Biomarkers, March 2008; 13(2): 184–200

informa
healthcare

The release of DNA into the plasma of mice following hepatic cell death by apoptosis and necrosis

TRINH T. TRAN[1], PAMELA GROBEN[2], & DAVID S. PISETSKY[1,3]

[1]*Department of Medicine, Division of Rheumatology and Immunology, Duke University Medical Center, Durham, NC, USA,* [2]*Department of Pathology, University of North Carolina, Chapel Hill, NC, USA and* [3]*Medical Research Service, Veterans Administration Hospital, Durham, NC, USA*

Abstract

The goal of these investigations was to measure levels of DNA in the plasma of mice following administration of hepatotoxic agents to induce apoptotic or necrotic cell death and determine any differences in the release of this marker depending upon death pathway. For this purpose, the effects of varying doses of anti-Fas, acetaminophen (APAP) or carbon tetrachloride (CCl_4) were assessed in normal mice. Plasma DNA was measured fluorometrically by the dye PicoGreen while lactate dehydrogenase (LDH) and caspase 3, other molecules released with cell injury or death, were measured by enzymatic assays. Histology was used to assess the occurrence of apoptosis or necrosis. Results of these experiments indicate that increased blood DNA levels occurred with all three agents and were highest with anti-Fas and CCl_4; caspase 3 levels were much higher with anti-Fas than the other agents. Histological examination confirmed the predominance of apoptotic death with anti-Fas and necrotic death with APAP and CCl_4. These results indicate that increased blood DNA is common in hepatotoxic injury and is a feature of both apoptotic and necrotic death.

Keywords: *Acetaminophen, anti-Fas, apoptosis, carbon tetrachloride, DNA, hepatotoxicity, necrosis, biomarker*

(Received 8 February 2007; accepted 27 September 2007)

Introduction

Cell death is a prominent feature of disease and occurs by two main mechanisms dichotomized as apoptosis or necrosis. In apoptosis, an elaborate molecular programme causes the systematic disassembly of cells mediated by cascades of enzymes called caspases. In contrast, in necrosis, physical or chemical trauma causes sudden or accidental death that culminates in cell lysis (Majno & Joris 1995). The distinction between apoptosis and necrosis can be made on the basis of morphological features as well as biochemical changes including caspase activation and the cleavage of proteins and nucleic acids (McConkey 1998, Renvoize et al. 1998, Van Cruchten & Van Den Broeck 2002). In addition to differences in the pattern of death, apoptotic and necrotic cells differ in their functional properties, including inflammatory

Correspondence: David S. Pisetsky, 151G Durham VA Medical Center, 508 Fulton Street, Durham, NC 27705, USA. Tel: +1-919-286-6835. Fax: +1-919-286-6891. E-mail: piset001@mc.duke.edu

ISSN 1354-750X print/ISSN 1366-5804 online
DOI: 10.1080/13547500701791719

potential (Fadok et al. 2001, Huynh et al. 2002, Scaffidi et al. 2002, Filaci et al. 2003, Brouckaert et al. 2004, Gregory & Devitt 2004, Martinon & Tschopp 2004, Fink & Cookson, 2005, Hoffmann et al. 2005).

Among tissues in which apoptosis and necrosis occur, the liver is a major focus of investigation into these processes and their relevance to disease pathogenesis. Liver cell death can result from a wide range of causes that encompass infection, toxin exposure and trauma among many others (Losser & Payen 1996, Hayashi & Mita 1999, Gujral et al. 2001, Lee 2003). In contrast to other tissues, the liver can regenerate, with the mode and extent of death determining not only the functional capacity at the time of injury but the eventual outcome in terms of healing and fibrosis (Black et al. 2004, Lieu et al. 2006). While a variety of proteins can be measured in the blood as indicators of hepatic cell injury, markers to distinguish mechanisms of cell death would be useful in evaluating these processes and their consequences.

In recent studies, our laboratory has been investigating the release of DNA from cells as a biomarker of the processes leading to cell death. As shown in studies in mice, DNA can appear in the blood of mice receiving agents that induce apoptosis (Jiang et al. 2003). Thus, mice treated with either lipopolysaccharide (LPS) or anti-Fas release DNA into the blood, with mice treated with anti-Fas also showing increases in blood levels of caspase 3. In a model in which cell death is induced *ex vivo*, infusion of apoptotic or necrotic human Jurkat cells into mice leads to a time and dose-dependent increase in DNA in the circulation (Jiang et al. 2003b, Jiang & Pisetsky 2004, Jiang & Pisetsky 2005). Together, these results suggest that the extracellular release of DNA may occur following both apoptosis and necrosis, with other analytes such as lactate dehydrogenase (LDH) and caspase 3 also useful in delineating the mechanism of cell death as well as its extent (Bicknell & Cohen 1995, Drent et al. 1996, Hentze et al. 2001).

In our prior experiments on the behaviour of necrotic cells, we used, as a model, Jurkat cells treated *in vitro* with either heat or ethanol (Jiang et al. 2003a, Jiang & Pisetsky 2004, Jiang & Pisetsky 2005). Such cells may differ in properties from cells in which necrosis occurs *in vivo* in a clinical or experimental setting. In the current experiments, we have therefore assessed a liver model and directly compared the appearance of DNA in the blood of mice treated with agents that induce either hepatic cell apoptosis or necrosis. For this purpose, we have used three agents: monoclonal anti-Fas antibody (Jo2), acetaminophen (APAP) and carbon tetrachloride (CCl_4), which induce hepatocyte cell death by different mechanisms (Ogasawara et al. 1993, Estaban et al. 1993, James et al. 2003, Weber et al. 2003, Cover et al. 2006, Jaeschke & Bajt 2006). We then measured levels of DNA, LDH and caspase 3 in the blood as well as liver pathology. We show that DNA appears in the blood of mice treated with all three agents, while levels of caspase 3 vary with the toxic agent. These findings indicate that increased levels of plasma DNA are a common feature of the death of hepatocytes as well other cell types and suggest its utility as a marker to assess apoptotic or necrotic death.

Materials and methods

Animals

Female BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, NE, USA) and housed under conventional conditions in the animal facilities of Durham

VAMC. Mice were used at 6–10 weeks of age. In these experiments, each experimental group had six to eight mice.

Hepatotoxic agents

4-Acetamidophenol (or *N*-Acetyl-*p*-aminophenol) (APAP) and carbon tetrachloride (CCl_4) were purchased from Sigma-Aldrich Inc. (St Louis, MO, USA) and stored at room temperature. Jo-2, a monoclonal anti-Fas antibody, was purchased from BD Pharmingen (San Diego, CA, USA) and stored at 4°C. Solutions used for treatments were prepared freshly prior to each experiment. APAP was dissolved in normal saline, pH 7.0, to a concentration of 20 mg ml^{-1}. CCl_4 was mixed with olive oil in 1:1 volume ratio and Jo-2 was diluted in phosphate-buffered saline (PBS).

Induction of hepatotoxicity

Mice were weighed and bled from the retro-orbital sinus under methoxyflurane anaesthesia prior to intraperitoneal injection with either APAP (0.65 mg g^{-1} of body weight) or CCl_4 (1.5 µl g^{-1} of body weight). For anti-Fas treatment, mice were injected with the Jo-2 at 1–5 µg per mouse in a volume of 100 µl in PBS. These doses were derived from preliminary dose–response experiments to define a dose necessary to induce liver injury without a high mortality rate. In these experiments, the intraperitoneal route was used because APAP and CCl_4 can cause significant hepatotoxicity as compared with the oral route, but with much less mortality as compared with the intravenous route (Esteban et al. 1993, Ogasawara et al. 1993, Janakat & Al-Merie 2002). Following the treatments, the mice were bled at 6 h (7 h for the anti-Fas-treated mice), 24 h and 48 h. Blood samples were anticoagulated using 5 µM EDTA and plasma was obtained by centrifugation at 14 000 rpm for 2 min. The levels of plasma DNA, LDH and caspase 3 activity were then measured using the assays described below. These experiments involved groups of varying size as indicated in the figure legends and were repeated on multiple occasions. Depending upon the agent and dose, the effects on animals varied, with mice treated with the highest doses of anti-Fas showing noticeable physical signs.

DNA assay

To measure plasma DNA, a fluorometric assay was used in which DNA is detected with the dye PicoGreen (Molecular Probes, Eugene, Oregon, USA). PicoGreen binds specifically to double-stranded (ds) DNA and allows detection of dsDNA in the pg–ng range. As a standard, native calf thymus DNA (CT DNA) was purchased from the Sigma Chemical Co and further purified by phenol-chloroform extraction and ethanol precipitation. The concentration of DNA was determined by OD260 readings with purity assessed by the OD260/OD280 ratio.

Twofold dilutions of native CT DNA in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) starting at 1000 ng ml^{-1} were prepared as standards. Dilutions of plasma samples (1:100) and of PicoGreen dye (1:200) were also made in TE buffer. Samples were added to duplicate wells (50 µl per well) in a Costar black polystyrene flat-bottomed 96-well plate (Costar Corning Incorporated, Costar, NY, USA), mixed with PicoGreen (50 µl per well). The plate was immediately read in a TECAN/GENios fluorometric microtitre plate reader (Salzburg, Austria) at an excitation wavelength of

485 nm and emission wavelength of 535 nm. DNA concentrations were calculated from a standard curve, with results expressed in μg ml^{-1}.

Enzyme assays

Plasma caspase activity was measured using the EnzChek Caspase-3 assay kit purchased from Molecular Probes (Eugene, Oregon, USA). Briefly, 50 μl of 1:100 plasma dilutions in PBS were added to duplicate wells containing 2x substrate working solution (50 μl per well) in a Costar black polystyrene flat-bottomed 96-well plate (Costar Corning Incorporated), covered with aluminum foil. After 30-min incubation at room temperature, the plate was then read in a TECAN/GENios fluorometric microtiter plate reader at an excitation wavelength of 485 nm and emission wavelength of 535 nm. Results are expressed in fluorescence units (FU).

Plasma LDH levels were measured with the CytoTox 96 assay purchased from Promega Corporation (Madison, WI, USA). A 1:500 plasma dilution in PBS/1%BSA was added to duplicate wells (50 μl per well) containing reconstituted substrate solution (50 μl per well) in a 96-well Styrene microtitre plate (ThermoLab System, Franklin, MA, USA), covered with aluminum foil. After 30-min incubation at room temperature, 50 μl of stop solution was added to each well and OD490 was determined with a microtitre plate reader (Molecular Dynamics, Menlo Park, CA, USA).

Liver histology

At 6 h and 24 h after injection of APAP and CCl_4, mice were sacrificed by cervical dislocation. Their livers were collected, fixed in 10% formaldehyde and embedded in paraffin. Sections of 5 μm were cut and stained with haematoxylin and eosin (H&E). Mice injected with Jo-2 were sacrificed at 7 h and their livers were similarly processed. Sections were analyzed by a pathologist blinded to the experimental group.

DNA electrophoresis

Plasma DNA was purified using MasterPure DNA purification kit from Epicentre (Madison, WI, USA). Purified DNA (0.2 μg) was loaded into each lane of an agarose 1% minigel and electrophoresis was performed at 60 V and 500 A in TBE buffer. After electrophoresis was completed, photographs of the gel were taken using the Alpha Innotech FluorChem™ 8900 photographic device purchased from Alpha Innotech Corp (San Leandro, CA, USA).

Statistical methods

The statistical significance of the results was evaluated using the Mann–Whitney *U* test. Correlation coefficient and linear regression analysis were used to assess the correlation between plasma DNA and caspase activity.

Results

The changes of plasma levels of DNA, LDH and caspase 3 resulting from treatment with the Jo-2 antibody, an agent that can cause apoptosis, were measured first. In

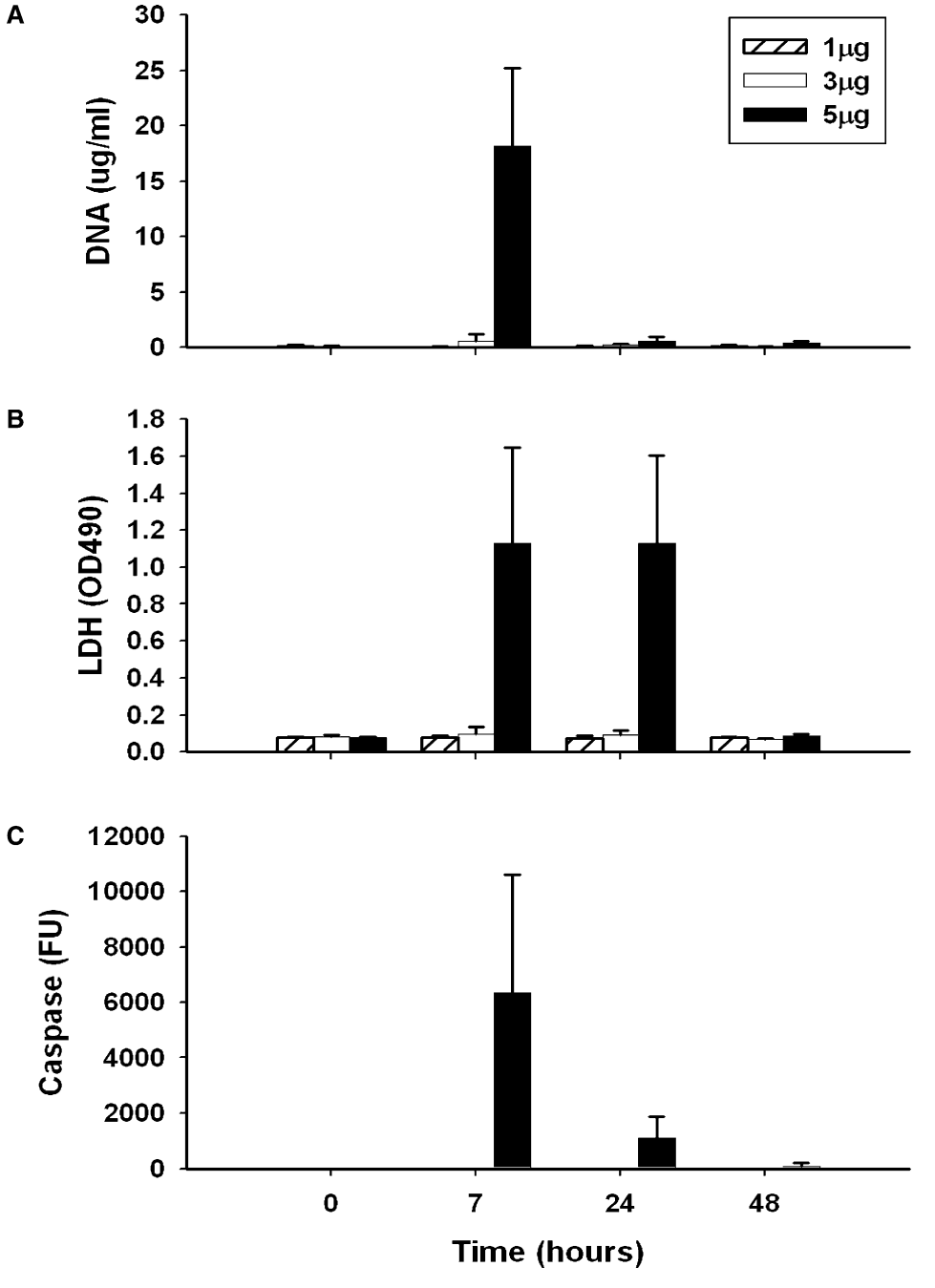

Figure 1. Effects of anti-Fas antibody treatment on plasma DNA, LDH and caspase 3 levels. Female BALB/c mice were injected intraperitoneally with different doses of Jo-2 anti-Fas antibody (1 μg, 3 μg and 5 μg per mouse) and plasma DNA, LDH, and caspase 3 activity were measured at 0, 7, 24 and 48 hours following the injections. The results are expressed in μg ml^{-1}, OD 490 and FU respectively. Groups contained 4–6 mice. Data presented are mean values ± standard deviations.

these experiments, LDH has been used as a general marker of cell death and injury; while it can be released from liver, it can also be released from other cell types. As these data indicate (Figure 1), at doses of Jo-2 of 1 μg and 3 μg per mouse, levels of the analytes tested did not change significantly. In contrast, at a dose of 5 μg per mouse, DNA, LDH and caspase 3 levels all increased significantly ($p < 0.01$). At doses higher than 5 μg per mouse, the mice showed high mortality and did not survive past 12 h post-injection (data not shown).

In these experiments, levels of plasma DNA and caspase 3 showed a similar time course following Jo-2 injection, with levels maximal at 7 h, and returning to baseline by 24 h after treatment. In contrast, LDH levels showed a more sustained elevation, for as long as 24 h. These results indicate that anti-Fas treatment causes an elevation of all three analytes, although the time course of plasma appearance may differ.

Having assessed responses to an agent inducing liver apoptosis, the effects of agents considered to result primarily in hepatocyte necrosis were tested next. In these experiments, mice were treated with either APAP at 0.65 mg g^{-1} of body weight, or CCl_4 at 1.5 μl g^{-1} of body weight, using as controls, 0.9% saline or olive oil, respectively. These doses were chosen on the basis of preliminary experiments which showed maximal DNA levels with ~100% survival rate. At higher doses, the mice had significant mortality (data not shown).

As shown in Figure 2, following APAP treatment, plasma DNA levels increased at 6 h and returned to baseline by 48 h while, following CCl_4 treatment, the highest levels were measured in the 24-h sample, with significant elevations also observed at 48 h (Figure 2A). Following APAP injection, elevations of LDH levels were observed at 6 h, peaking at 24 h and returning to baseline by 48 h (Figure 2). In comparison, in CCl_4-injected mice, LDH levels peaked at 24 h and showed elevations at 48 h. As in the case of mice treated with anti-Fas, DNA and LDH responses showed differences in the duration of elevation.

Although APAP and CCl_4 are considered to induce primarily necrotic cell death (James et al. 2003, Weber et al. 2003), the release of caspase under these conditions was nevertheless measured and compared with that induced by anti-Fas. As data presented in Figure 3 indicate, although caspase 3 levels induced by APAP and CCl_4 were much lower than those resulting from anti-Fas ($p < 0.01$), they were nevertheless elevated when compared with the baseline pre-injection levels ($p < 0.05$).

These results indicate that, similar to anti-Fas treatment, APAP and CCl_4 can cause release of caspase 3 as well as DNA and LDH into the blood although the time course and magnitude may differ depending on the inducing agent. Table I presents results of these determinations for all three agents.

To investigate the effects of these treatments on liver pathology, histological examination was performed. Figure 4 shows representative H&E sections of a liver of an untreated BALB/c mouse (Figure 4A–C) and a BALB/c mouse treated with anti-Fas at 7 h after injection (5 μg) (Figure 4D–F). As these micrographs indicate, features of apoptosis in the liver are present in the anti-Fas-treated mouse as shown by cell shrinkage, chromatin condensation, margination and apoptotic bodies. Cells at early and late stages of apoptosis were present in same section (Figure 4F), with hepatocytes around the portal tracts showing more change than hepatocytes around the central veins and venules (Figure 4D).

Figures 5 and 6 show sections of the livers of BALB/c mice treated with APAP or CCl_4 at 6 and 24 h. In this study, at the early time point, sections showed centrilobular

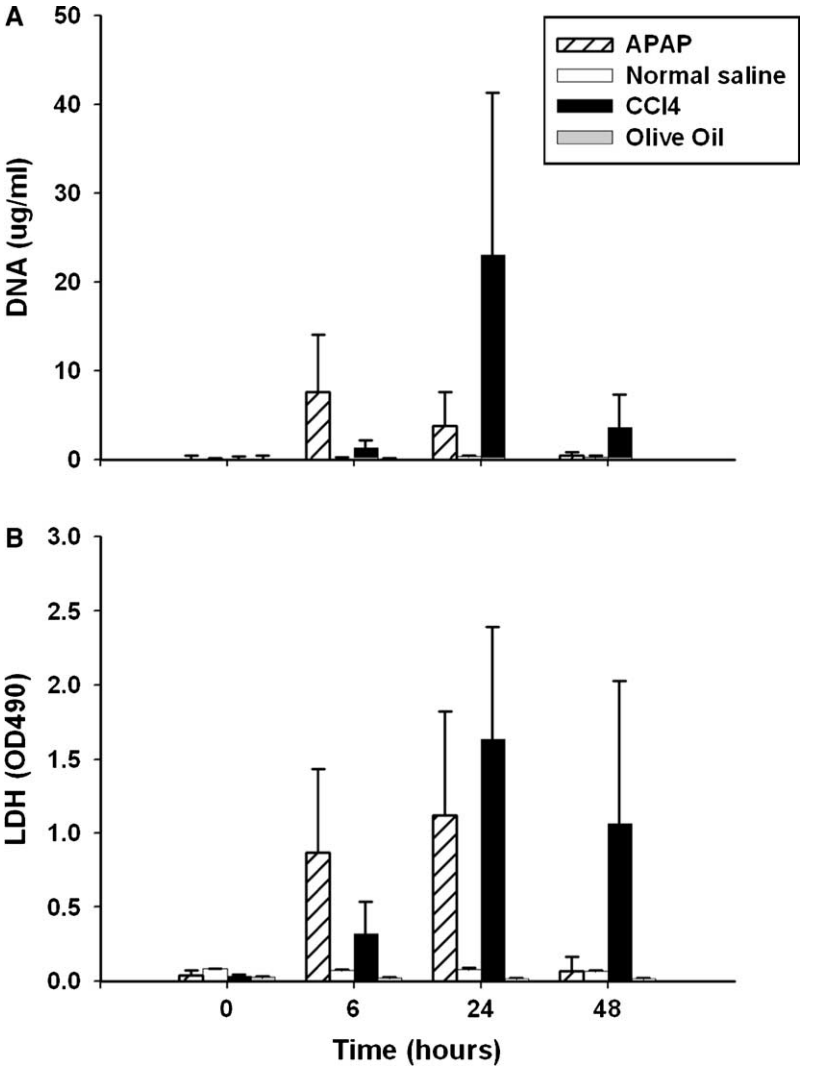

Figure 2. Plasma DNA and LDH levels following treatment with APAP (0.65 mg g^{-1}) or CCl_4 (1.5 µl g^{-1}). Female BALB/c mice were given IP injections of APAP or CCl_4, using 0.9% saline and olive oil for negative controls for APAP and CCl_4 respectively. Plasma DNA and LDH levels were measured at 0, 6, 24 and 48 hours and results are expressed in µg ml^{-1} and OD 490 respectively. Data presented are mean values ± standard deviations. There were 6–8 mice in each group.

pallor while, at the later time point, a centrilobular zonal pattern of cell death was present with both agents. Although features of necrosis were dominant, occasional apoptotic cells were present. Other features observed in these sections include ballooning of cells, karyorrhexis and loss of nuclear contents to produce ghosts in the areas of severe coagulative necrosis. These findings confirm the extensive necrosis induced by APAP and CCl_4, although they suggest the presence of apoptosis as well, consistent with the caspase 3 elevations measured.

Apoptotic and necrotic death, in addition to the pathology, can differ in the nature of DNA released from cells (Jahr et al. 2001). Because of cleavage during apoptosis,

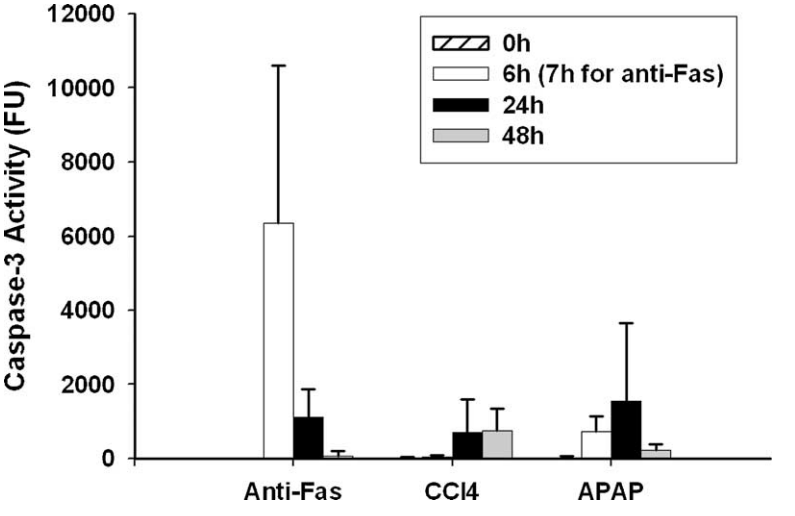

Figure 3. Plasma caspase 3 levels following treatment with anti-Fas (5 μg/mouse), APAP (0.65 mg g^{-1}) or CCl_4 (1.5 μl g^{-1}). Plasma were collected at 0, 6 (7 hours for anti-Fas), 24 and 48 hours and caspase 3 levels measured. Results are expressed in FU. Anti-Fas treated mice were bled at 7 rather than 6 hours because previous experiment had shown peak DNA release at that time point. Data presented are mean values ± standard deviations.

the released DNA shows size laddering, with low molecular weight fragments. In contrast, DNA from necrotic cells can display high molecular weight because nucleases are not activated (Wyllie 1980, Wyllie et al. 1992). This DNA, however, could be reduced in size if cells are taken up by macrophages or blood DNA is digested by nucleases. The size of the DNA in the blood of mice treated with the three agents was therefore measured. As shown in Figure 7, most of the plasma DNA existed as low molecular weight fragments, predominantly the size of mononucleosomes, regardless of the treatment. These findings indicate that the size of DNA found in the plasma is similar following both apoptotic and necrotic cell death.

Since the administration of both apoptotic and necrotic cells can lead to an elevation of plasma DNA levels but only apoptotic cells release caspase 3, these

Table I. Peak levels of plasma DNA, lactate dehydrogenase (LDH) and caspase 3 following treatment with hepatotoxic agents.

	DNA (μg ml^{-1}) (±SD)	LDH (OD490) (±SD)	Caspase 3 (FU) (±SD)
Anti-Fas	18.2 (±7.0)	1.13 (±0.5)	6356 (±4258)
CCl_4	23.0 (±18.2)	1.64 (±0.8)	705 (±899)
APAP	7.5 (±6.5)	1.23 (±0.7)	1557 (±2094)
Normal saline	.09 (±0.10)	0.08 (±0.01)	39 (±48)
Olive oil	<0.01	0.02 (±0.01)	42 (±126)

These results indicated that caspase 3 activity induced by anti-Fas is significantly higher than that induced by APAP or CCl_4 ($p < 0.01$). Caspase 3 activity induced by APAP is not statistically different from that induced by CCl_4 ($p > 0.05$). DNA levels in mice injected with anti-Fas and CCl_4 are similar ($p > 0.05$) whereas DNA levels in mice injected with APAP are significantly less than those of mice injected with anti-Fas or CCl_4 ($p < 0.01$). There is no statistically significant difference in the LDH activities in the three groups.

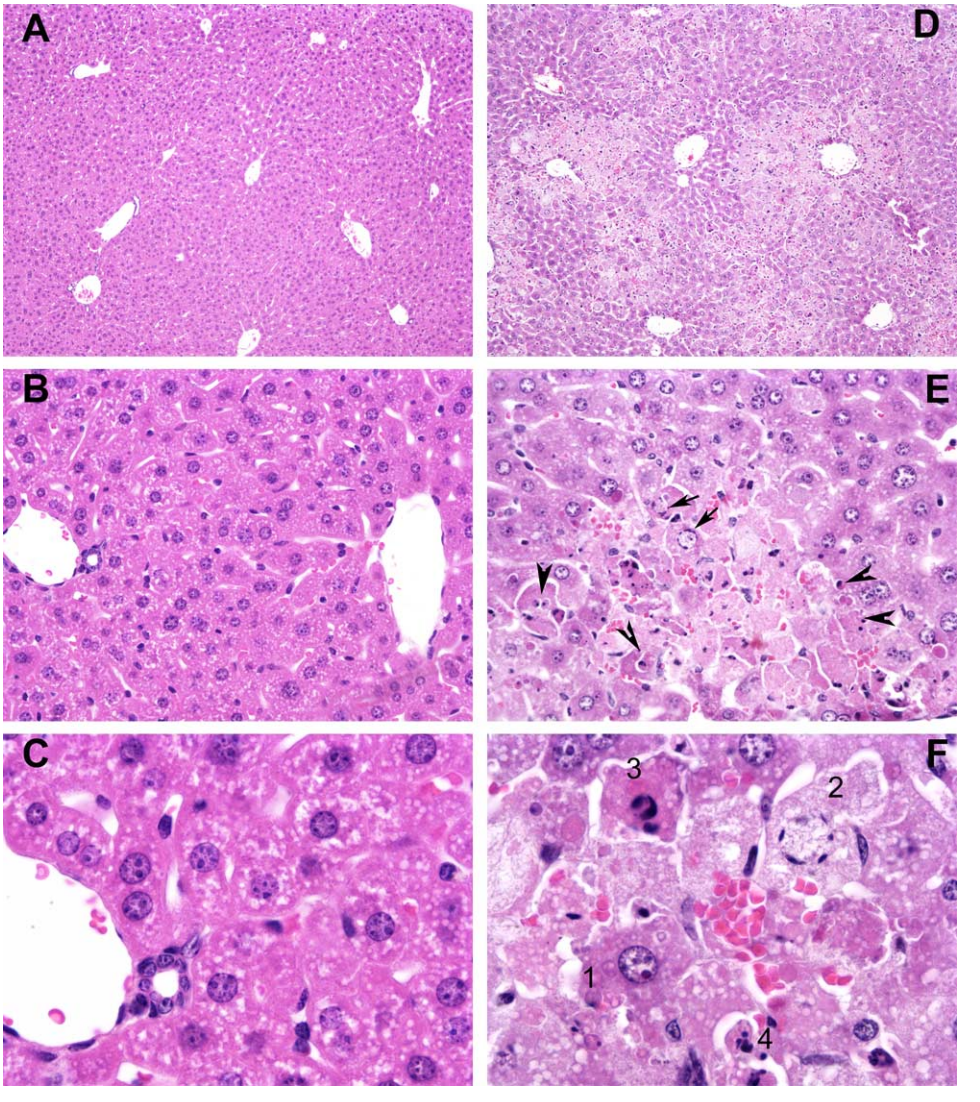

Figure 4. Pathological changes in mouse liver following treatment with anti-Fas antibody. Livers of three anti-Fas injected BALB/c mice were collected at 7 hours and H&E sections were prepared (Figures 4D, E and F). Livers of normal untreated BALB/c mice were used as controls (Figures 4A, B and C). Representative sections are shown at various magnifications. At low magnification, the centrilobular zonal pattern of hepatic injury induced by anti-Fas is evident as shown in Figure 4D. Typical features of apoptotic cell death such as nuclear margination (long arrows) and apoptotic body formation (short arrows) are shown in Figure 4E. Different stages of apoptosis can be seen at the same time as shown in Figure 4F: early cell shrinkage with preserved nucleus (#1 cell), nuclear margination (#2 cell), chromatin condensation (#3 cell) and the formation of apoptotic bodies (#4 cell).

findings suggest that relative amounts of DNA and caspase 3 can be used to delineate the pattern of hepatocyte death (Jiang et al. 2003b). To investigate the relationship between plasma DNA and caspase 3 levels, values of peak plasma DNA were plotted against the corresponding plasma caspase 3 activities for the three agents. Using a linear regression model, correlation coefficients were calculated.

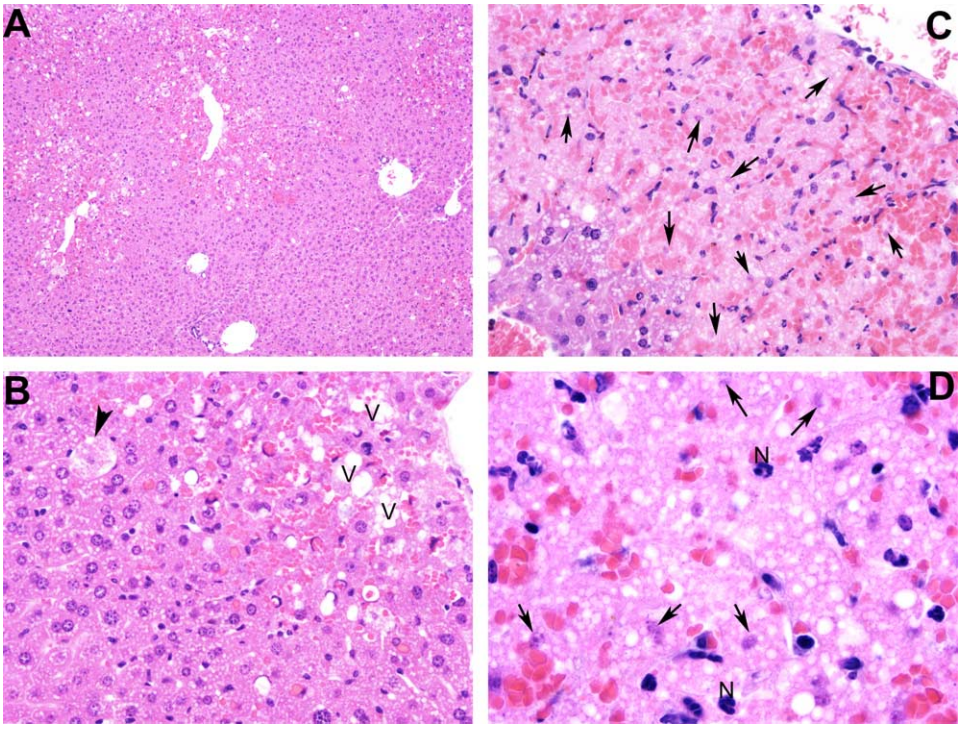

Figure 5. Pathological changes in mouse liver following treatment with APAP. Livers of mice injected with APAP were collected at 6 and 24 hours and H&E sections were prepared. Livers from three mice were collected for each time point. Histologically, APAP causes a centrilobular zonal pattern of hepatic injury as shown at low magnification in Figure 5A. Balloon degeneration of hepatocytes (short arrow) and cytoplasm vacuolization (V) are seen at 6 hours, as shown in Figure 5B. At 24 hours, loss of nuclear details (long arrows) and the appearance of neutrophils (N) in the necrotic areas can be seen as shown in Figure 5C and D.

As shown in Figure 8, the plasma DNA and caspase 3 levels showed a strong correlation in mice injected with anti-Fas.

For APAP- or CCl_4-injected mice, however, no correlations were observed. Indeed, in these experiments, two of the highest concentrations of DNA occurred in the plasma of mice treated with CCl_4; these two mice had only a limited elevation of caspase 3. These results suggest that the mechanism of cell death can be inferred from the relative amounts of DNA and caspase 3 in the blood, with apoptotic death associated with a strong correlation in the magnitude of these responses.

Discussion

Results of these studies provide new insight into the origin of plasma DNA and its utility as a biomarker for *in vivo* cell death. Thus, we have shown that DNA appears in the blood of mice treated with anti-Fas antibody, carbon tetrachloride or acetaminophen. With agents that induce predominantly either apoptosis or necrosis, plasma DNA levels rose significantly, although the relative expression in comparison to LDH and caspase 3 varied. With all three agents, levels of LDH increased to similar levels while levels of caspase 3 were much greater in anti-Fas-treated mice than either of the

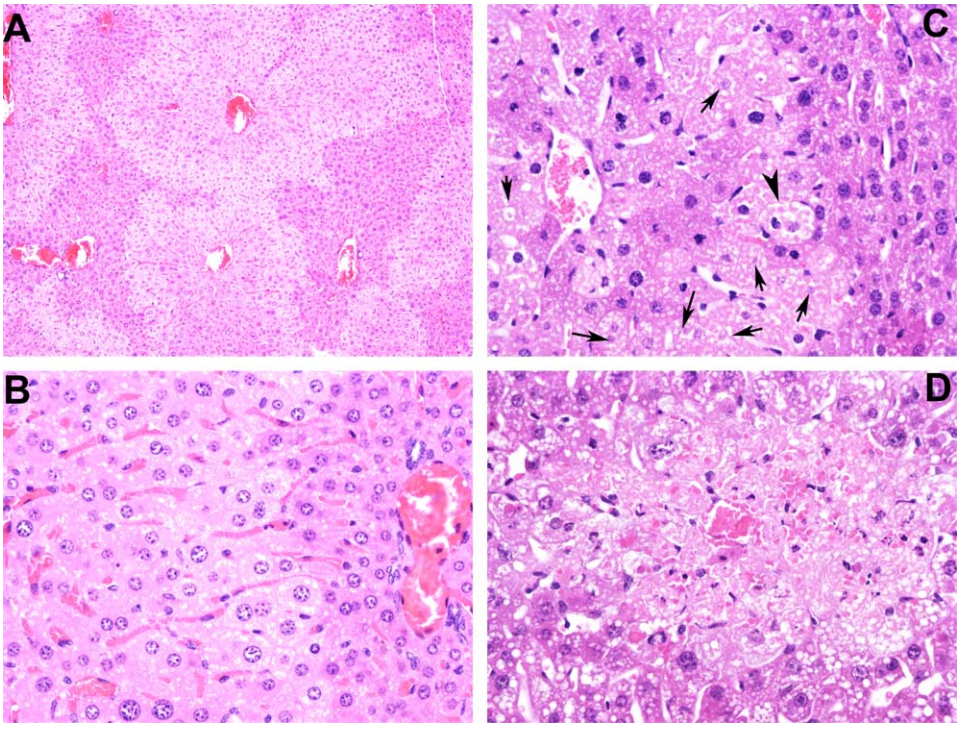

Figure 6. Pathological changes in mouse liver following treatment with CCl_4. Livers of mice injected with CCl_4 were collected at 6 and 24 hours and H&E sections were prepared. Livers from three mice were collected for each time point. Centrilobular zonal pattern of hepatic injury is seen similar to that seen with anti-Fas and APAP (Figure 6A). Centrilobular palor and some balloon degeneration are seen early at 6 hours (Figure 6B). Loss of nuclear details and nuclear contents leading to the appearance of "nuclear ghosts" (long arrows) are seen at 24 hours along with some balloon degeneration at the periphery at the junction between normal and necrotic areas (Figure 6C). Massive eosinophilic necrosis is evident in Figure 6D.

other two agents. Since the mechanism of hepatotoxicity differs among these inducing agents, these results suggest that both necrosis and apoptosis culminate in substantial release of nucleic acid from the cell, despite differences in molecular and cellular events during these death processes.

Given the importance of hepatic cell death in liver disease whether induced by viruses, toxins or xenobiotics, there has been extensive investigation into the mechanism of this process in both patients as well as animal models of disease (Neuman 2001, Feldstein et al. 2003, Riordan & Williams 2003, Ribeiro et al. 2004, Seidel et al. 2005, Wieckowska et al. 2006). In rodent models, a variety of agents have been used to induce hepatic cell death (Trautwein et al. 1998a,b, Koteish et al. 2002, Tzirogiannis et al. 2003, Taniguchi et al. 2004). These agents can induce apoptosis or necrosis, although both processes may occur concomitantly depending on the extent of death and the metabolic state of the animal (Nicotera et al. 1999, Shi et al. 1998, Gujral et al. 2002, Nicotera & Melino 2004, Toyoshiba et al. 2006). Furthermore, even though these agents can induce specific functional abnormalities in hepatocytes, other cell types such as macrophages can affect these processes, with cytokines secreted by Kupffer cells sensitizing the liver to injury induced by other triggers

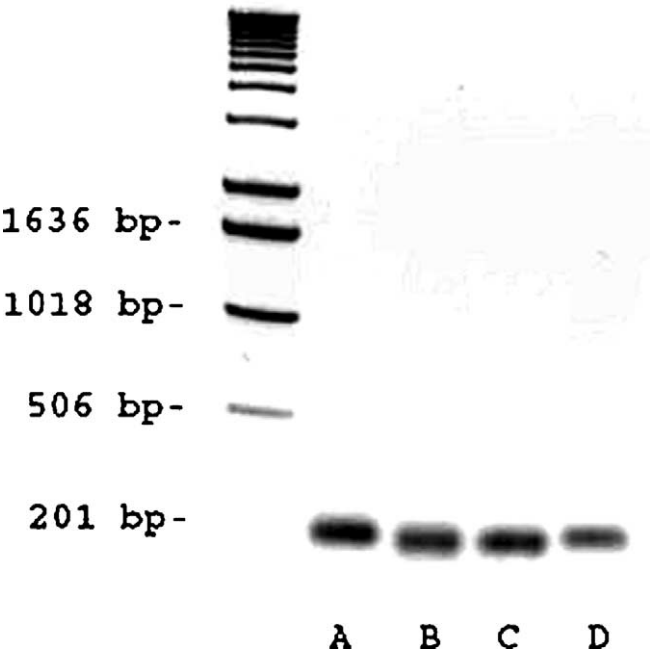

Figure 7. Size of plasma DNA following treatment with anti-Fas, APAP or CCl_4. 0.2 μg of DNA purified from mouse plasma following treatment with anti-Fas (A), CCl_4 (B) or APAP (C) was subjected to gel electrophoresis analyzed for size. Plasma DNA from mice that received apoptotic Jurkat cells was used as a positive control (D). DNA marker is shown in the first lane on the left. Under these conditions, DNA from normal plasma is not visualized on these gels. Under the conditions of this assay, plasma from untreated mice did not show detectable DNA when stained with ethidium bromide. The plasma was obtained at 6–7 h depending upon the treatment.

(Bradham et al. 1998, Hamada et al. 1999, Canbay et al. 2003, Duffield et al. 2005, Corazza et al. 2006).

Of the agents we have studied, anti-Fas antibody has the most direct mode of action. Anti-Fas can bind to the Fas molecule on cells and trigger subsequent apoptotic cascade by receptor cross-linking. This process entails the activation of caspases and as well as cleavage of DNA (Feldman et al. 1998). Since anti-Fas may induce endothelial cell death, the resulting lesion may reflect both vascular events as well as targeting of hepatocytes (Cardier et al. 1999, Cardier & Erickson-Miller 2002, Janin et al. 2002). Furthermore, while apoptosis may be the predominant mode of anti-Fas-mediated death in the liver, necrosis may also be observed especially when massive liver cell death occurs. In addition to its effects on the liver, anti-Fas can affect other cell populations, including lymphocytes and monocytes. There is therefore uncertainty concerning the amount of DNA in the blood that arises from the liver as well as the amount that arises from apoptotic compared with necrotic cells.

In contrast to anti-Fas-induced apoptosis, APAP-induced death is more complex and involves the metabolism of APAP to *N*-acetyl-*p*-benzoquinone-imine (NAPQI) via cytochrome P4510E1 (CYP2E1) (James et al. 2003). This agent can deplete liver cell glutathione and lead to the formation of protein adducts. The generation of reactive oxygen species as well as cytokine expression in the liver contributes to the hepatic cell death. While necrosis is usually considered the mechanism of APAP-induced cell death,

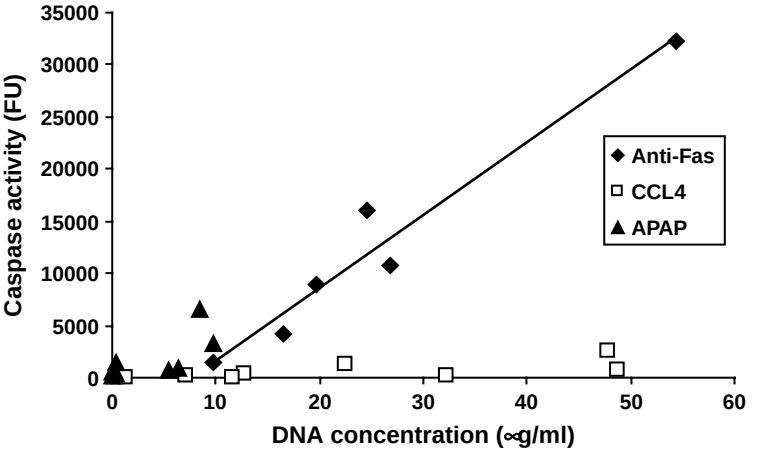

Figure 8. Correlation between plasma DNA and caspase 3 levels in mice following treatment with anti-Fas, APAP or CCl_4. Peak plasma DNA values are plotted against the corresponding caspase 3 activities. The correlation coefficient values (R^2) for anti-Fas, APAP and CCl_4 are 0.95, 0.51 and 0.40 respectively.

biochemical and histological analysis of liver exposed to this agent have suggested that apoptosis may also occur (Ray & Jena 2000, Boulares et al. 2002, Gujral et al. 2002, Kass et al. 2003). The elevation of caspase 3 and our histological studies are consistent with the occurrence of some apoptosis.

Similar to APAP-induced cell death, death by CCl_4 is bought about by a chain of complex events that begins with the activation of carbon tetrachloride by cytochrome CYP2E1, CYP2B1 or CYP2B2 and possibly CYP3A to form reactive radicals (Shi et al. 1998, Janakat & Al-Merie 2002, Weber et al. 2003, Taniguchi et al. 2004). Subsequent activation of cytokines such as tumour necrosis (TNF)-α, nitric oxide and transforming growth factor (TGF)-α and TGF-β and induction of interleukins (IL) such as IL-1, IL-6 and IL-10 play an important role in determining whether cells die by apoptosis or necrosis, or regenerate. In this regard, while this toxin can induce hepatocyte injury, effects on other tissues can occur. Our findings therefore do not exclude the possibility that some of the analytes in the blood result from death to other organs (e.g. the kidney).

In our prior studies assessing DNA as a marker of cell death, we infused either apoptotic or necrotic Jurkat cells into normal mice and observed a prompt blood response with both (Jiang et al. 2003b, Jiang & Pisetsky 2004, Jiang & Pisetsky 2005). Furthermore, with both types of cells, the DNA appearing in the blood showed low molecular weight, with a pattern consistent with cleavage to nucleosomes. The presence of low molecular weight DNA with necrotic cells is notable since necrosis does not involve the nucleases, with intracellular DNA in necrotic cells showing high molecular weight (Wyllie 1980, Wyllie et al. 1992, Jahr et al. 2001). The current findings suggest that DNA is also cleaved following necrosis induced *in vivo*. Our studies, however, do not identify the origin of the cleaving enzymes nor determine whether they operate following uptake of dead cells by macrophages or following the release of DNA into the blood where it could be digested by extracellular nucleases.

The studies presented extend our prior results on infused cells and, importantly, show the presence of low molecular weight DNA in blood when cell death is induced *in vivo* as opposed to *ex vivo*. Thus, for all three agents, levels of blood DNA increased

at the time of hepatocyte death, with DNA from all three treatments showing low weight bands of the size range of nucleosomes. In these experiments, CCl_4 and anti-Fas treatment produced the highest levels of DNA, with levels in fact similar although the pattern of death was different. In contrast, levels of DNA following treatment with APAP were in general lower than either of these other treatments. As such, these findings suggest that the size of DNA is not necessarily indicative of the mode of cell death.

In this model system of hepatic cell death, levels of caspase 3 in the blood showed a pattern different from than that of DNA. Thus, levels of caspase 3 were much higher in mice treated with anti-Fas than with either APAP or CCl_4 even though levels of DNA with anti-Fas and CCl_4 were similar. Indeed, as shown in Figure 8, levels of DNA and caspase 3 were closely related in mice treated with anti-Fas while mice treated with CCl_4 showed high levels of blood DNA in the presence of much lower amounts of circulating caspase 3. This result is consistent with apoptosis as the predominant mechanism of anti-Fas death and necrosis for APAP and CCl_4 death. Thus, to the extent the behaviour of hepatocytes is similar to that of other cells, a combination of DNA and caspase 3 blood levels could provide information relevant to the operation of apoptotic vs necrotic death mechanism. Unless an additional analyte (e.g. creatinine phosophokinase) is measured, however, these determinations would not distinguish the cell type or types dying.

In addition to measuring DNA and caspase 3 in the blood, we also measured LDH, which has been commonly used as marker of cellular injury and death (Decker & Lohmann-Matthes 1988, Lappalainen et al. 1994, Drent et al. 1996), although its presence is not specific for hepatic cells. Our data indicate that, whether the predominant mode of death was apoptosis or necrosis, plasma LDH levels are increased. Interestingly, the levels were similar with all three agents, although the time courses differed from that of DNA release in each case. Thus, LDH levels showed more sustained levels and were present at the time points (24 and 48 h) when the DNA levels have returned to baseline. Furthermore, in the case of anti-Fas treatment, LDH levels were present at a time (24 h) when both DNA and caspase 3 levels were down.

For DNA, caspase 3 and LDH and other molecules released during cell death or activation, levels depend on both the rate of extracellular release and the rate of clearance. Studies on LDH have indicated that this enzyme is cleared by a receptor-mediated process that is influenced by both genetic and environmental factors. Clearance involves macrophages and occurs over days (Hayashi et al. 1988, Hayashi & Notkins 1994). In contrast, DNA in the form of nucleosomes is rapidly cleared via Kupffer cells. For free DNA, clearance depends on strandedness, although it is rapid and occurs in minutes (Emlen & Mannik 1984, Gauthier et al. 1996, Du Clos et al. 1999). While we measured DNA in the blood by fluorimetry, it is likely that this molecule is bound to proteins, most probably in the form of nucleosomes. The clearance of this material would depend on the relative amounts of protein and nucleic acid which are unknown at this time. In this regard, it is possible that the form of the DNA (and hence its clearance) would differ depending upon whether the cell died by apoptosis or necrosis. The clearance of caspase 3 has not been well studied.

Together, these studies indicate that both apoptosis and necrosis culminate in the release of DNA into the blood from hepatocytes and potentially other cells dying in response to toxin exposure. While DNA can be readily assessed as a marker by

fluorometric assays, neither the amount of DNA nor its size in the blood allows distinction between death mechanisms. The similarity in DNA release into the blood with both death mechanisms is notable in view of the marked differences in cellular events in apoptosis and necrosis as well as the differences in the handling of apoptotic and necrotic cells by macrophages. Future studies are ongoing to assess DNA release in other forms of *in vivo* tissue injury and to increase the utility of DNA as a biomarker through analysis of associated protein components.

Acknowledgements

This research was supported by the VA Medical Research Service, National Institutes of Health grant AI44808 and the Lupus Research Institute.

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