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Modulation of Fas-FasL related apoptosis by PBN in the early phases of choline deficient diet-mediated hepatocarcinogenesis in rats

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Abstract
This study focused on the detection of apoptosis related events in very early phases of choline-deficient (CD)-induced hepatocarcinogenesis (at 2–5 weeks). Flow cytometry of isolated intact primary hepatocytes from CD diet fed rats indicated increased expression of the apoptosis-associated protein Fas. Increased apoptosis in CD-treated livers was confirmed by Western blot analyses of caspases and cytochrome c. This study was also able to detect differences in apoptotic events following phenyl butyl nitrone (PBN) treatment. Fas expression was inhibited by PBN, indicating that PBN is anti-apoptotic. It is speculated that in the early stages of CD-induced hepatotoxicity, PBN is involved in inhibiting pro-inflammatory factor-driven apoptosis of normal hepatocytes, which protects against the initiation of carcinogenesis. The CD diet model is also considered as a model for non-alcoholic steatohepatitis (NASH) in humans and early expression of Fas could also be a good index of the progression of NASH.

Keywords: *Choline-deficient diet, hepatocarcinogenesis, apoptosis, phenyl butyl nitrone (PBN), rats*

Introduction

Long-term feeding of a choline-deficient (CD) diet has been shown to cause the development of hepatocellular carcinomas in a rat model [1]. In this model, 8-hydoxydeoxyguanosine (8-OHdG) levels in rat liver DNA were significantly increased at the early stages of feeding, suggesting that this diet generates intracellular oxidative damage [2]. In addition, numerous other changes at the molecular level have been shown to occur. Because choline is a major methyl group donor for the biosynthesis of methyl group-bearing molecules, this naturally results in a feeding period-dependent global loss of 5-methylcytosine in liver DNA [3,4]. The loss in 5-methylcytosine is believed to be due to elevated mRNA of proto-oncogenes, including c-myc [5], c-Ha-ras [6] and c-fos [7]. Also, in previous studies, CD diet fed rats were found to induce caspase activation [8], cyclooxygenase 2 (COX2) expression [9], serum TNF-α elevation [10], hepatocyte transformation [11], protein kinase C activation [12], oxidative DNA damage [13] and the activation of p53 and the Nuclear Factor kappaB

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(NF-κB) [14]. However, the relationship between these changes and carcinogenesis mechanisms is not well understood [15].

A more potent hepato-carcinogenicity was found in rats fed a modified CD diet, the choline deficient L-amino acid-defined (CDAA) diet [15]. Following CDAA diet consumption for 1 year, more than 90% of rats form hepatocellular carcinomas (HCC), while only 30% of rats develop HCC with the conventional Lombardi's CD diet [9]. The difference in the ingredients in these two diets is that the milk protein in the CD diet is substituted by a mixture of amino acids in the CDAA diet. The CDAA diet is rich in free L-arginine which is a substrate for nitric oxide (NO) synthase and thus NO is detected in liver cells isolated from CDAA diet fed rats [16]. NO is believed to play either a cancer-promotional or -preventive role depending on its production level [17–20]. It is speculated that excess NO production in the liver of CDAA diet fed rats may be the reason for enhanced hepatocarcinogenesis and tumour development in this model.

In a rat CDAA diet model, several chemical reagents including anti-inflammatory agents and antioxidants have been shown to possess cancer-preventive capacity [21]. The reagent that showed the most potent cancer-preventive action in this model is a synthetic antioxidant and free radical trapping compound, phenyl N-*tert*-butyl nitrone (PBN). Co-feeding of PBN or its hydroxylated analogues (less than 0.03% of a total food or water weight) with a CDAA diet for more than 1 year almost completely inhibited HCC formation in rats [22,23]. In the early stages of feeding, such as at 16 weeks, PBN was found to prevent against pre-cancerous changes, by suppressing the size of the pre-neoplastic legions in rat livers [9]. In the past, a variety of pharmacologic activities of PBN in animal models of inflammatory disease has been reported, including the prevention of endotoxin-mediated death, neuro-protetive and anti-diabetogenic actions, and the extension of life-span [24,25]. At the molecular level, PBN has been shown to inhibit NF-κB and activator protein-1 (AP-1) activation, consequently down-regulating the expression of various pro-inflammatory cytokines and inducible nitric oxide synthase (iNOS) [26–28]. In *in vitro* and *in vivo* rat neuro-degeneration models, PBN was found to inhibit p-38 mitogen-activated protein kinase (p38) activation [25,29] and stress-activated protein kinase/c-Jun N-terminal protein kinase (SAPK/JNK) activation [29] and also promote extracellular-response kinase (ERK) activation [29]. In CDAA diet fed rats at 12 weeks, cyclo-oxygenase2 (COX2) activity was found to be down-regulated by PBN treatment [9].

Other important changes detected in the livers of PBN-treated CDAA diet fed rats included the modulation of apoptotic processes. Apoptotic changes in hepatocytes in CDAA rats (16 weeks) have been reported and PBN treatment was found to enhance apoptosis in the pre-neoplastic lesion cells in these studies [30]. Therefore, PBN can have an influence on apoptosis, inflammation and malignancy. In the very early stages of CDAA diet feeding (less than 2 weeks), severe fatty degeneration is readily recognized even by visual inspection. In the human disease, non-alcoholic steatohepatitis (NASH) is known to progress into tumour formation after chronic fatty liver and, thus, the CDAA model is considered to be a good model for NASH. In human steatohepatitis, some investigators have indicated that Fas (CD95/APO-1) and/or tumour necrosis factor receptor 1 (TNF-R1)-related apoptosis occurs [31–33]. Also, apoptosis was found to be induced by reactive oxygen species (ROS) resulting from fatty degeneration [34].

We hypothesize that the CDAA diet induces apoptosis, especially in the early phases, through the expression of apoptosis-related cell-surface proteins and that PBN is able to inhibit the expression of these proteins. Microscopic inspection indicates that there is an obvious macroscopic distinction between CDAA livers and CDAA plus PBN treated livers at very early stages of feeding (2–5 weeks). In order to detect the cell-surface specific expression of apoptosis-associated proteins such as Fas, we isolated mixed liver cells from the livers of rats fed a CDAA diet, for a specified period, using *in situ* collagenase digestion, which was followed by flow cytometry detection with a fluorescence-labelled antibody to Fas. We also determined the levels of apoptosis-related proteins in bulk liver using Western blot analyses. As a result, we found a dramatic difference in the effect of PBN on apoptosis when comparing 2–5 week and 16 week feeding protocols, i.e. at 2–5 weeks, PBN was found to be anti-apoptotic and at 16 weeks it acts as a pro-apoptotic agent.

Materials and methods

Animals

Animal experiments were performed by strictly following the animal use protocol that had been approved by the Institutional Animal Care and Use Committee (IACUC) of the Oklahoma Medical Research Foundation. Male Fisher rats were purchased from Harlan (Indianapolis, IN) and maintained in the Oklahoma Medical Research Foundation Laboratory Animal Resource Center (LARC). CSAA (choline sufficient, amino acid defined) and CDAA diets were purchased from Dyets Inc (Bethlehem, PA). PBN was synthesized and purified in our laboratory. Rats were given PBN through the drinking water which containing 0.130% PBN. PBN was synthesized and purified to 99% purity by vacuum sublimation while checking the melting point (71–75°C) [35].

Histological analysis

Liver tissues were fixed in 10% buffered formalin and embedded in paraffin wax. Sections were cut to a thickness of 5 μm using a RJ microtone, stained with haematoxylin and eosin, and microscopy images were recorded on a Nikon Eclipse E200 microscope.

Isolation of rat hepatocytes

The rats' hepatocytes were isolated using a method described by Nagaoka et al. [36] and Miyazaki et al. [37] with slight modifications. After the rats were anaesthetized with 2.5% isoflurane, 500 USP units of heparin sodium (American Pharmacentical Partners Inc, Schaumburg, IL) were injected through the external jugular vein. An incision was performed on the ventral midline and the viscus was displaced to reveal the inferior vena cava and the portal vein. The vena cava posterior to the renal veins and proximal to the portal vein was gently tied with a surgical thread. The portal vein was cannulated and perfused with a PBS buffer at a rate of 10 ml/min for 5 min. The inferior vena cava around the renal vein was sectioned and a thoracotomy was performed. The inferior vena cava was cannulated from the right atrium and the inferior vena cava around the renal vein was tied and clamped. After perfusion with PBS, the liver was perfused with a collagenase buffer (Hanks, 3.7 mm $CaCl_2$, 4 mm $NaHCO_3$, Collagenase 0.5 g/L (Wako, Osaka, Japan), a trypsin inhibitor 0.1 g/L (Sigma, St. Louis, MO), pH 7.5 adjusted with 1 N NaOH) at a rate of 17 ml/min for 10 min. The liver was removed onto a dish containing PBS and gently diced. The liver cells were mechanically dispersed and filtered though a 60 μm mesh filter. Hepatocytes were isolated by centrifugation ($50 \times g$) for 2 min twice at 4°C. The cells were counted and viability was determined using a trypan blue-exclusion method (0.02% final concentration). Hepatocytes with viability greater than 70% were used for the following experiments.

Flow cytometry analysis

The cells were harvested and incubated on ice for 30 min with a monoclonal antibody for Fas or Fas-L (MBL, Nagoya, Japan). Cells were washed with PBS, centrifuged ($200 \times g$ for 3 min) and re-suspended in PBS containing the secondary antibody FITC-F (ab') 2 fragment of a goat anti-mouse IgG or a FITC-goat anti-rat IgG (control). Flow cytometry analyses were performed in a FACS caliber (BD Biosciences, San Diego, CA). Dot plots (forward-scattering vs side-scattering plots) were first obtained and hepatocytes were selected based on size and subsequently fluorescence histograms were recorded. The data were analysed with Cell Quest software (BD Biosciences, San Diego, CA).

Western bolt analysis

Rat livers were perfused with a PBS buffer for 5 min (10 ml/min) and kept frozen at −80°C until used. Frozen liver tissues were sliced into thin pieces using a razor blade and thawed in a lysis buffer containing an EDTA free Halt TM Protease Inhibitor Cocktail (PIERCE, Rockford, IL). Tissue was further disrupted and homogenized at 4°C, incubated on ice, transferred to a micro-centrifuge tube and centrifuged ($10\,000 \times g$, 5 min, 4°C) twice. The supernatant fluid was the total cell lysate. The total protein concentration was measured using a Quant-it protein assay kit, with BSA as a standard (Invitrogen, Carlsbad, CA). The protein was separated on a 12% Tris-HCl SDS-PAGE gel (Bio-Rad, Emeryville, CA) with a 0.25 m Trizma base containing 1.92 m Glycine and 1% SDS, and transferred to nitrocellulose membranes (Bio-Rad, Emeryville, CA) with a 0.25 m Trizma base containing 1.92 m glycine and 20% methanol. The membrane was blocked in 5% non-fat milk for 1 h at room temperature and incubated overnight at 4°C with the previously indicated primary antibodies. After hybridization with primary antibodies, the membrane was washed with TBS-T (Tris buffered saline Tween-20). Western analyses was performed using primary antibodies against caspase-3 ($\times 100$ dilution), caspase-8 ($\times 100$ dilution), caspase-9 ($\times 1000$ dilution) and cytochrome *c* ($\times 1000$ dilution) (Cell Signaling Inc, Danvers, MA) and β-actin ($\times 1000$ dilution) (Abcam, Cambridge, MA). Secondary antibodies were labelled with a peroxidase conjugated anti-rabbit antibody (Amersham Biosciences, Piscataway, NJ) or a HRP conjugated donkey anti-mouse antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA). The ECL Advance Western Blotting Detection Kit (Amersham Biosciences, Piscataway, NJ) was used to detect immunoreactive proteins. A positive control was obtained from the cytosolic extract of 2 μm camptothecin treated Jurkat cells (BioVision Research Products, Mountain View, CA). Ratios of the densities of the β-actin band, from each individual gel, to individual proteins (cleaved caspase 8, pro-caspase 9, cleaved caspase 9 (37 kDa and 35 kDa), cytochrome c and cleaved caspase 3) were obtained by measuring band densities using the NIH ImageJ software program (National Institutes of Health, Bethesda, MD).

Results

Microscopic comparison between CDAA livers with CDAA plus PBN treated livers in early phases

Figure 1 shows microscopic images of liver sections obtained from rats fed CDAA or CSAA diets with or without PBN for 10 days. In CDAA livers (Figure 1B), the appearance of numerous fatty cells, nuclear

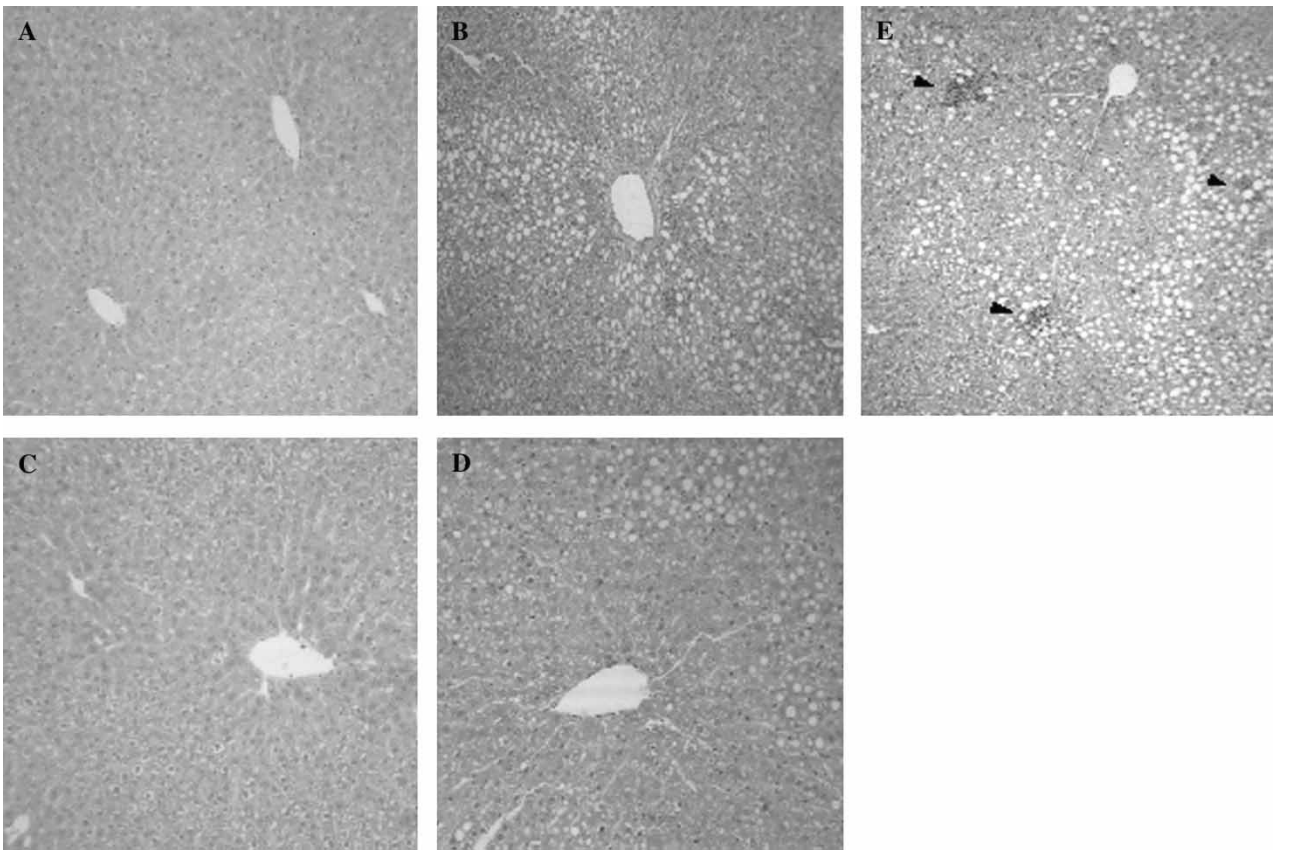

Figure 1. Haematoxylin and eosin staining of livers of rats fed CSAA (A), CDAA (B), CSAA+PBN (C) and CDAA+PBN (D) diets at day 10. CDAA livers (B) depict severe fatty degeneration and scattered inflammatory cells (indicated with arrows; panel E) as compared with other treatment samples. Livers of PBN treated CDAA diets fed rats indicate less fatty changes and the presence of no inflammatory cells (D). Magnifications are ×100.

shift and deformation was clear as compared to CSAA livers (Figure 1A). In some sections of CDAA livers there were scattered inflammatory cells (Figure 1E). There was a decrease in the appearance of inflammatory cells and nuclear changes with PBN treatment, but still the appearance of fatty cells (Figure 1C and D).

Expression of Fas in CDAA diet fed rats

We measured Fas expression on the hepatocytes isolated from rats fed CSAA, CDAA, CSAA+PBN and CDAA+PBN diets for various periods (2–5 weeks). By using size selection with forward/side-scattering, we obtained fluorescence histograms from fairly homogeneous hepatocytes. From CSAA, CDAA, CSAA+PBN and CDAA+PBN livers at 2 weeks, Fas and Fas-L expression was not detected (Figure 2A). However, in 4-week livers there was a high level expression of Fas, but not Fas-L (Figure 2B). Fas expression increased as a function of feeding time (Figure 3), but there was no Fas-L expression for any feeding period. Fas expression was inhibited in CDAA+PBN livers (Figures 2 and 3).

Fas and Fas-L related apoptosis in CDAA fed rats

In order to confirm that expression of Fas on hepatocytes leads to a Fas mediated cell death, we analysed caspase-8, pro- and cleaved-caspase-9, cytochrome *c* and caspase-3 as apoptosis related proteins with Western blotting. Cleaved caspase-8, as the initiator caspase, was detected in CDAA fed rats at 4 weeks (Figure 4). It was found to decrease in liver tissues from CDAA+PBN fed rats (Figure 4). Cleaved caspase-3, as an effector caspase, showed a similar tendency, although not as much as detected for cleaved-caspase-8. Cytochrome *c* and cleaved caspase-9 (37 kDa and 35 kDa) were detected in liver tissues from CDAA fed rats at 4 weeks and were also slightly decreased in CDAA+PBN livers.

Discussion

The primary objective of this study was to detect the expression of apoptosis-related proteins in very early stages (2–5 weeks) of carcinogenesis associated with CDAA-diet feeding in rats. CD diet induced hepatocarcinogenesis is a unique model in which no known carcinogen is involved. In previous studies, the appearance of diffuse fatty liver was reported within

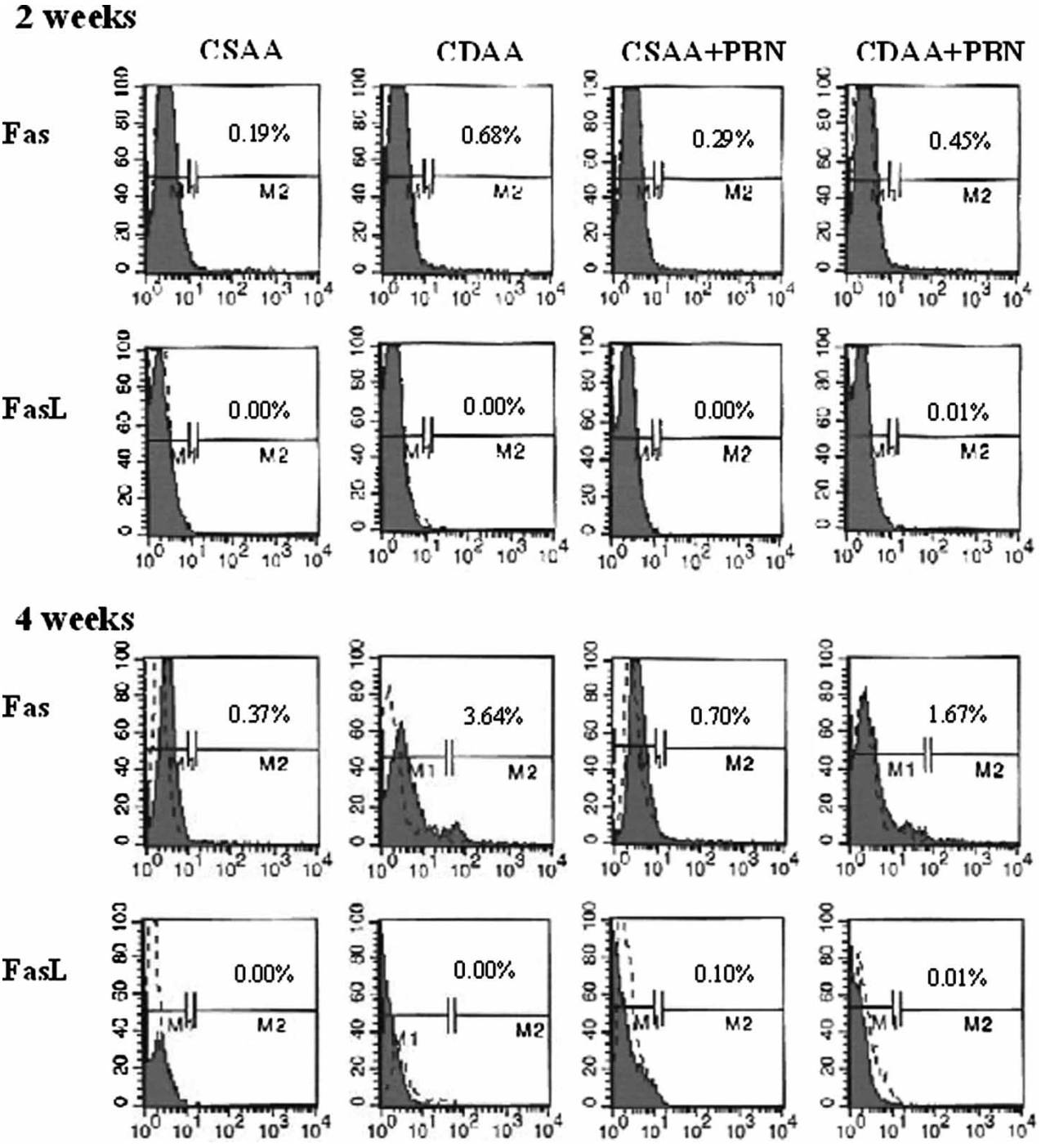

Figure 2. Flow cytometry fluorograms of hepatocytes isolated from livers of rats fed CSAA, CDAA, CSAA+PBN and CDAA+PBN diets for 2 and 4 weeks. Cells were immunolabelled with a fluorophor-conjugated Fas or Fas-L antibody. A shift of the Fas peak to right hand side, as compared with IgG labelled or non-stained cells (negative control), indicates expression of Fas. The *Y*-axis represents the cell number stained with mAbs and the *X*-axis represents logarithmic scale of fluorescence intensity.

1 week after CDAA diet feeding. After 2 weeks, a significant increment of regenerative hepatocyte proliferation was detected [15], but it was only after a 4-week feeding that a significant number of apoptotic cells were detected with immuno-histochemistry using an anti-single-stranded DNA (anti-ssDNA) antibody [15]. Liver cirrhosis was present after feeding for 12 weeks [15] and GST-P positive lesions also appeared at this time. Although the appearance of apoptotic cells at 4 weeks after CDAA diet feeding was reported, the authors found that the appearance of fat cells preceded the appearance of apoptotic cells, suggesting that steatosis and apoptosis are sequential but not simultaneous events.

Our present observations using primary heaptocytes obtained from rats fed a CDAA diet for 2–5 weeks do not suggest that steatosis and apoptosis are sequential events. Histological examination of a

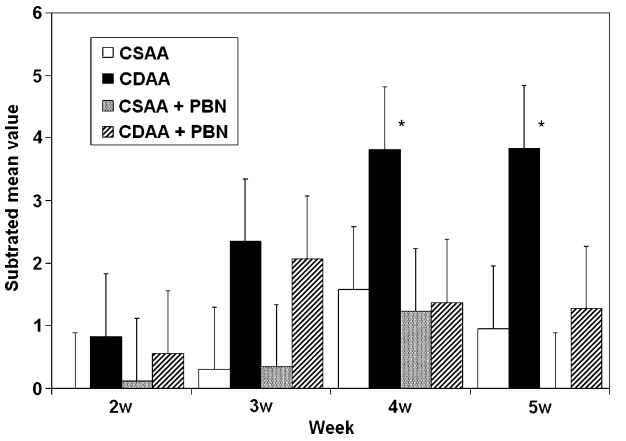

Figure 3. A time course of Fas surface expression in hepatocytes isolated from CSAA, CDAA, CSAA+PBN and CDAA+PBN fed rats. Mean values were calculated by subtracting the mean of the negative control (isotype control) value from the mean of Fas fluorescence intensity ($n=2$). Error values are indicated as SE ($p<0.01$ for columns marked with an asterisk).

10-day CDAA liver depicted many fatty cells, as well as cells with a nuclear shift and deformation, and macroscopic signs of apoptosis (Figure 1). Although microscopic examination of cell shape gives only a qualitative measure of the apoptotic event, we were able to also observe molecular events. The expression of the cell surface molecular marker of apoptosis, Fas, was slightly increased at week 3 and very clear at weeks 4 and 5 in the CDAA treated hepatocytes (Figures 2 and 3). The role of Fas in cancer has been investigated and, for instance in thyroid carcinoma cells *in vitro*, Fas signalling was shown to lead to apoptosis and then proliferation [38]. In the same study, it was demonstrated that deactivation of Fas affects promoted mitogen-activated protein (MAP) kinase, ERK kinase, NF-κB and AP-1 pathways, whereas it stimulated proliferation in thyroid carcinoma cells *in vitro* and protected cells from apoptosis [38]. A mutation in the death domain (DD) within the cytoplasmic tail of Fas has been shown to abrogate induction of apoptosis [39]. Stimulation of Fas has been shown to result in the activation of the transcription factor, NF-κB and MAP kinase [40,41].

Regarding Fas expression, two types of Fas-signalling pathways have been proposed, both of which were implicated in carcinogenesis; (1) Fas signalling activates caspase-8 and directly promotes caspase-3 (immune cells) [42]; and (2) Fas signalling activates apoptosis related proteins in the mitochondria via caspase-8 cleavage and promotes caspase-3 (hepatocytes) [43]. For instance, it has been shown that the addition of peptide caspase inhibitors to cells blocks Fas-induced apoptosis, which indicates that caspases are mediators of Fas-induced apoptosis [42]. In our data, cleaved caspase-8 and cleaved caspase-3 were detected in CDAA fed rats and these proteins were inhibited by PBN (Figure 4). Caspase-9 and cytochrome *c* were detected in CDAA fed rats and were slightly decreased by PBN co-feeding. In a NASH model, Feldstein et al. [31] reported that Fas signalling promotes caspase-8; entering the mitochondria and finally evoking apoptosis. Mitochondrial dysfunction is suggested to generate reactive oxygen species, activating the caspase cascade and apoptosis. This assumption is consistent with our Western blot results and PBN may play a role in decreasing reactive oxygen species and inhibiting apoptosis.

The effect of the free radical trapping compound PBN on apoptosis in 2–5 weeks CDAA livers was different than what was detected in 16-week CDAA livers previously. As shown in Figures 2 and 3, PBN co-feeding inhibits cell-surface expression of Fas at early stages of CD-induced hepatotoxicity. A previous report indicated that 16-week PBN-CDAA fed rats had enhanced apoptosis in the liver, by using double staining with GST-P immunostaining and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labelling (TUNEL) [30], suggesting that PBN's apoptosis-promoting activity can prevent neoplastic changes in cells at later stages of carcinogenesis. It should be noted that in the 16-week study PBN was administered via the diet, not the drinking water as done in our study, however comparisons conducted in our laboratories between administration of PBN in the food or the drinking water has not resulted in any significant differences in the anti-cancer activity of PBN (unpublished data). PBN seems to have a protective role against apoptosis in stressed normal cells, including early stages of CD-induced hepatotoxicity, whereas it takes on a pro-apoptotic role against transformed neoplastic cells during later stages of hepatocarcinogenesis. Indeed, a CDAA diet in rats has been shown to cause persistent oxidative stress in the liver as evidenced by an increase in 8-hydroxygunanosine (8-OHdG) levels in DNA and 2-thiobarbituric acid–reacting substances (TBARS) as early as 1–3 days after feeding [15]. In very early phases of feeding, these oxidative products may cause the attraction of inflammatory cells (Figure 1). PBN's inhibitory function against the pro-inflammatory transcription factor NF-κB in rat liver has been shown in LPS-administered rats, where PBN dramatically decreased LPS-mediated mortality [27,28]. In the redox activation of doxorubicin induced apoptosis in endothelial cells and cardiomyocytes, PBN was found to protect apoptosis pathways by inhibiting caspase-3 activation [44]. PBN also suppresses apoptosis by inhibiting caspase-3 activity, AP-1 activation and c-fos in light induced retinal degeneration in rats [45]. PBN treated U937 cells showed a significant inhibition of apoptotic features such as activation of caspase 3, up-regulation of Bax and p53 and down-regulation of Bcl-2 compared to control cells following exposure to

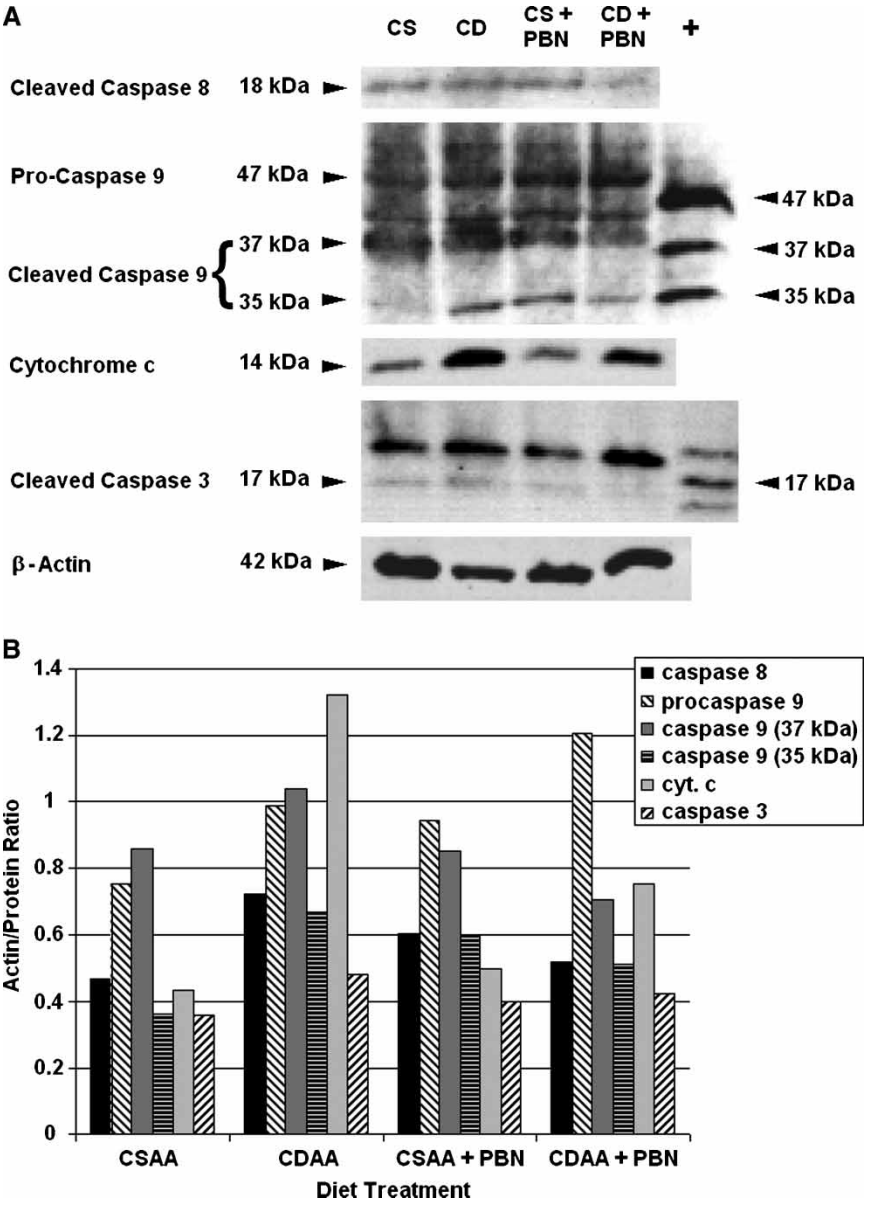

Figure 4. (A) Western blot analyses of liver proteins in CDAA fed rats. Cleaved-caspase8 (18 kDa), pro- (47kDa) and cleaved caspase-9 (37 kDa and 35 kDa), cytochrome *c* (14 kDa) and cleaved caspase-3 (17 kDa) for Fas/FasL signalling were detected in liver tissues from CDAA fed rats; lane A is from liver of CSAA fed rats, lane B is from liver of CDAA fed rats, lane C is from liver of CSAA+PBN fed rats and lane D is from liver of CDAA+PBN fed rats. β-actin was used to ensure equal loading of proteins. The bands depicted in the figure are representative for one of the gels. (+) Positive control of caspase-3 and caspase-9 (2 μm camptothecin treated Jurkat cells). (B) Histograms of the density ratios of the β-actin band (42 kDa), obtained from each individual gel runs to the protein bands (cleaved caspase 8 (18 kDa), pro-caspase 9 (47 kDa), cleaved caspase 9 (37 and 35 kDa), cytochrome c (14 kDa) and cleaved caspase 3 (17 kDa)) in (A).

ionizing radiation which produces reactive oxygen species (ROS) [46]. It is well known that ROS play an important causative role in apoptotic cell death. S-PBN (N-tert-butyl-2-sulpo-phenyl nitrone) has also been found to protect cortical neurons from oxidative damage (hydrogen peroxide treatment) and apoptosis [47]. In the presence of neoplastic cells, PBN has a pro-apoptotic activity. For instance, a derivative of PBN, CQPN (C-(2-chloroquinoline-3-yl)-N-phenyl nitrone), was found to inhibit cell proliferation and induce apoptosis of breast carcinoma MCF-7 cells [48]. Likewise, another COX2 inhibitor, SC-236, has been found to increase apoptosis in a CD-induced hepatocarcinogenesis model in mice [49]. It was thought that the results from this study were suggestive that the mechanism of SC-236 occurred via a COX-2 dependent inhibition of Akt phosphorylation and induction of apoptosis [49]. It may be possible that PBN's pro-apoptotic capability may be attributed to the protection ability of an apoptosis-related protein against oxidative damage, such as PTEN (unpublished data).

The CDAA diet model is considered a good model for NASH, where Fas and caspase-3 generated cytokeratin 18 fragments are used as a novel marker of NASH [31,50]. Also, there are many reports

indicating that hepatocyte Fas expression is related to a liver inflammation state, such as in virus-related hepatitis [51], steatohepatitis [31–33] and hepatocellular carcinoma [51]. From the effect of PBN on the CDAA model, the present study was able to show that there is a close relationship between apoptosis and inflammation, which is also closely associated with carcinogenesis. Although we do not understand the true nature of this relationship at present, there is a possibility that Fas expression could become an early marker of carcinogenesis and that PBN could be a potential therapeutic drug that can inhibit Fas expression and could result in it is use as a cancer-preventive agent.

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