNA, which was used as a negative control, had no effect on either EndoG mRNA or protein levels (Fig. 7, A and B).

Decreases in the Number of TUNEL-positive Nuclei Caused by Transfection of EndoG siRNA—Cells transfected by control or EndoG siRNA showed DNA laddering in response to treatment with ATZ+MS. However, the density of DNA laddering in cells transfected by EndoG siRNA seemed to be weaker than that in cells transfected by control siRNA (Fig. 8A). Given that agarose gel electrophoresis was not suitable for quanti-
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In response to treatment with ATZ, almost all cells transfected by control siRNA exhibited positive nuclei was calculated. Values are given as the mean ± S.E. of five separate experiments. Data were analyzed using the Student’s t test. p values less than 0.05 were considered significant. **p < 0.01.

Shrinkage and nuclear condensation were noted at 12 h by phase contrast microscopy. We have previously shown that chromatin condensation occurs at 12 h and increases in a time-dependent manner thereafter and that DNA laddering is observed after 24 h of incubation (37). These results indicate that apoptotic cell death occurred at 12 h of incubation. The finding that pretreatment with SKF or vitamin C suppressed the appearance of TUNEL-positive nuclei as well as DNA fragmentation further supported the previous idea that this apoptotic cell death is mediated by endogenous oxidative stress resulting from accumulated ROS derived from cytochrome P450 (36, 37).

Nucleosomal DNA fragmentation as an index of morphological change in apoptosis is known to stem from the action of CAD; this normally exists as a heterodimer with ICAD inside cells and its DNase activity is suppressed in such a state. However, activated caspase releases CAD from the heterodimer via ICAD cleavage, and the CAD causes nucleosomal DNA fragmentation. Although we have previously shown that caspase-3 is not related to this apoptotic cell death, there remained the possibility that other caspases could cleave ICAD, followed by CAD-dependent DNA fragmentation. In fact, caspase-1, caspase-3, caspase-6, caspase-7, and caspase-8 are all reported to be able to cleave ICAD directly (38, 39). In the current study, however, we revealed that a pan-caspase inhibitor, z-VAD-fmk had no effect on DNA laddering and that ICAD was not cleaved in apoptotic cell death induced by ATZ+MS. In this regard, our results provide further evidence for a lack of participation of caspase systems in this apoptotic cell death.

As described above, treatment with ATZ+MS induced clear nucleosomal DNA fragmentation without ICAD cleavage, which was suppressed by pretreatment with ATA (a general DNase inhibitor). These results imply the involvement of DNase(s) other than CAD. To date, those candidate DNases that have been linked to apoptotic processes are as follows: DNase I, DNase II, DNase γ, apoptosis-inducing factor (AIF), and EndoG. DNase I is a secretory protein that is highly expressed in the kidney and small intestine, rather than the liver, in rats (49); it also cleaves both the core region and the linker region of chromatin (50). DNase II has been reported to act under acidic conditions (51, 52); however, it cleaves chromatin DNA with 3′-phosphoryl (P)/5′-hydroxyl (OH) ends, which are different from TUNEL-positive cleavage, 3′-OH/ 5′-P (53). The DNase γ proenzyme is normally located in the perinuclear region; apoptotic stimuli process its N-terminal precursor peptide and the mature form is translocated into nuclei, followed by nucleosomal DNA fragmentation (49, 54). AIF is normally localized in the mitochondria intermembrane space and is translocated from mitochondria to nuclei in response to apoptotic stimuli; in the latter, AIF can induce DNA large fragmentation (~50 kbp), but not nucleosomal DNA fragmentation, in a caspase-independent manner (55, 56). EndoG translocates from mitochondria to nuclei in response to apoptotic stimuli, followed by nucleosomal DNA fragmentation; all of these processes (translocation and nucleosomal DNA fragmentation) occur caspase-independently (40). As mitochondria are one of the main targets of ROS, which can evoke the release of mitochondrial pro-apoptotic proteins through changes in mitochondrial permeability transition (MPT) (57–60), EndoG is appeared to be the most likely candidate DNase to be involved in apoptotic cell death induced by ATZ+MS. Therefore, we focused on investigating the role of EndoG in this study.

BLAST analysis of the open reading frame sequence of rat EndoG revealed 84 and 95% identity with human and mouse EndoG, respectively, at the nucleotide level, and 90 and 97% identity with human and mouse EndoG at the amino acid level (61, 62). In particular, the active site regions of EndoG from Ser131 to Asn189 in rats were similar among...
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all three species (63). While human EndoG was reported to induce nucleosomal DNA fragmentation by itself (40), cooperation with other DNases seemed to be required to form DNA laddering (42). Thus, an argument has arisen as to whether EndoG alone can induce nucleosomal DNA fragmentation. In the current study, we have shown for the first time that recombinant rat EndoG can digest rat hepatic DNA to nucleosomal fragments, thereby revealing that rat EndoG has the ability to induce nucleosomal DNA fragmentation by itself. In addition, recombinant EndoG retained its DNase activity against plasmid or isolated nuclei, even in the presence of 10 mM hydrogen peroxide, suggesting that EndoG can function under oxidative stress.

In this apoptotic cell death, the mitochondrial membrane potential decreased prior to the release of cytochrome c and EndoG from mitochondria. Because COX IV, which is localized in the mitochondrial inner membrane, was not detected in the cytosol despite EndoG and cytochrome c release, the mitochondrial inner membrane was intact. Therefore, it is conceivable that EndoG and cytochrome c were released from the mitochondria through changes in MPT. Although changes in MPT are common when pro-apoptotic molecules are released from mitochondria (for reviews, see Refs. 64 and 65), the mechanism for change in MPT is not fully understood. In this regard, the report by Baies et al. (66) that changes in MPT and apoptosis induced by hydrogen peroxide were suppressed in cyclophilin D-knockout MEF cells implies the possibility of the involvement of cyclophilin D in this apoptotic cell death. Further studies to investigate the participation of cyclophilin D in this apoptotic cell death will be needed.

Although cytochrome c was released from mitochondria, the caspase pathway was not activated during this apoptotic cell death. It is well known that in response to various kinds of cell death stimuli, changes in MPT result in cytochrome c release into the cytosol. The released cytochrome c together with dATP binds to apoptosis protease-activating factor-1, which subsequently recruits and activates pro-caspase-9 (67). The processed and activated caspase-9 in turn activates downstream effector caspases, including caspase-3. However, the active site cysteine residue of caspases is the most likely target of hydrogen peroxide and would be sensitive to oxidation, as discussed in a previous article (37). In that sense, caspase-9 appeared not to be activated based on the observation that caspase-3 was not processed (37). One potential reason for this finding is the oxidation of the SH residue of caspase-9, as was reported for caspase-3 in the previous article (37). To clarify the involvement of EndoG in this apoptotic cell death more directly, we applied an RNAi technique. The extent of the EndoG knockdown had substantial effects on this DNA fragmentation. Thus, EndoG appears to be involved in the DNA fragmentation induced by AT2+M5, although we cannot exclude the possibility of the involvement of other DNase(s).

In conclusion, the inhibition of ROS elimination by treatment with AT2+M5 induces sustained oxidative stress, resulting in the translocation of EndoG from mitochondria to nuclei, followed by nucleosomal DNA fragmentation, whereas the caspase family members are thought to be present in an oxidized form.

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