Evidence That 1,1-Dichloroethylene Induces Apoptotic Cell Death in Murine Liver

Erik J. Martin and Poh-Gek Forkert

Department of Anatomy and Cell Biology, Queen’s University, Kingston, Ontario, Canada

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ABSTRACT

1,1-Dichloroethylene (DCE) causes dysfunction of hepatic mitochondria. As mitochondria have been implicated in apoptosis through opening of the permeability transition pore (PTP), we have undertaken studies to test the hypothesis that DCE induces apoptosis, in addition to necrosis, in murine liver. Our primary objective was to identify the biochemical events associated with DCE-induced apoptosis. Female CD-1 mice were treated with a mildly hepatotoxic dose of DCE (125 mg/kg, i.p.). Using the fluorescent dye JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide), decreased mitochondrial membrane potential was detected at 2 h. Western blotting of liver cytosolic proteins showed greater immunoreactivity for cytochrome c in fractions from mice treated with DCE for 4 h than in controls. Furthermore, caspase-9 activity was significantly increased 6 h after DCE exposure. Immunohistochemical studies with an antibody to activated caspase-3 and terminal deoxynucleotidyl transferase dUTP nick-end labeling staining were used to detect apoptotic cells. In both experiments, positive reactivities were observed in centrilobular hepatocytes 12 and 24 h after DCE. Additionally, centrilobular hepatocytes showing morphological criteria of apoptosis were observed at 24 h. Apoptosis and all apoptotic events were inhibited by pretreatment for 20 min with cyclosporine A (CyA) (50 mg/kg), a specific inhibitor of the mitochondrial PTP. To determine a major role for mitochondrial permeability transition (MPT) in DCE hepatotoxicity, serum alanine aminotransferase (ALT) activity was evaluated. ALT activity was significantly elevated 2 to 24 h after DCE, and CyA failed to inhibit this activity. These data suggested that DCE produces apoptosis by inducing MPT, causing release of cytochrome c into the cytosol and caspase activation.

1,1-Dichloroethylene (DCE; vinylidene chloride) is a synthetic chemical used in the production of plastics and textiles and, as a result of its release during manufacture and use, has become a widespread environmental contaminant (U.S. Environmental Protection Agency, 2002). Previous studies have shown that exposure to DCE causes liver injury selectively in centrilobular hepatocytes (Kanz and Reynolds, 1986; Forkert and Boyd, 2001). The lesion predominantly involved plasma membranes, nuclei, and mitochondria, whereas the endoplasmic reticulum (ER) appeared unaffected (Reynolds et al., 1984; Kanz and Reynolds, 1986). Early morphological changes in mitochondria were manifested as swelling, disruption of cristae, and loss of mitochondrial matrix density (Kanz and Reynolds, 1986). Functional alterations in mitochondria also occur early in the toxic response to DCE. Dysfunction of these mitochondria was documented as reduced ADP-stimulated (state-3) respiration, decreased respiratory control ratio, and ADP:O ratio (Martin et al., 2003). Collectively, these findings suggested that mitochondria are a primary site of damage in DCE-mediated hepatotoxicity.

Mitochondria play a pivotal role in necrotic and apoptotic cell death through opening of the permeability transition pore (PTP) (Halestrap et al., 1998; Lemasters et al., 1998). Opening of this high-conductance inner membrane channel requires elevated cytosolic calcium levels in addition to an “inducing agent”, such as organic peroxides and substances that oxidize sulfhydryl groups (Crompton, 1999). Activation of the PTP causes mitochondrial swelling and uncoupling of oxidative phosphorylation that, when unrestrained, leads to necrosis (Halestrap et al., 1998; Lemasters et al., 1998). Alternatively, transient PTP opening may be involved in the induction of apoptosis by initially causing mitochondrial

ABBREVIATIONS: DCE, 1,1-dichloroethylene; PTP, permeability transition pore; ER, endoplasmic reticulum; ALT, alanine aminotransferase; CyA, cyclosporine A; Gal/ET, galactosamine/endotoxin; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide; BSA, bovine serum albumin; MPT, mitochondrial permeability transition; TBS, Tris-buffered saline; T/TBS, Tween 20-TBS; RT, room temperature; PBS, phosphate-buffered saline; TdT, terminal deoxynucleotidyl transferase; ΔΨm, mitochondrial membrane potential; AMC, 7-amino-4-methylcoumarin.
swelling followed by rupture or permeabilization of the outer membrane, thus releasing cytochrome c into the cytosol (Halestrap et al., 1998, 2000). Once released, cytochrome c triggers the assembly of the apoptosome, a multimeric structure composed of cytochrome c, apoptotic protease activating factor 1 (APAF-1), and ATP or dATP. This complex recruits and activates procaspase-9, a prolife initiator caspase, that subsequently cleaves and activates procaspase-3 (Hengartner, 2000). Caspase-3, a potent downstream effector caspase, induces the proteolytic cleavage of a range of target proteins responsible for the rearrangement of the cytosol, nucleus, and plasma membrane characteristic of apoptosis (Kluck et al., 1997; Li and Yuan, 1999; Zimmermann et al., 2001).

A number of compounds including acetaminophen (Ray et al., 1996) and galactosamine (Gujral et al., 2003) are postulated to cause hepatocellular death by both necrosis and apoptosis. Although the precise mechanism(s) of DCE-induced liver injury has not been clearly delineated, centrilobular necrosis is well documented, whereas studies characterizing apoptosis are rudimentary. Previous investigations have demonstrated covalent binding of DCE metabolites in fractions of mitochondria, endoplasmic reticulum, and plasma membranes (Okine et al., 1985), organelles responsible for maintenance of calcium and cellular energy homeostasis. Additionally, it has been shown that DCE inhibits calcium sequestration by the Ca$^{2+}$/Mg$^{2+}$ ATPase in rat liver ER (Moore, 1982). Moreover, cytosolic calcium accumulation was observed in hepatocyte suspensions after DCE exposure (Long and Moore, 1987). Increased cytosolic calcium concentrations and mitochondrial dysfunction are both conditions intrinsic to apoptotic cell death. Furthermore, following DCE administration scattered individual hepatocytes were observed in rat livers showing nuclear and cytoplasmic alterations typical of apoptosis (Reynolds et al., 1984). In this investigation, we have undertaken studies to test the hypothesis that apoptosis is an important and currently unexplored process in DCE-mediated hepatotoxicity. Our primary objective was to examine the biochemical events associated with this mode of cell death, including mitochondrial permeability transition (MPT) induction, cytochrome c release, and activation of caspase-9 and -3. Additionally, we have investigated the occurrence of DCE-induced apoptosis and its relationship to necrosis using the TUNEL assay and histopathological evaluation. Furthermore, we have examined the role of MPT in DCE-mediated apoptosis using cyclosporine A, a specific inhibitor of PTP.

**Materials and Methods**

**Chemicals and Reagents.** Chemicals and reagents were purchased from suppliers as follows: 1,1-dichloroethylene (vinylidene chloride; ≥99% purity) (Aldrich Chemical Co., Montreal, Quebec, Canada), anti-cytochrome c (BD Biosciences Canada, Mississauga, ON, Canada), Bio-Rad protein assay dye reagent concentrate (Bio-Rad, Hercules, CA), cyclosporine A (Calbiochem, San Diego, CA), peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA), 5,5′,6,6′-tetrachloro-1',3',3′,4'-tetraethylbenzimidazolocarbocyanine iodide (JC-1) (Molecular Probes Inc., Eugene, OR), bovine serum albumin (BSA), collagenase type I, taurocholate, and streptavidin-peroxidase (Zymed Laboratories Inc., South San Francisco, CA). Other chemicals were of reagent grade and were obtained from standard commercial suppliers.

**Animal Treatment.** Female CD-1 mice (25–30 g) were obtained from Charles River Canada (St. Constant, Quebec, Canada). They were maintained on a 12 h light/dark cycle and given free access to food (Purina Rodent Chow;Ralston Purina International, Strathroy, ON, Canada) and drinking water. Following acclimatization to laboratory conditions for at least 5 days, mice were randomly assigned to control or treatment groups (n = 3). In all experiments, mice were treated intraperitoneally (i.p.) with 125 mg/kg DCE in corn oil. This dose has previously been shown to produce mitochondrial dysfunction and mild centrilobular necrosis in murine liver (Forkert and Boyd, 2001; Martin et al., 2003). For studies that examined the effects of CyA (50 mg/kg in corn oil, i.p.), CyA was administered 20 min before treatment with DCE for 24 h (CyA + DCE). In other investigations, this dose of CyA has been shown to inhibit apoptotic cell death (Supie et al., 2002). In flow cytometry studies, mice were treated with DCE, DCE and CyA, or 700 mg/kg galactosamine and 100 mg/kg endotoxin (Gal/ET, i.p.) for 2 h, as mitochondrial dysfunction has been shown to peak at this time (Martin et al., 2003). Mice were then anesthetized for perfusion and hepatoctye isolation. Gal/ET has been shown to produce extensive hepatocellular apoptosis in mice (Jaeschke et al., 1998; Gujral et al., 2002) and was used as a positive control. For detection of cytochrome c in cytosol and measurement of caspase-9 protease activity, mice were treated for 4 and 6 h, respectively, with DCE or DCE and CyA. These mice were sacrificed by cervical dislocation. For measurements of serum alanine aminotransferase (ALT) activity, mice were treated with DCE or DCE and CyA for 0.5, 1, 2, 4, 8, 12, 18, and 24 h. Mice were then anesthetized with sodium pentobarbital (120 mg/kg, i.p.), and intra-cardiac blood was obtained for assessment of ALT activity. For immunohistochemical, hematoxylin and eosin (H&E), and TUNEL staining experiments, mice were treated with DCE for 12 or 24 h, CyA + DCE or Gal/ET for 6 h, and then anesthetized for perfusion. In all studies, control mice were treated with equivalent volumes of the appropriate vehicle and were sacrificed at times corresponding to those in the experimental groups.

**Preparation of Isolated Hepatocytes.** Hepatic parenchymal cells were isolated by the method of Klauing et al. (1981) with modifications. Briefly, mice were perfused with calcium- and magnesium-free Hanks’ balanced salt solution containing 0.5 mM EGTA and 50 mM HEPES (pH 7.3) and then with Leibovitz modified L15 medium (pH 7.4) containing collagenase (120 U/mg, ICN), and in some instances, calcium was omitted. Cardiac blood was obtained for assessment of ALT activity. For immunohistochemical, hematoxylin and eosin (H&E), and TUNEL staining experiments, mice were treated with DCE for 12 or 24 h, CyA + DCE or Gal/ET for 6 h, and then anesthetized for perfusion. In all studies, control mice were treated with equivalent volumes of the appropriate vehicle and were sacrificed at times corresponding to those in the experimental groups.

**Measurement of Hepatocyte Mitochondrial Membrane Potential.** Mitochondrial membrane potential in intact liver cells was assessed using the mitochondrial-specific fluorescent probe JC-1, based on the method of Reers et al. (1995). JC-1 exists as a green-fluorescing monomer at low membrane potentials (<120 mV) and as a red-fluorescing dimer (J-aggregate) at membrane potentials greater than 180 mV. Following excitation at 488 nm, the ratio of red (595-nm emission) to green (525-nm emission) fluorescence measures the ratio of high-to-low mitochondrial membrane potential (Reers et al., 1995). Hepatocytes (10⁶ cells/ml L15 medium containing BSA and horse serum (10% v/v)). Initial cell viability was measured in a hemocytometer by trypan blue dye (0.4%) exclusion and was found to be greater than 90%.

**Measurement of Hepatocyte Mitochondrial Membrane Potential.** Mitochondrial membrane potential in intact liver cells was assessed using the mitochondrial-specific fluorescent probe JC-1, based on the method of Reers et al. (1995). JC-1 exists as a green-fluorescing monomer at low membrane potentials (<120 mV) and as a red-fluorescing dimer (J-aggregate) at membrane potentials greater than 180 mV. Following excitation at 488 nm, the ratio of red (595-nm emission) to green (525-nm emission) fluorescence measures the ratio of high-to-low mitochondrial membrane potential (Reers et al., 1995). Hepatocytes (10⁶ cells/ml L15 medium containing BSA and horse serum) were incubated with JC-1 (5.0 μM) for 25 min at 37°C in the dark with gentle agitation. Cells were then washed twice with fresh media and resuspended in a total volume of 10 ml. To measure the basal fluorescence of each cell preparation, an
aliquot was removed before the addition of JC-1 and measured cytofluorometrically. Thus, each mouse liver yielded 1 aliquot (10 ml; 10^6 cells/ml) for sample reading and 1 aliquot (5 ml; 10^6 cells/ml) for reading of basal fluorescence and then biotin to block any endogenous biotin. Tissue sections were subsequently incubated overnight in rabbit antiactive caspase-3 polyclonal antisera (Cell Signaling Technology Inc., Beverly, MA). The antisera was diluted 1:200 in PBS containing 2.5% normal goat serum. The sections were rinsed thoroughly to remove unbound antibodies and were reacted with biotinylated goat anti- rabbit IgG for 10 min at RT. Endogenous peroxidase activity was blocked by incubating tissue sections for 30 min with 1% hydrogen peroxide in nanopure water. Sections were then reacted with streptavidin conjugated to horseradish peroxidase for 10 min, and the immunoperoxidase color reaction was developed using a DAKO liquid diaminobenzidine (DAB) kit (DAKO Corp., Carpinteria, CA). The tissue sections were then counterstained in hematoxylin, dehydrated, cleared, and mounted. Incubations were also performed in the absence of the specific antibody to control for the specificity of the immunohistochemical reaction.

**TUNEL Assay.** DNA fragmentation characteristic of apoptosis was examined using a TdT (terminal deoxynucleotidyl transferase)-FragEL kit (Oncogene Research Products, San Diego, CA). Briefly, 4% paraformaldehyde-fixed tissue samples were embedded in paraffin and 5-μm sections were obtained. Replicate sections were rehydrated and permeabilized with proteinase K (20 μg/ml) for 20 min at RT. Next, endogenous peroxidases were inactivated by covering the sections with 3% H2O2 for 5 min. After incubation for 5 min in TdT buffer (200 mM sodium-cacodylate, 30 mM Tris, 0.3 mg/ml BSA, 0.75 mM CoCl2, pH 6.6), the slides were covered with TdT and biotinylated dUTP and incubated for 1.5 h at 37°C in a humidified chamber. Negative controls were incubated with biotinylated dUTP in TdT buffer in the absence of enzyme. The reaction was terminated by covering the sections with stop buffer (0.5 M EDTA, pH 8.0) for 5 min at RT. After blocking in BSA (4%), the slides were incubated with a streptavidin-horseradish peroxidase conjugate for 30 min. The sections were then incubated in DAB for 12 min at RT and counterstained with methyl green. Apoptotic cells were identified by staining properties and by morphological criteria (cell shrinkage, chromatin condensation and/or margination, and apoptotic bodies).

**Histopathology.** Liver tissue was prepared for histopathological evaluation as previously described (Forkert and Boyd, 2001). Briefly, livers were fixed by vascular perfusion through the left ventricle with 4% paraformaldehyde in 0.1 M Sorenson’s phosphate buffer, pH 7.4. Tissues were processed and embedded in paraffin using standard procedures. Liver sections (5 μm) were stained with hematoxylin and eosin.

**Statistical Analysis.** Data are expressed as mean ± S.D. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey’s test to determine significant differences between experimental groups (p < 0.05).

**Results**

**Measurement of Mitochondrial Membrane Potential.** Hepatocyte mitochondrial transmembrane potential (ΔΨm) was assessed 2 h after treatment with DCE or DCE and CyA using the fluorescent probe JC-1. As indicated by JC-1 fluorescence ratios, DCE caused substantial disruption of mitochondrial membrane potential (Fig. 1). The alterations in fluorescence were similar to those caused by Gal/ET treatment for 4 h. Pretreatment with CyA significantly inhibited DCE-induced disruption of mitochondrial membrane potential.

**Immunohistochemical Detection of Cytochrome c.** Protein immunoblotting of liver cytosol for cytochrome c revealed a single band of approximately 15 kDa in all samples (Fig. 2).
This is similar to the apparent molecular mass of cytochrome c identified in other studies (Knight and Jaeschke, 2002). At 4 h, immunoreactive protein was augmented in cytosolic fractions from DCE-treated mice compared with fractions from untreated animals; however, these increases were abrogated by pretreatment with CyA. Densitometric analysis of the protein bands showed that the amounts of cytochrome c in cytosolic fractions from DCE-treated mice were approximately 3-fold higher than in those from untreated mice. Pretreatment with CyA diminished the DCE-induced increases of cytochrome c to approximately 2-fold of control levels.

**Measurement of ALT Activity.** DCE-induced hepatotoxicity and the protective effects of CyA were temporally assessed by measurement of serum ALT activity. ALT activity was found to be significantly elevated from 2 to 24 h after DCE, compared with controls (Fig. 3). Pretreatment with CyA failed to inhibit DCE-induced increases in serum ALT activity at any time point.

**Measurement of Caspase-9 Protease Activity.** Caspase-9 protease activity was assessed by cleavage of the peptide substrate LEHD-AMC in modified liver homogenates. Conjugated AMC emits in the UV range (\(\lambda_{max} = 380\) nm); however, after proteolytic cleavage by caspase-9, free AMC fluoresces green (460 nm). Protease activity was found to be significantly increased at 6 h after DCE (125 mg/kg) (Fig. 4). Pretreatment with CyA inhibited the elevations in caspase-9 activity observed after DCE treatment. To verify that the signal detected was due to caspase-9 protease activity, samples from DCE-treated animals were incubated with caspase-9 inhibitor. Here, caspase-9 activity was similar to that in control samples.

**Immunohistochemical Localization of Activated Caspase-3.** Immunohistochemical studies were performed on liver tissues to detect activated caspase-3, one of the key executioners of apoptosis (Hengartner, 2000). Staining of cleaved caspase-3 is characteristically localized in the cytoplasm and perinuclear region of apoptotic cells. In liver sections from control mice, specific staining was not apparent (Fig. 5A). However, in sections from mice treated with DCE (125 mg/kg) for 12 or 24 h, staining was observed in centrilobular hepatocytes, although a few individual cells in other areas of the hepatic lobule were also stained (Fig. 5, C and D). Moreover, staining for activated caspase-3 appeared to increase from 12 to 24 h. In liver sections from mice treated with CyA + DCE, sparse hepatocyte staining was observed in centrilobular regions (Fig. 5E). In sections from mice treated with Gal/ET for 6 h, numerous positively-stained hepatocytes were seen scattered throughout the parenchyma (Fig. 5B). In all tissue sections, the majority of stained cells showed some morphological characteristics of apoptosis (i.e., cell shrinkage, chromatin condensation and/or margination, and apo-
Histopathology. Histopathological evaluation was performed in liver tissue 12 and 24 h after treatment with DCE (125 mg/kg). Hepatocellular injury was not observed in mice treated with DCE for 12 h compared with liver structure of the controls (Fig. 7, A and B); however, treatment with DCE for 24 h elicited mild centrilobular necrosis and eosinophilia (Fig. 7, C and D). Additionally, some centrilobular hepatocytes showed typical morphological characteristics of apoptosis, including cell shrinkage, retraction of cell borders, and chromatin condensation and margination (Fig. 7D). In liver sections from mice treated with CyA + DCE, apoptotic and necrotic hepatocytes were observed in centrilobular regions; however, CyA pretreatment greatly reduced the number of hepatocytes undergoing apoptosis (Fig. 7E). In sections from mice treated with Gal/ET for 6 h, many apoptotic hepatocytes were observed scattered throughout the parenchyma (Fig. 7F).

Discussion

The hepatotoxic lesion induced by DCE is manifested in centrilobular hepatocytes and involves several cellular organelles, including plasma membranes, nuclei, and mitochondria (Reynolds et al., 1980; Kanz and Reynolds, 1986; Forkert and Boyd, 2001). Here, we document the occurrence of hepatocellular apoptosis and its relationship to necrosis following acute exposure to DCE. Several approaches were applied to elucidate the molecular mechanisms involved in this mode of cell death. Our results suggested that DCE produces apoptotic cell death by inducing MPT causing release of cytochrome c into the cytosol and caspase activation.

Apoptosis is mediated through two major pathways, the extrinsic ‘death receptor’ pathway (Hengartner, 2000) and the intrinsic mitochondrial pathway (Kroemer and Reed, 2000). The extrinsic pathway is initiated by ligand binding and subsequent clustering of plasma membrane death receptors. This event leads to sequential recruitment of adaptors and initiator caspases (i.e., caspase-8 and -10) into the death-inducing signaling complex that ultimately mediates cell death (Hengartner, 2000). Alternatively, the intrinsic mitochondrial pathway is triggered by various extracellular and/or internal stress cues, such as perturbations of calcium homeostasis, DNA damage, and oxidative stress. These signals can directly or indirectly induce MPT setting the apoptotic cascade in motion (Kroemer and Reed, 2000). Previous investigations have demonstrated early increases of cytosolic calcium levels following DCE exposure (Long and Moore, 1987). In other studies, structural and functional perturbations of mitochondria have been reported (Kanz and Reynolds, 1986; Martin et al., 2003). Furthermore, morphological alterations characteristic of apoptosis were observed in scattered hepatocytes of rats after DCE administration (Reynolds et al., 1984). Taken together, these data suggest that DCE induces the intrinsic mitochondrial pathway, and several features of this apoptotic pathway were investigated in this study

MPT reflects a sudden increase in permeability of the inner mitochondrial membrane resulting in membrane depolarization, uncoupling of oxidative phosphorylation, mitochondrial swelling, and release of various soluble intermembranous proteins including cytochrome c (Lemasters et al., 1998). MPT is regulated by a voltage-dependent channel that is inhibited by nanomolar concentrations of cyclosporine A (Seaton et al., 1998). To assess DCE-mediated induction of MPT, $\Delta V_m$ and release of cytochrome c to the cytosol were determined. The $\Delta V_m$, measured using the cationic dye JC-1, was found to be significantly decreased 2 h after DCE (Fig. 1). Interestingly, this is the same time-point at which uncoupling of oxidative phosphorylation was maximal (Martin et al., 2003). Additionally, cytosolic levels of cytochrome c were 3-fold greater in livers from mice treated with DCE for 4 h than in controls (Fig. 2). Moreover, CyA pretreatment significantly inhibited DCE-induced disruptions of $\Delta V_m$ and release of cytochrome c (Figs. 1 and 2). These findings are
consistent with the premise that DCE activates the intrinsic mitochondrial pathway of apoptosis in which MPT precedes release of cytochrome c to the cytosol.

To support a major role for MPT in the mechanism of DCE hepatotoxicity, serum ALT activity was temporally assessed following DCE or DCE and CyA treatment. DCE treatment significantly increased ALT activity from 2 to 24 h indicative of hepatocellular damage (Fig. 3). Pretreatment with CyA failed to inhibit these increases in serum ALT activity at any time-point. Thus, these data do not support a major role for MPT in overall DCE hepatotoxicity. In recent studies, elevated levels of serum ALT activity have been reported following in vivo induction of hepatic apoptosis (Segawa et al., 2001). These studies are intriguing as increases in serum ALT activity have classically been associated with necrotic cell death. However, in the current investigation, CyA had no effect on DCE-induced increases in serum ALT activity, suggesting that elevations in activity are the result of necrotic cell death and not apoptosis.

Extensive investigation into the signaling mechanisms of the mitochondrial pathway of apoptosis demonstrated a critical role for the caspase family of cysteine proteases (Hengartner, 2000). The proteolytic activity of these enzymes accounts for many of the characteristic morphological features of apoptosis, including nuclear shrinking, chromatin condensation, and cytoplasmic blebbing (Rao et al., 1996; Liu et al., 2001).

Fig. 5. Immunohistochemical detection of activated caspase-3 in murine liver. Staining was performed with a rabbit antiactive caspase-3 polyclonal antiserum. Immunoreactivity in representative liver sections from mice treated with vehicle (A) or Gal/ET (B) or from mice treated with DCE for 12 h (C) or 24 h (D). Some mice were pretreated with CyA for 20 min before administration of DCE for 24 h (E). cv, central vein; pv, periportal vein. Scale bar = 200 μm.
Previous studies have shown that mitochondrial release of cytochrome c triggers the assembly of the apoptosome, a multimeric structure that recruits and activates procaspase-9, a prolific initiator caspase (Hengartner, 2000). The primary function of caspase-9 is processing of procaspase-3 to generate caspase-3. Once activated, this executioner caspase initiates a chain of reactions from which there is no return to the nonapoptotic state of the cell (Cohen, 1997). In the present study, levels of caspase-9 activity were significantly elevated 6 h following DCE administration (Fig. 4). Furthermore, activated caspase-3 was detected primarily in centrilobular hepatocytes, whereas a few individual cells were stained in other regions of the hepatic parenchyma (Fig. 5). As CyA pretreatment inhibited caspase-9 activity and decreased staining for caspase-3, these data again implicated MPT in the sequence of events leading to DCE-induced apoptosis. Because apoptosis is an ongoing physiological process, tissues often display basal levels of activated caspases.

Fig. 6. Histochemical analysis of DNA fragmentation using the TUNEL assay. A, control; liver structure was histologically normal. TUNEL-positive cells were rare. B, Gal/ET; numerous positively stained apoptotic hepatocytes scattered throughout the tissue. C, DCE 12 h; TUNEL staining in some centrilobular hepatocytes. D, DCE 24 h; TUNEL staining in centrilobular regions. Some TUNEL-positive hepatocytes were also observed in other areas of the parenchyma. E, CyA + DCE 24 h; TUNEL staining was observed in centrilobular hepatocytes. Staining was confined to centrilobular regions. cv, central vein; pv, periportal vein. Scale bar = 200 μm.
enzyme. This contention is supported by the results of this study, wherein basal levels of caspase-9 activity and -3 staining were observed. Collectively, these data supported a role for caspase-9 and -3 in the elaboration of DCE-induced apoptosis.

Additional insight into the pathogenesis of DCE-mediated hepatotoxicity was gained from TUNEL staining and histopathological assessment. TUNEL staining can be used to detect both apoptosis and necrosis as both modes of cell death involve internucleosomal DNA cleavage (Tateyama et al.,...
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However, apoptotic cells exhibit distinct nuclear staining, whereas necrotic cells display diffuse staining in the cytosol and nucleus (Gujral et al., 2003). TUNEL staining in the cytosol of necrotic cells may result from nuclear degradation and leakage of cleaved DNA to the cytosol. In mice treated with DCE for 12 or 24 h, TUNEL staining was observed predominantly in centrilobular hepatocytes, whereas a few cells were stained in other areas of the tissue (Fig. 6). All of the TUNEL-positive cells showed diffuse cytoplasmic staining although nuclear staining varied from moderate to intense, suggesting a necrotic mode of cell death. However, when H&E-stained liver sections were evaluated 24 h after DCE, apoptotic and necrotic hepatocytes were observed concomitantly in the centrilobular region (Fig. 7). Apoptotic cells exhibited characteristic morphological features of apoptosis, including cell shrinkage and chromatin condensation and margination. CyA pretreatment greatly reduced the number of histologically observable hepatocytes undergoing apoptosis, supporting the notion that MPT induction is an important event in DCE-induced apoptosis. These data are consistent with previous ultrastructural studies in rats in which morphological features of both apoptotic and necrotic cell death were observed after DCE administration (Reynolds et al., 1984). Thus, it is possible that TUNEL-positive centrilobular hepatocytes demonstrating intense nuclear staining represent a population of damaged cells undergoing apoptosis.

Recent debate has questioned whether apoptosis and necrosis are indeed two separate modes of cell death. As evidence for a unified cell death pathway, studies have shown that MPT may be a critical event in both apoptosis and necrosis (Lemasters, 1999). Since apoptosis is an energy-requiring process, ATP depletion may interrupt the apoptotic signaling cascade leading to secondary necrosis (Leist et al., 1997). Thus, it was postulated that cellular ATP content following MPT induction determines whether a cell dies by apoptosis or necrosis (Lemasters, 1999); however, data from the current study suggested that DCE induces apoptosis independent of necrosis. This conclusion is supported by the observation that CyA, despite its effectiveness in preventing apoptosis, had no significant effect on necrosis.

Although the present investigation indicates that DCE induces both apoptosis and necrosis, the initiating events leading to either mode of cell death are unclear. It is conceivable that DCE-derived metabolites arylate and/or oxidize organellar proteins essential for the maintenance of calcium homeostasis. Indeed, studies have demonstrated covalent binding of DCE metabolites in fractions of mitochondria, ER, and plasma membranes (Okine et al., 1985). Furthermore, it has been shown that DCE inhibits calcium sequestration by the Ca\(^{2+}\)/Mg\(^{2+}\) ATPase in rat liver ER (Moore, 1982). Moreover, cytosolic calcium accumulation was observed in hepatocyte suspensions after DCE exposure (Long and Moore, 1987). Because high cytosolic calcium concentrations are closely associated with induction of MPT, DCE may initiate apoptosis through this mechanism. Alternately, an increase in cytosolic calcium levels may activate a number of calcium-dependent hepatic enzymes, including lipases, proteases, and endonucleases, that could cause irreversible damage to cell constituents and ultimately lead to cell necrosis. Studies to date have excluded endonuclease activation as an early event in DCE hepatotoxicity (Long et al., 1989); however, further investigation is necessary to determine whether other degradative enzymes contribute to toxicity. Thus, a calcium-mediated mechanism of cell injury could explicate both apoptotic and necrotic cell death following DCE exposure.

In conclusion, this investigation provides for the first time mechanistic data supporting hepatocellular apoptosis as a mode of cell death in DCE hepatotoxicity. Here, MPT induction, cytochrome c release, and caspase activation appear to be important events in the apoptotic cascade. Additionally, apoptotic cell death was shown to occur concurrently with necrosis, the mode of cell death classically associated with DCE hepatotoxicity.

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**Address correspondence to:** Dr. Poh-Gek Forkert, Department of Anatomy and Cell Biology, Queen’s University, Kingston, ON, Canada K7L 3N6. E-mail: forkertp@post.queensu.ca