Nuclear Translocation of Endonuclease G and Apoptosis-Inducing Factor during Acetaminophen-Induced Liver Cell Injury

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Received April 18, 2006; accepted August 4, 2006

Mitochondrial dysfunction and internucleosomal DNA fragmentation are well-recognized features of acetaminophen (AAP)-induced hepatocyte cell death. However, the endonucleases responsible for this effect have not been identified. Apoptosis-inducing factor (AIF) and endonuclease G are nucleases located in the intermembrane space of mitochondria. AIF is thought to trigger chromatin condensation and induce cleavage of DNA into high molecular weight fragments (50–300 kb), and endonuclease G can produce oligonucleosomal DNA fragments. Therefore, the objective of this investigation was to test the hypothesis that endonuclease G and AIF could be involved in AAP-induced nuclear DNA fragmentation. Using immunofluorescence microscopy, it was shown that in primary cultured mouse hepatocytes, endonuclease G and AIF translocated to the nucleus between 3 and 6 h after exposure to 5 mM AAP. In contrast, other mitochondrial intermembrane proteins such as cytochrome c or the second mitochondria-derived activator of caspases (Smac) did not accumulate in the nucleus. The translocation of AIF and endonuclease G correlated with mitochondrial dysfunction as indicated by the progressive loss of the mitochondrial membrane potential (measured with the JC-1 assay) and the appearance of nuclear DNA fragments in the cytosol (determined by an anti-histone ELISA). Pretreatment with 20 mM N-acetylcysteine prevented mitochondrial dysfunction, the nuclear translocation of endonuclease G and AIF, and the nuclear DNA fragmentation. The data support the conclusion that endonuclease G and AIF could be involved in AAP-induced nuclear DNA fragmentation. Using immunofluorescence microscopy, it was shown that in primary cultured mouse hepatocytes, endonuclease G and AIF translocated to the nucleus between 3 and 6 h after exposure to 5 mM AAP. In contrast, other mitochondrial intermembrane proteins such as cytochrome c or the second mitochondria-derived activator of caspases (Smac) did not accumulate in the nucleus. The translocation of AIF and endonuclease G correlated with mitochondrial dysfunction as indicated by the progressive loss of the mitochondrial membrane potential (measured with the JC-1 assay) and the appearance of nuclear DNA fragments in the cytosol (determined by an anti-histone ELISA). Pretreatment with 20 mM N-acetylcysteine prevented mitochondrial dysfunction, the nuclear translocation of endonuclease G and AIF, and the nuclear DNA fragmentation. The data support the conclusion that endonuclease G and AIF could be involved in AAP-induced mitochondrial dysfunction and may be responsible, at least in part, for the initial DNA fragmentation during AAP hepatotoxicity.

Key Words: acetaminophen hepatotoxicity; endonucleases; N-acetylcysteine; cultured hepatocytes; DNA fragmentation.

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Acetaminophen (AAP) overdose is the most frequent cause of drug-induced liver failure in the United States and the United Kingdom (Lee, 2004). Central to the pathophysiology is the formation of a reactive metabolite, presumably N-acetyl-p-benzoquinone imine (NAPQI), which first depletes hepatocellular glutathione and subsequently covalently binds to cellular proteins (Nelson, 1990). However, it is not the total protein binding but the alkylation of mitochondrial proteins that correlates with the toxicity (Qiu et al., 2001; Tirmenstein and Nelson, 1989). Consequences of the modifications of mitochondrial proteins by NAPQI are impaired mitochondrial respiration (Meyers et al., 1988), ATP depletion (Jaeschke, 1990; Tirmenstein and Nelson, 1990), a specific mitochondrial oxidant stress (Jaeschke, 1990; Tirmenstein and Nelson, 1990), and generation of peroxynitrite inside mitochondria (Cover et al., 2005b). These events, which clearly occur before cell death (Bajt et al., 2004), lead to the opening of the mitochondrial membrane permeability transition (MPT) pore and breakdown of the mitochondrial membrane potential (Kon et al., 2004) followed by oncotic necrosis (Bajt et al., 2004; Gujral et al., 2002; Kon et al., 2004).

In addition to the mitochondrial dysfunction, there are dramatic nuclear changes after AAP overdose with extensive DNA damage as indicated by morphology (karyolysis) and DNA fragmentation (Ray et al., 1990). The DNA modifications include DNA ladders on agarose gels (Ray et al., 1990; Shen et al., 1991), staining of hepatocytes with the terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) assay (Lawson et al., 1999), and detection of DNA fragments in the cytosol and plasma (Jahr et al., 2001; Lawson et al., 1999). DNA fragmentation correlated with nuclear Ca2+ accumulation (Shen et al., 1991) and was inhibited by a general endonuclease inhibitor (Shen et al., 1992). These data and the fact that no nitrotyrosine, a footprint of peroxynitrite activity, was detectable in the nucleus (Cover et al., 2005b) suggested that DNA fragmentation after AAP overdose was an enzymatically catalyzed process (Cover et al., 2005b; Shen et al., 1992). However, the identity of the endonucleases involved remained unclear. Since scavenging of mitochondrial peroxynitrite with GSH effectively prevented...
DNA fragmentation (Cover et al., 2005b), there appears to be a connection between mitochondrial dysfunction and DNA fragmentation.

Apoptosis-inducing factor (AIF) and endonuclease G are located in the mitochondrial intermembrane space (Daugas et al., 2000; van Loo et al., 2001). Both enzymes can be released from mitochondria, translocate to the nucleus, and may participate in nuclear DNA fragmentation as shown in a variety of cell lines (Daugas et al., 2000; van Loo et al., 2001; Widlak and Garrard, 2005). AIF is thought to be mainly responsible for chromatin condensation and cleavage of DNA into high molecular weight fragments (50–300 kb) (Susin et al., 1999). On the other hand, endonuclease G and caspase-activated DNase (CAD) can produce the oligonucleosomal DNA fragments responsible for the DNA ladder on agarose gels (Widlak and Garrard, 2005; Zhang et al., 2003). Since there is evidence for generation of high and low molecular weight DNA fragments after AAP overdose (Cover et al., 2005b; Jahr et al., 2001; Ray et al., 1990), the objective of this investigation was to test the hypothesis that AIF and endonuclease G are released from mitochondria, translocate to the nucleus, and may be involved in DNA fragmentation in primary cultured murine hepatocytes after AAP overdose.

MATERIALS AND METHODS

Animals. Male C3Heb/FeJ mice with an average weight of 18–20 g were purchased from Jackson Laboratory (Bar Harbor, ME). All animals were housed in an environmentally controlled room with 12-h light/dark cycle and allowed free access to food (certified rodent diet no. 8640, Harlan Teklad, Indianapolis, IN) and water. The experimental protocols were approved by the Institutional Animal Care and Use Committee of University of Arizona and followed the criteria of the National Research Council for the care and use of laboratory animals in research. All chemicals were purchased from Sigma Chemical Co. (St Louis, MO) unless stated otherwise.

Mouse hepatocyte isolation. Primary hepatocytes were isolated from mice anesthetized with pentobarbital sodium solution (Nembutal, Abbott Laboratories, North Chicago; 50 mg/kg, ip) as previously described (Bajt et al., 2004). Briefly, the inferior vena cava was cannulated, and the liver was first perfused in situ with an oxygenated Hanks’ buffer solution (HBSS) containing penicillin/streptomycin (100 U/ml) pH 7.4 (8 ml/min, 37°C for 10 min), followed by perfusion with oxygenated HBSS containing 1 mM Ca²⁺ and Mg²⁺, penicillin/streptomycin (100 U/ml), and 0.04% collagenase D (Roche Molecular Biochemicals, Mannheim, Germany), pH 7.4 for 10 min. The liver was removed and then gently minced in HBSS containing penicillin/streptomycin (100 U/ml) (Gibco, Grand Island, New York) and 1 x 10⁻³ M insulin (Sigma), pH 7.4. The liver cell suspension was then filtered with Falcon cell strainers (40, 70, and 100 mm; Becton Dickinson, Bedford, MA) and centrifuged at 50 x g for 2 min. From the isolation of one mouse liver, a typical yield was about 5 x 10⁷ to 6 x 10⁸ hepatocytes. Hepatocytes were plated on 6-well plates (6 x 10⁴ cells per well) or 24-well plates (8 x 10⁵) Biocoat collagen I cellware plates (Becton Dickinson) in Williams’ Medium E (Gibco) containing 10% fetal bovine serum (Gibco), 100 U/ml penicillin/streptomycin, and 1 x 10⁻³ M insulin and cultured at 37°C with 5% CO₂. After an initial 4-h attachment period, cultures were washed with phosphate-buffered saline (PBS) and then the media were changed to plain culture medium (controls) or medium containing 20mM N-acetylcysteine (NAC). Thirty minutes later, 5mM AAP was added to some of the wells. After the initial attachment period, cell viability, as determined by trypan blue exclusion, was generally > 90%, and cell purity was > 95% hepatocytes.

Methods. Cell viability was assessed by trypan blue uptake. After removal of the cell medium, hepatocytes were incubated with 0.8% trypan blue solution for 3 min at room temperature. Trypan blue–positive cells were counted in four different fields (x10; a total of approximately 1500 cells). For the DNA fragmentation assay, cells were removed from wells with a cell scraper and placed into a test tube. After freeze-thawing, the lysate was centrifuged at 15,000 rpm for 20 min at 4°C. Aliquots of the supernatant were diluted 1:286 in sample buffer, and cytosolic DNA fragments were measured by an anti-histone ELISA according to the manufacturer’s instruction (Cell Death Detection ELISA; Roche Diagnostics, Indianapolis, IN). In this assay, the kinetics (Vmax) of product generation is a measure of DNA fragmentation. The Vmax values obtained for untreated controls (100%) are compared with those in treated groups. The assay allows the specific quantitation of cytoplasmic histone–associated DNA fragments. The mitochondrial membrane potential was assayed with the cationic dye 5,5’,6,6’-tetrachloro-1,1’,3,3’-tetrathylbenzimidazolylcarbocyanine iodine using the JC-1 Mitochondrial Membrane Potential Kit (Cell Technology, Mountain View, CA) according to the manufacturer’s directions. Briefly, hepatocytes were washed once and then incubated with the JC-1 reagent for 25 min at 37°C. Cells were washed with 1 ml assay buffer and then resuspended in 400 μl assay buffer. Cells were removed from wells with a cell scraper, and 100 μl of the cell suspension was placed into each of three wells of a black 96-well plate. The dye, which exists as green fluorescing monomer (excitation 485 nm; emission 535 nm) in the cytosol, is taken up into healthy mitochondria and forms a red fluorescing aggregate (excitation 550 nm; emission 600 nm). Red and green fluorescence were measured using the Spectramax Gemini fluorescence plate reader (Molecular Devices, Sunnyvale, CA). Loss of the mitochondrial membrane potential is indicated by the loss of red fluorescence and the increase of green fluorescence.

Immunohistochemistry. Primary mouse hepatocytes (1.5 x 10⁵ cells) were plated on collagen I–coated coverslips (BD Biosciences, Bedford, MA). After an initial 4-h attachment period, cells were washed with PBS and then incubated in culture medium (controls) or culture media containing 5mM AAP. After various times of AAP treatment, medium was removed, and cells were washed briefly with PBS. Cells were then fixed for 20 min with 1% formaldehyde (4% formaldehyde) for 4°C. After washing cells briefly with PBS, cells were permeabilized with PTB (PBS containing 0.3% Triton X-100 and 0.1% bovine serum albumin [BSA]) for 10 min. Cells were then rinsed with PBS containing 0.1% BSA. To inhibit nonspecific binding, cells were then incubated for 30 min in PBS containing 8% BSA. After washing with PBS, cells were incubated overnight at 4°C with primary antibody rabbit anti-AIF monoclonal antibody (Epitomics, Burlingame, CA), rabbit anti–endonuclease G polyclonal antibody (Chemicon International, Temecula, CA), rabbit anti–cytochrome c polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or an antibody against second mitochondria–derived activator of caspases (5mac)/direct inhibitor of apoptosis–binding protein with low pi (DIABLO) (BD Biosciences PharMingen, San Jose, CA). Cells were then washed three times with PTB and incubated with secondary antibody conjugated with fluorescein isothiocyanate (FITC) (Alexa Fluor 488 goat anti-rabbit, Molecular Probes, Eugene, OR) or Texas Red (Alexa Fluor 594 goat anti-rabbit, Molecular Probes) for 2 h at room temperature. Cells were then washed three times with PTB and incubated at room temperature with 4’,6-diamidino-2-phenylindole, dihydrochloride (DAPI) for nuclear visualization (300nM). After 10 min, cells were briefly washed with PTB, covered with mounting media, and then the coverslips were adhered to the slides. Visualization of the staining was performed using an inverted fluorescence microscope (Axiovert 200, Carl Zeiss GmbH, Germany) with excitation and emission filters 495/519 for FITC, 590/615 for Texas Red, and 358/461 for DAPI, respectively. The images were analyzed with Axiovision 4.5 software (Carl Zeiss GmbH).

Statistics. All results were expressed as mean ± SE. Comparisons between multiple groups were performed with one-way analysis of variance (ANOVA)
or, where appropriate, by two-way ANOVA, followed by a post hoc Bonferroni test. If the data were not normally distributed, we used the Kruskal-Wallis test (nonparametric ANOVA) followed by Dunn’s multiple comparisons test. The $p$ value $< 0.05$ was considered significant.

RESULTS

The potential nuclear translocation of AIF and endonuclease G was evaluated at 1.5, 3, and 6 h after AAP exposure. These time points were selected based on our previous results showing no mitochondrial dysfunction at 1.5 h, mitochondrial dysfunction but no significant cell death at 3 h, and moderate ($< 20\%$) hepatocellular necrosis at 6 h (Bajt et al., 2004).

Staining of control hepatocytes with antibodies against AIF or endonuclease G showed extensive granular staining throughout cells consistent with mitochondrial localization of these proteins (Fig. 1A and 1B). Nuclei were stained blue with DAPI. At 6 h after treatment with 5mM AAP, the red (AIF) or green (endonuclease G) fluorescence in the body of many cells was diminished, and the overall fluorescence in the nuclei of these cells was increased (Fig. 1C and 1D). The change of color in the nuclei from blue in controls to purple (AIF) or white (endonuclease G) after AAP treatment suggests that the blue fluorescence of DAPI was overlaid with red or green fluorescence of AIF or endonuclease G, respectively. These findings indicate that part of the mitochondrial AIF and endonuclease G translocated to the nucleus in response to AAP treatment. To further support this conclusion, we analyzed the fluorescence composition through cross sections of individual cells. In control hepatocytes stained for endonuclease G, green fluorescence showed progressively increasing intensity towards the center of the cells with the highest levels in the area around the nucleus (Fig. 2A). The nuclei had reduced green fluorescence compared with its surrounding area (Fig. 2A). Since this is not confocal microscopy, the green fluorescence in the area over the nucleus is most likely derived from mitochondria, which are located above or below the nucleus. On the other hand, the body of the cells did not show relevant blue fluorescence. There was only a sharp peak of blue fluorescence marking the nucleus (Fig. 2A). However, 6 h after AAP treatment, the green fluorescence outside the nucleus was substantially diminished (Fig. 2B). In contrast, there was a peak of green fluorescence, which overlaid with the peak of blue fluorescence outlining the nucleus (Fig. 2B). These measurements reflect the nuclear translocation of endonuclease G during AAP exposure. To verify the conclusion that these changes in fluorescence distribution within the cell actually reflect nuclear translocation rather than unspecific accumulation of proteins released from mitochondria, the potential nuclear accumulation of cytochrome c and the second mitochondria-derived activator of caspases (Smac) was evaluated (Fig. 2C–2F). Both proteins are located in the intermembrane space of mitochondria and can be released similar to AIF and endonuclease G (Li et al., 2002; Liu et al., 1996). In control cells, the highest levels of cytochrome c and Smac were found in the body of the cell (outside the nucleus) (Fig. 2C and 2E). Again, blue DAPI fluorescence sharply marked the position of the nucleus. At 6 h after AAP exposure, cells were rounding up. In contrast to endonuclease G (Fig. 2B), neither cytochrome c (Fig. 2D) nor Smac (Fig. 2F) showed a relevant increase in green fluorescence associated
with the blue nuclear fluorescence of DAPI. These data support
the conclusion that there is a specific nuclear translocation of
endonuclease G and AIF.

To provide a quantitative analysis, green and blue fluores-
cence were determined in 10 randomly selected cells of control
hepatocytes and 10 cells treated with AAP (6 h), which showed
nuclear condensation and increased overall nuclear fluores-
cence. Green fluorescence (endonuclease G) showed a signifi-
cant decline in the extranuclear space and a significant increase
in the nuclei after AAP exposure (Fig. 3A). On the other hand,
the blue DAPI fluorescence, which was almost absent in the
cytosol, significantly increased in the nuclei after AAP exposure
(Fig. 3B). The DAPI effect is most likely caused by nuclear
condensation in the AAP-treated cells. In fact, measurements of
nuclear diameters demonstrated a 45% reduction in AAP-
treated cells compared with controls (Fig. 4). Analysis of the
red (AIF) fluorescence in control versus AAP-treated cells
showed quantitatively similar results with AIF (Fig. 5A and 5B)
as observed with endonuclease G (Fig. 3A and 3B).

Assessment of the number of AIF-positive and of endonu-
clease G–positive nuclei at different times after AAP exposure
showed no significant difference between controls and AAP-
treated cells at 1.5 h (Fig. 6). On the other hand, approximately
25–30% and 45–55% of hepatocytes had AIF- and endonucle-
clease G–positive nuclei at 3 and 6 h after AAP exposure,
respectively (Fig. 6). Later time points could not be evaluated
because dying cells were washed off the coverslips during the
staining procedure. Treatment with 20mM NAC, which was
previously shown to protect under these experimental con-
ditions (Bajt et al., 2004), significantly attenuated the nuclear
translocation of AIF and endonuclease G (Figs. 3, 5, and 6) and
the reduction of the nuclear diameter (Fig. 4).
To investigate if nuclear translocation of AIF and endonuclease G correlated with mitochondrial dysfunction, the mitochondrial membrane potential was measured with the JC-1 assay. The cationic dye exists as green fluorescing monomer in the cytosol and as red fluorescing aggregates in mitochondria with intact membrane potential. Thus, a decline of the red-to-green fluorescence ratio indicates a loss of the mitochondrial membrane potential. At 1.5 h, the red-to-green fluorescence ratio in hepatocytes exposed to AAP was similar as in cells treated with vehicle only (Fig. 7). However, at 3 and at 6 h, the ratio decreased by 43 and by 64%, respectively, indicating the progressive loss of the mitochondrial membrane potential (Fig. 7). Treatment with NAC completely prevented the reduction of the red-to-green fluorescence ratio, which suggests that NAC preserved mitochondrial function (Fig. 7).

To verify that the nuclear translocation of AIF and endonuclease G actually correlates with nuclear DNA damage, cytosolic DNA fragments were measured with the anti-histone ELISA. At 1.5 h after AAP exposure, there was no significant DNA fragmentation compared with time-matched controls (Fig. 8). However, DNA fragmentation was significantly increased by 253 and 445% at 3 and at 6 h after AAP exposure, respectively. Treatment with NAC completely prevented any DNA fragmentation (Fig. 8).

**DISCUSSION**

Nuclear DNA fragmentation is a well-established phenomenon in AAP hepatotoxicity *in vivo* and in primary cultured hepatocytes (Cover *et al.*, 2005b; Gujral *et al.*, 2002; Lawson *et al.*, 1999; Ray *et al.*, 1990; Shen *et al.*, 1991,1992). Although some of the DNA fragments generated during AAP-induced cell death may be rather large (Cover *et al.*, 2005b; Jahr *et al.*, 2001), the presence of internucleosomal DNA cleavage products, as shown by the characteristic DNA ladder on agarose gels (Cover *et al.*, 2005b; Ray *et al.*, 1990; Shen *et al.*, 1991), suggests the involvement of nucleases in the process. This hypothesis is further supported by the reduced DNA damage after treatment with aurintricarboxylic acid, which is a broad-spectrum nuclease inhibitor (Shen *et al.*, 1992). In addition, the fact that Ca$^{2+}$ chelators and Ca$^{2+}$ channel blockers reduced nuclear Ca$^{2+}$ accumulation and
prevented DNA damage after AAP overdose is consistent with the participation of a Ca\(^{2+}\)-dependent endonuclease in the pathophysiology (Ray et al., 1993; Shen et al., 1992).

We previously reported that scavenging of mitochondrial peroxynitrite effectively attenuated nuclear DNA fragmentation (Cover et al., 2005b). Since we did not observe nitrotyrosine adducts in the nucleus, other mediators must have been responsible for the signaling between mitochondria and the nucleus (Cover et al., 2005b). It is well established that AAP overdose induces the release of cytochrome c from the mitochondrial intermembrane space (Adams et al., 2001; El-Hassan et al., 2003; Jaeschke and Bajt, 2006; Knight and Jaeschke, 2002). Consistent with these earlier observations, we now provide evidence for the nuclear accumulation of other known mitochondrial proteins, i.e., endonuclease G and AIF. Using fluorescence microscopy, we showed that in control cells, both proteins are located outside the nucleus. Since this is conventional microscopy, the fluorescence intensity is integrated over the entire light path through the cell. Therefore, the green or red fluorescence in the center of the nucleus of control cells represents endonuclease G or AIF, respectively, which are present in mitochondria located above or below the nucleus. Since the nucleus occupies some of the space, the green or red fluorescence is reduced compared with the space surrounding the nucleus. On the other hand, both the change in nuclear color as well as the direct analysis of green or red fluorescence distribution indicates that 6 h after AAP treatment, a substantial amount of endonuclease G and AIF...
translocated into the nucleus. Since there was no nuclear accumulation of other intermembrane proteins, i.e., cytochrome c and Smac, which are released also from mitochondria, the nuclear translocation was a specific effect of AIF and endonuclease G. The translocation of AIF and endonuclease G started between 1.5 and 3 h after AAP exposure and affected 50% of all hepatocytes by 6 h. Our previous analysis of events under identical experimental conditions showed that exposure of murine hepatocytes to 5mM AAP depleted cellular glutathione levels by 2–3 h and triggered an oxidant stress and mitochondrial dysfunction (XTT assay) between 3 and 6 h (Bajt et al., 2004). Based on enzyme release and trypan blue uptake, there was no relevant loss of cell viability before 6 h (Bajt et al., 2004). About 20% of cells were necrotic at 6 h after AAP, and the number of dead cells increased rapidly over the next hours resulting in almost 90% necrosis by 15 h (Bajt et al., 2004). Furthermore, the opening of the MPT pore and loss of the mitochondrial membrane potential were observed between 4 and 6 h (Kon et al., 2004). Assessment of the mitochondrial membrane potential with the JC-1 assay supported this time course in the present experiments with a significant loss at 3 and 6 h. At the same time, there was a progressive increase of nuclear DNA fragmentation. Furthermore, pretreatment with NAC triggers a rapid resynthesis of hepatocellular GSH, which, under these conditions, mainly scavenges NAPQI and thus effectively prevents mitochondrial dysfunction, oxidant stress, and cell injury (Bajt et al., 2004). Therefore, NAC eliminated the loss of the mitochondrial membrane potential, prevented the nuclear translocation of endonuclease G and AIF, and inhibited DNA fragmentation.

Together, these data strongly support the conclusion that after the initial metabolic activation of AAP, which induced mitochondrial dysfunction and oxidant stress, endonuclease G and AIF translocated to the nucleus and caused nuclear DNA fragmentation. Thus, endonuclease G and AIF appear to be at least in part responsible for the initial DNA damage observed after AAP exposure.

CAD, the endonuclease responsible for DNA fragmentation during apoptosis, requires the cleavage of its inhibitor ICAD by caspase-3 for its activation (Nagata et al., 2003). However, many different investigators consistently were unable to detect relevant caspase activation during AAP hepatotoxicity (Adams et al., 2001; El-Hassan et al., 2003; Gujral et al., 2002; Jaeschke et al., 2006; Lawson et al., 1999; Napirei et al., 2006). Moreover, the pan-caspase inhibitor ZVAD-fmk, which is highly effective in preventing DNA fragmentation and liver injury after Fas and tumor necrosis factor receptor–mediated apoptosis (Jaeschke et al., 1998; Lawson et al., 1999), has no protective effect against DNA damage or liver injury after AAP overdose (Jaeschke et al., 2006; Lawson et al., 1999). The recently described protection by ZVAD-fmk in the absence of caspase activation (El-Hassan et al., 2003) could be explained by the effect of the solvent dimethyl sulfoxide on AAP bioactivation (Jaeschke et al., 2006). These data are consistent with the lack of apoptotic morphology in liver cells of AAP-treated animals (Gujral et al., 2002; Kon et al., 2004). Thus, there is no reliable evidence to support the role of CAD in the pathophysiology of AAP-induced liver injury.

In a very recent paper, Napirei et al. (2006) reported reduced liver injury in deoxyribonuclease 1 gene knockout (DNase1−/−) mice compared with wild-type animals after AAP overdose. DNase1 is a Ca2+/Mg2+-dependent secreted endonuclease located in the endoplasmic reticulum (Napirei
et al., 2005). Secreted DNase1 enters necrotic cells and causes internucleosomal DNA degradation (Napirei et al., 2004). However, since DNase1−/− had reduced DNA strandbreaks (TUNEL assay) in the liver and reduced expansion of necrotic cell death in DNase1−/− mice, it was concluded that release of DNase1 by necrotic cells within the inner centrilobular area contributes to the expansion of necrosis (Napirei et al., 2006). Thus, secreted DNase1 may enter either stressed or dying hepatocytes and contribute to their cell death.

It was postulated that the DNase1-mediated necrosis may be related to NAD+ and energy depletion due to excessive activation of poly(ADP-ribose)-polymerase-1 (PARP-1) (Napirei et al., 2006). However, PARP-1 activation occurs late during AAP hepatotoxicity (Cover et al., 2005a), and neither PARP-1−/− mice (Cover et al., 2005a) nor cultured hepatocytes treated with a PARP inhibitor (Shen et al., 1992) were protected against AAP hepatotoxicity. Thus, the mechanism of injury by DNase1 needs to be further investigated. Interestingly, even in DNase1−/− mice, DNA damage and necrosis were only partially prevented (Napirei et al., 2006), suggesting the involvement of additional endonucleases in the process. Taken together, the emerging concept appears to be that endonuclease G and AIF released from mitochondria may initiate nuclear DNA fragmentation, a process which may be further aggravated by DNase1 released from necrotic cells.

In summary, our data with primary cultured murine hepatocytes exposed to AAP indicate that after the metabolic activation of AAP and depletion of glutathione, the developing mitochondrial dysfunction leads to the release of endonuclease G and AIF from mitochondria and to their nuclear translocation. These endonucleases are then involved in the initial nuclear DNA fragmentation, a process which contributes to oncocytic necrosis of hepatocytes.

ACKNOWLEDGMENTS

This investigation was supported in part by National Institutes of Health Grants R01 AA12916 (H.J.), R01 DK70195 (H.J.), R01 AA009156 (J.J.L.), and P01 DK059349 (J.J.L.).

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