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Generation of aberrant forms of DFF40 concurrent with caspase-3 activation during acute and chronic liver injury in rats

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Abstract

DNA fragmentation factors (DFF) form protein complexes consisting of nuclease DFF40/CAD and inhibitory chaperon DFF45/ICAD. Although activated caspase-3 has been shown to cleave DFF complexes with the release of active DFF40 and DNA fragmentation, the organ-specific mechanisms of DFF turnover during liver injury accompanied by massive apoptosis are unclear. In this study, we investigated hepatic profile of DFF40-immunopositive proteins in two models of liver injury in rats: acute ischemia/reperfusion (I/R) and chronic alcohol administration. We show that DFF40-like proteins occur in intact rat liver mainly as a 52 kDa protein. Hepatic I/R-induced caspase-3 activation and a time-dependent accumulation of DFF40-positive protein fragments (40 and 20 kDa), most likely via specific caspase-3 cleavage as evidenced by *in vitro* digestion of intact liver tissue with recombinant caspase-3. In addition, immunoprecipitation with DFF40 followed by Western blot with active caspase-3 antibody revealed the presence of active caspase-3 in DFF40-immunopositive 20 kDa proteins. Chronic alcohol administration in rats also resulted in a dose-dependent fragmentation of DFF40 proteins similar to I/R injury. Collectively, these data demonstrate that DFF40 immunopositive proteins exist in the liver as distinct, tissue-specific molecular forms that may be processed by caspase-3 during both acute and chronic liver injury.

Keywords: DNA fragmentation factors; Caspase-3; Liver injury; Ischemia/reperfusion; Alcohol

DNA fragmentation factors (DFF), also known as CAD/ICAD family proteins, occur normally in the cells as a complex consisting of DNAase DFF40 with its inhibitor DFF45 [1,2]. Active caspase-3, and possibly caspase-7, can cleave the DFF complexes upon induction of apoptosis through the extrinsic (death receptor) and/or intrinsic (mitochondria) pathways [3–5]. The mechanism of DFF activation and DNA cleavage is not well understood. It has been suggested that during progression of apoptosis, DFF complexes are cleaved into two fragments: DFF40 and DFF45 [6–8]. The cleaved DFF45 fragments dissociate from DFF40 allowing DFF40 to translocate into the nucleus, oligomerize to DNA and histone H1, and finally form a large functional complex that fragments DNA by introducing double strand breaks [2]. Therefore, DFF40-mediated DNA breakdown is one of the last steps in apoptosis execution and is a true hallmark of apoptosis [3].

It has been also reported that DFF45 can be further cleaved into 24, 22, and 12 kDa fragments by caspase-3, caspase-7, and cytochrome *c* in dATP-treated cytosol [3]. In addition, DFF45 is increased during brain ischemia/reperfusion revealing bands of 32 and 11 kDa [9]. It has been

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shown that CAD/DFF40 that mediates DNA breakdown is associated with nuclear alterations in ischemic neurons [10]. In contrast, molecular pathways processing DFF upon execution of cell death during acute and chronic liver injury have not been investigated.

The liver is a vital target for many toxins such as endotoxin, xenobiotics, drugs, and alcohol. The liver is also particularly susceptible to hypoxia and ischemia due to the low oxygen tension in the venous blood, which constitutes the majority of hepatic flow. Apoptosis plays an important role in pathogenesis of liver diseases [11,12], especially, in early stage of liver injury [12]. We hypothesized that DFF40-like proteins exhibit a high molecular heterogeneity and liver specificity, and may play a role in hepatic cell death during both acute and chronic liver injury.

In this study, we profiled the hepatic expression of DFF40 proteins in two rat models of liver injury both accompanied by extensive hepatic cell death: acute ischemia/reperfusion (I/R) and chronic alcohol administration. We show that during both acute and chronic damage to the liver, DFF40 is co-expressed with active caspase-3 predominantly with nuclear localization and is extensively cleaved to a fragment of 20 kDa in a time-dependent manner.

Experimental procedures and methods

Materials. Protein assay kits were obtained from Bio-Rad (Hercules, CA); Polyclonal anti-rabbit DFF40 antibody was purchased from eBioscience (San Diego, CA); active caspase-3 (Asp175) antibody was acquired from Cell Signaling (Beverly, MA); argininosuccinate synthase (ASS) monoclonal antibody was a product of BD Pharmingen. Protein A–agarose was obtained from Sigma (St. Louis, MO). Other chemicals and solvents used were of the highest analytic grade commercially available.

Rat ischemia/reperfusion injury. Experiments were performed using male of Sprague–Dawley rats weighing 250–300 g. Laparotomy was performed via a midline incision; ligamentous attachments of the liver were divided to the diaphragm and neighboring organs; the hepatoduodenal ligament was dissected to identify the common bile duct, portal branches, and hepatic artery. Ischemia in the median and left lobes was induced by clamping the proper hepatic artery, portal branches, and the common bile duct, 30 min of ischemia followed by 30 min, 1 h, or 3 h of reperfusion at which time the median and left lobes of the liver were excised. Sham control rats received the same anesthesia and surgical procedures performed on the experimental rats except I/R. Naïve control rats were anesthetized and their liver lobes were excised by simple surgical procedures. All animal research was performed according to University of Florida institutional guidelines which are in compliance with the National Institutes of Health guidelines. At the end of experiments, the liver was briefly perfused with cold PBS (no calcium, magnesium) and homogenized in RIPA buffer consisting of 20 mM Hepes, 1 mM EDTA, 2 mM EGTA, 150 mM NaCl, 0.1% SDS, 1.0% IGEPAI, 0.5% deoxycholic acid (pH 7.5) containing 1 mM PMSF, and protease inhibitor cocktail (Roche, Inc.).

Rat chronic alcoholic disease. Sprague–Dawley rats were kept on a nutritionally complete liquid diet for 28 weeks. The liquid diet consisted of ethanol (8–9.4% by volume) mixed with Boost high protein drink (Mead Johnson, Evansville, IN). Rats in the sucrose (control) group received an equivalent diet to their counterparts in the ethanol group. Average blood alcohol levels (BAL) of 150–175 mg/dl were achieved during this experiment. For the first 10 weeks, rats received a 36% alcohol or sucrose diet after which the alcohol/sucrose content was raised to 39%. The stock ethanol solution was prepared with 2 L of 95% ethanol and 1 L deionized water; and the stock sucrose solution with 1950 g dixie crystals sugar and deionized water to a total volume of 2250 mlg. At the end of the first week and then once a week during treatment, rats were sacrificed, liver was taken for analysis and processed as described above.

Purified caspase-3 digestion of liver extract. Total protein was extracted from naïve rat liver by a Triton X-100 method. The extraction buffer consisted of 20 mM Tris–HCl, 1 mM DTT, 5 mM EDTA, 5 mM EGTA, 150 mM NaCl, and 1% Triton X-100, pH 7.4 [13]. Naïve rat liver extract (50 μg of protein) was digested with or without 1 μg/μl of human caspasae-3 (Chemicon, Temecula, CA) in digestion buffer containing 100 mM Tris–HCl, 1 mM DTT and 5 mM EDTA, pH 7.4 at 37 °C for 4 h [13]. The digestion was stopped by the addition of SDS-containing sample buffer for PAGE. Samples were subjected to SDS–PAGE, transferred to PVDF membrane, and probed with the indicated antibodies (DFF40).

Purified calpain digestion of liver extract. Total protein was extracted from naïve rat liver by a Triton X-100 method as described above for caspase-3. Naïve rat liver extract (50 μg of protein) was digested with or without 0.25 μg/μl of porcine calpain-2 (Calbiochem, San Diego, CA) in digestion buffer containing 100 mM Tris–HCl, 1 mM DTT, and 10 mM $CaCl_2$ at room temperature for 30 min [14]. The digestion was halted by the addition of SDS-containing sample buffer for PAGE. Samples were subjected to SDS–PAGE, transferred to PVDF membrane, and probed with the indicated antibodies (DFF40).

Western blot analyses. Proteins (20–25 μg protein) were separated by SDS–PAGE and transferred to a nitrocellulose membrane. Membranes were incubated with the specific antibodies against DFF40 (1:1000) active Caspase-3 (1:1000), ASS (1:2000) followed by HRP-labeled secondary antibodies. The bands were visualized using enhanced chemiluminescence Plus reagents (ECL Plus, Amersham; Arlington Heights, IL) according to the manufacturer's instructions. Semi-quantitative, densitometric analysis was performed using ImageJ software (NIH, Bethesda, MD). Data were expressed as means ± SEM and compared by t test using PRISM software.

Immunoprecipitation. Protein A–agarose/Sepharose (Sigma, St. Louis, MO) beads were washed twice with PBS, restored to a 50% slurry with PBS, then incubated with DFF40 antibody (1:1) for 1 h at room temperature. Two hundred micrograms of cell extract protein was added to 400 μl of immunoprecipitation buffer and 20 μl of protein A–agarose beads/DFF40 antibody mixture. This mixture was gently mixed overnight at 4 °C on a rocker. The agarose/Sepharose A beads were collected by pulse centrifugation (5 s in the microcentrifuge at 14,000 rpm), the supernatant fraction was discarded, and beads were washed three times with 800 μl ice-cold modified RIPA buffer. Finally, immunoprecipitated proteins were subjected to SDS–PAGE protein separation followed by Western blot with DFF40, active caspase-3 or ASS as described above.

Protein assay. Protein concentrations in liver tissue and cell lysates were determined by bicinchoninic acid method using protein assay kits (Bio-Rad; Hercules, CA).

Statistical analysis. All experiments, except where stated otherwise, were performed four times ($n = 4$). Data were expressed as means ± SEM. Data were analyzed using a one-way analysis of variance (ANOVA) and individual-group-means were then compared with a t test. Differences were considered significant when P values were less than 0.05.

Results

DFF40-immunopositive proteins occur primarily as a 52 kDa entity in normal rat liver and may be cleaved to a 20 kDa fragment by active caspase-3 following I/R

In intact rat liver, DFF40-immunopositive proteins were migrated as a large 52 kDa protein band (DFF52) as indicated by Western blot analyses (Fig. 1A, upper panel). In sham-operated rat liver, DFF40 protein was found in both 52 and 25 kDa protein form (Fig. 1A). By contrast, in acute liver ischemia/reperfusion (I/R) injury, hepatic expression profile of DFF40 by Western blot analysis using a