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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 kDa 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. Liver immunohistochemistry showed an up-regulation of DFF40-immunopositive proteins with nuclear co-localization of DFF40 with histone H1 following acute I/R. 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

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Introduction

DNA fragmentation factors (DFF), also known as CAD/ICAD family proteins, occur normally in the cells as a complex consisting of DNA-ase 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 kDa, 22 kDa 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 kDa and 11 kDa [9]. It has been 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 to 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 250–300g. 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 minutes of ischemia followed by 30 minutes, 1 hour

or 3 hours 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. Naive 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 % IGEPAL, 0.5% Deoxycholic Acid, (pH 7.5) containing 1 mM PMSF and protease inhibitor cocktail (Roche, Inc). For immunohistochemistry, liver specimens were frozen in OCT using histobath and stored at -80°C until use.

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 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 mL. 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 naive rat liver by a Triton X-100 method. Naive rat liver extract (50 μg of protein) was digested with or without 1 $\mu\text{g}/\mu\text{l}$ of human caspase-3 (Chemicon, Temecula, CA) in digestion buffer including 1M Tris-HCl, 1 M DTT, and 100 mM CaCl_2 at 37°C for 4 hours. 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). The lysis buffer consisted of 20 mM Tris-HCl, 1 mM DTT, 5 mM EDTA, 5 mM EGTA, 150 mM NaCl, and 1% Triton 100, pH 7.4 [13].

Purified calpain digestion of liver extract

Total protein was extracted from naive rat liver by a Triton X-100 method. Naive rat liver extract (50 μg of protein) was digested with or without 0.25 $\mu\text{g}/\mu\text{l}$ of porcine calpain-2 (Calbiochem, San Diego, CA) in digestion buffer including 1M Tris-HCl, 1 M DTT, and 100 mM CaCl_2 at room temperature for 30 minutes. 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). The lysis buffer consisted of 20 mM Tris-HCl, 1 mM DTT, 5 mM EDTA, 5 mM EGTA, 150 mM NaCl, and 1% Triton 100, pH 7.4 [14].

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 mean $\pm$ 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 Anti-DFF 40 antibody (1:1) for 1 hour at room temperature. Two hundred micrograms of cell extract protein was added to 400 μ l of immunoprecipitation buffer and 20 μ l of protein A agarose beads/DFF-40 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 seconds in the microcentrifuge at 14,000 rpm), the supernatant fraction was discarded and beads were washed 3 times with 800 μ l 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.

Immunohistochemistry

Frozed liver samples were sectioned (5 μ m), placed on round glass cover slips, dried at 4°C for at least overnight and rinsed with cold PBS to remove excess of OCT. After fixation with 1 ml/well of -20°C methanol for 20 min on ice, samples were permeabilized with 1 ml 0.5 % Triton X-100 in 2% sucrose/PBS and blocked with 20 % of goat serum in PBS for 1 hour at room temperature. Samples were then incubated with primary rabbit polyclonal DFF40 antibody (1:100) in 20 % goat serum/PBS at 4 °C overnight. Primary antibodies were removed, the samples were thoroughly washed five times with cold PBS, at least 5 min each, and then incubated for 1 hour with anti-rabbit secondary antibody conjugated with AlexaFluor™ red (574 nm) at room temperature. After washing five times, the samples were incubated overnight with primary mouse monoclonal H1 antibody (1:100) in 20 % goat serum/PBS at 4° C. Slips were washed 5 times with PBS and incubated with secondary anti-mouse antibody conjugated with AlexaFluor™ green (488 nm) for 1 hour at room temperature. The cover slips were washed several times with PBS, dried and mounted on slides. The slides were analyzed using fluorescent microscope (Carl Zeiss, Inc) using appropriate filters.

Protein Assay

Protein concentrations in liver tissue and cell lysates were determined by bicine caninic acid method using protein assay kits (Biorad; Hercules, CA).

Statistical Analysis

All experiments, except where stated otherwise, were performed 4 times (n=4). Data was expressed as mean $\pm$ SEM. Data was 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 kDa 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 polyclonal DFF40 antibody revealed DFF40-

immunopositive bands at 52 kDa, 40 kDa, 25 kDa, and 20 kDa (Fig. 1 A). Concurrently, there was an increase in active caspase-3 immunoreactivity following I/R that correlates with the appearance of these fragments (Fig. 1A, lower panel).

To determine whether the generation of these fragments (40 kDa and 20 kDa) was due to a cleavage by active caspase-3, we employed *in vitro* caspase-3 and calpain-2 treatment of normal liver tissue. Liver tissue lysates treated with active caspase-3 exhibited additional bands at 25 kDa and 20 kDa, while those treated with calpain showed only the 25 kDa band (Fig. 1B). These data support the idea that the appearance of the 20 kDa fragment may be the result of active caspase-3 cleavage of DFF40. Densitometric measurement of DFF20 revealed a significant accumulation of this protein *in vivo* under I/R condition (Fig. 1C) and *in vitro* in caspase-3 treated liver tissue (Fig. 1D). No significant differences were observed in the levels of DFF52 under either condition. Collectively, these data suggest that caspase-3 activation contributes to the formation of DFF20, possibly by cleavage of DFF52.

Temporal profile of DFF40 proteins in rat liver following I/R

To determine the temporal profile of DFF20 and active caspase-3 accumulation following I/R injury, three time points were used; 30 minutes of ischemia followed by either 30 minutes, 1 hour or 3 hours of reperfusion. Western blot analysis revealed DFF40-immunopositive bands at 52 kDa, 40 kDa, 25 kDa and 20 kDa in rat liver tissue (Fig. 2). As reperfusion time was extended, hepatic I/R elicited an increase in the DFF40-positive 40 kDa and 20 kDa fragments starting at 30 minutes of reperfusion with concomitant accumulation of active caspase 3 (17 kDa and 19 kDa). These data further support a possible role for caspase-3 in the formation of DFF40-positive fragments but do not rule out the involvement of other proteases early in the hepatic apoptosis.

DFF40 proteins interact with caspase-3 in rat liver following I/R

To determine the possible interaction between DFF40-like proteins and active caspase-3, liver tissue samples were immunoprecipitated with a polyclonal anti-DFF40 antibody and analyzed by Western blotting using antibodies against DFF40 and active caspase-3. Immunoprecipitation and western blotting using anti-rabbit DFF40 antibody for naïve, sham-operated, and I/R samples (Fig. 3A) revealed a strong DFF20 bands in both I/R samples, with or without immunoprecipitation. A very faint DFF20 band was also seen in sham-operated liver but no band was present in naïve tissue (Fig. 3A).

Active caspase-3 immunostaining of DFF40-immunoprecipitated proteins revealed a strong immunoreactivity in 17 to 20 kDa bands. In contrast, no ASS-positive immunoreactivity was observed in DFF40-precipitated samples. These data suggest that DFF40-like proteins can form complexes with caspase-3 with the concomitant cleavage, activation of DFF40 and further execution of apoptosis.

DFF20 increases in the rat liver following chronic alcohol treatment

To determine if DFF-like proteins also play a role in liver injury caused by chronic alcohol treatment, we examined the hepatic profile of DFF40-like proteins using a chronic alcohol model. As shown in Fig. 4, DFF52 is ubiquitously expressed in naïve and alcohol conditions (A1 and A2), whereas DFF40 and DFF20 were only expressed following alcohol treatment. Similarly, the active forms of caspase-3 (17 kDa and 19 kDa) increased following alcohol treatment but not in naïve tissues (Fig. 4). These results suggest that liver injury due to alcohol abuse, similarly to I/R, may be mediated by caspase-3 activation and DFF52 cleavage.

Discussion

The principal findings of the present study demonstrate for the first time that DFF40-like proteins exist in the intact rat liver mainly as a 52 kDa protein. Both acute liver injury caused by ischemia/reperfusion (I/R) and chronic alcohol-induced hepatic damage were accompanied by accumulation of DFF40-positive protein fragments of 40 kDa, 25 kDa and 20 kDa. Of these, the DFF20 protein fragment, appears to be produced by active caspase-3 and increases in a dose-dependent and time-dependent manner following liver injury.

DFF40 is a nuclease with a full-length of 338 amino acid (aa) residues, while its inhibitor DFF45 is a protein with full-length of 331 aa residues. Folding of both proteins adjusts the formation of the DFF40/DFF45 compound [16]. It has been shown that inhibitory subunit (DFF45) can be cleaved by caspase-3 to 30 kDa and 11 kDa fragments during brain ischemia [9], and to 22–24 kDa and 12 kDa by caspase-3 and caspase-7 in Jurkat and MCF7 cell extracts [10]. We observed similar results: caspase-3 cleaved DFF45 to 28 kDa and 25 kDa in liver tissue (data not shown). The free nuclease is able to participate in both high molecular weight and nucleosomal apoptotic DNA fragmentation. The complexes produced by DFF40 are very large and principally occur in cells undergoing apoptosis [17].

Western blot analysis using anti-rabbit DFF40 antibody recognized the bands at 52 kDa in the liver of normal, sham-operated and acutely and chronically injured rats (Fig. 1–3). This suggests that DFF-like protein 52 kDa may be a super-molecular complex of DFF40 with a fragment of DFF45, thereby representing a liver-specific form of DNA fragmentation factor. We have shown the appearance of 25 kDa and 20 kDa DFF40-positive bands during both acute and chronic liver injury. (Fig. 1–3). The expression of 20 kDa band was significantly stronger than the 25 kDa band (Fig. 1 and 2). In contrast, treatment of intact liver tissue with recombinant caspase-3 *in vitro* for 4 hours produced predominantly 25 kDa DFF40-positive band (Fig. 1B), while the accumulation of 20 kDa band was minor. A prominent hepatic 25 kDa band, (but not 20 kDa) of DFF40, also appeared in sham-operated rats (Fig. 1A). Taken together, these data suggest two possibilities. The first one is that the 25 kDa fragment is the primary cleavage fragment by caspase-3, which can be further converted to 20 kDa fragment by other protease(s) *in vivo* during acute or chronic liver injury. The second possible explanation may be that DFF52 is cleaved upon hepatic injury via other, as yet identified protease(s) directly to 20 kDa fragment. It was reported that DFF40/CAD is resistant to proteolysis [3,18], and that the 40 kDa subunit of DFF is not easily cleaved by caspase-3 *in vitro* using MCF7 extracts [3], but DFF40 could be cleaved to a 35 kDa species by Granzyme B [3]. The patterns of DFF40 and DFF45 in the liver in normal and pathological conditions have not been studied previously. Therefore, the observed difference in hepatic DFF proteins turnover can be explained by cell/tissue-specific profile of DFF-immunopositive proteins involved in apoptosis during liver damage.

Moreover, we performed immunoprecipitation with DFF40 antibody followed by western blot with DFF40, active caspase-3, and argininosuccinate synthase (ASS) as a liver specific control (Fig. 3). These data showed a significant 20 kDa band only in liver injury samples. A prominent immunoreactivity at 52 kDa, 40 kDa and 20–17 kDa corresponding to DFF52, DFF40, DFF20 and two active forms of caspase-3, 17 kDa and 19 kDa, was detected by immunoprecipitation with DFF40 and cross-western blot with DFF40 and caspase-3 (Fig. 3 A and B), but not with ASS-specific antibody (Fig. 3 C). These data strongly suggest that (i) hepatic accumulation of DFF20 is due to liver injury, (ii) activation of caspase-3 is necessary for DFF20 kDa release, and (iii) caspase-3 can physically associate with DFF40-like proteins and promote their activation. Although both fragments (20 kDa and 25 kDa) appeared in the alcohol abuse model and under I/R treatment conditions, the hepatic expression of the 20 kDa band was more pronounced. The 20 kDa fragment appears to be

produced specifically by active caspase-3 and may represent a more terminal signal of apoptosis as opposed to the 25 kDa band which may be a very early signal of liver injury.

Castilla R. *et al* reported that alcohol increased dose-dependently DNA fragmentation, iodide propidium-DNA staining, caspase-3 activity and annexin V binding in human and rat hepatocytes [19]. Thus, our data in conjunction with these and other observations [19, 20] suggest that similar terminal apoptotic pathways operate during both acute I/R liver injury and chronic alcoholic liver disease.

In conclusion, we found that DFF40-like proteins occur in the liver mainly as 52 kDa proteins, which are slightly up-regulated with concomitant apoptotic cleavage during both acute and chronic liver injury. Accumulation of DFF40-positive 40 kDa, 25 kDa and 20 kDa fragments was dependent on caspase-3 activation as indicated by both in vivo and in vitro experiments. Detailed mechanism of DFF40-induced DNA fragmentation, and particularly the roles for 20 kDa and 25 kDa of DFF40-like proteins remains to be investigated.

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Fig 1. Expression of DFF40 proteins in rat liver following hepatic ischemia/reperfusion (I/R) Liver tissues were obtained from untreated rats (Naïve, N), sham operated animals (S), or rats subjected to 30 min of total hepatobiliary triade occlusion (Pringle maneuver) followed by 30 min reperfusion (I/R). **A:** Proteins were separated by SDS-PAGE and immunoblotted with rabbit DFF40 antibody (1:1000, upper panel) as described in detail in Materials and Methods. The membranes were stripped and re-probed with rabbit cleaved caspase-3 antibody (lower panel). The arrows indicate 52 kDa, 40 kDa, 25 kDa and 20 kDa of DFF40-positive proteins, and 19 kDa and 17 kDa of cleaved caspase-3 positive proteins. Representative western blot of DFF and active caspase-3 out from 4 performed is shown. **B:** Rat liver tissue from naïve rats was treated in vitro with recombinant caspase-3 for 3 hours (Cas), calpain-2 for 30 min (Cal) or buffer (N) as described in Materials and Methods in detail. Proteins were separated by SDS-PAGE and subjected to immunoblotting with rabbit DFF40 antibody (1:1000). The arrow indicates 52 kDa, 40 kDa, 25 kDa and 20 kDa of DFF40-like proteins. Representative western blot of DFF out from 4 performed is shown. **C:** Densitometry analysis of 20 kDa and 52 kDa DFF-immunopositive bands was performed using ImageJ software. Data were expressed as mean $\pm$ SEM and differences analyzed using t-test in GraphPad software. **D:** Densitometry analysis of 20 kDa and 52 kDa DFF-

immunopositive bands was performed liver tissue treated in vitro with recombinant caspase-3 (Cas) or calpain-2 (Cal). Data are expressed as mean $\pm$ SEM and compared using t-test in GraphPad software.

Fig 2. Time-course of hepatic DFF40 proteins and active caspase-3 expression following I/R in rat liver

Liver tissues were obtained from naive rats (N), sham operated animals (S), or rats subjected to 30 min of total hepatic ischemia followed by 30 minutes, 1 hour and 3 hours reperfusion (I/R). **A:** Proteins were separated by SDS-PAGE and probed with rabbit DFF40 antibody (1:1000, upper panel). Membranes were stripped and re-probed with rabbit cleaved caspase-3 antibody lower panel). The arrows indicate 52 kDa, 40 kDa, 25 kDa and 20 kDa of DFF40-immunopositive proteins, and 19 kDa and 17 kDa forms of cleaved caspase-3. Representative western blot out from 4 performed is shown. **B:** Densitometry analysis of 20 kDa and 40 kDa DFF-immunopositive bands, and 17 kDa & 19 kDa of caspase-3 was performed using ImageJ software.

Fig 3. DFF40 immunoprecipitation combined with Western blot analysis following I/R in rat liver

Liver tissues were obtained from naive rats (N), sham operated animals (S), or rats subjected to 30 min of total ischemia followed by 30 min reperfusion (I/R). **A:** Hepatic proteins were immunoprecipitated with DFF-40 antibody as described in Materials and Methods in detail. Proteins were separated by SDS-PAGE and probed with rabbit DFF40 antibody (1:1000, upper panel). Membranes were stripped and re-probed with rabbit cleaved caspase-3 antibody (1:1000, lower panel). Representative western blot of DFF out from 3 performed is shown. **B:** DFF40-immunoprecipitated hepatic proteins were separated by SDS-PAGE and probed with mouse monoclonal antibody against hepatic ASS protein (1:1000). The arrows indicate approximately 46 kDa of ASS protein.

Fig 4. Western blot analysis of the DFF40-immunopositive proteins in rat liver following chronic alcohol treatment

Liver tissues were obtained from rats subjected to chronic alcohol administration for 28 weeks as described in materials and Methods and age-matched controls. Proteins were separated by SDS-PAGE and probed with DFF40 antibody (1:1000). Membranes were stripped and re-probed with rabbit active caspase-3 antibody (1:1000). The data are shown from two different rats chronically treated with alcohol (A1 and A2) out of total 6 alcohol rats.