



High-fat-cholesterol diet mainly induced necrosis in fibrotic steatohepatitis rat by suppressing caspase activity

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ABSTRACT

Aim: Apoptosis and necrosis occur in nonalcoholic steatohepatitis (NASH) and are thought to be related to fibrosis. A stroke-prone spontaneously hypertensive (SHRSP5/Dmcr) rat fed a high-fat-cholesterol (HFC) diet exhibited similar pathological features to human NASH with severe liver fibrosis. We aimed to reveal the molecular pathway and to confirm the relationship between cell death, fibrosis and K18Asp396 levels, a neoepitope generated during cleavage of keratin 18 by caspases, as a candidate for biomarker of hepatic damage in this animal model.

Main methods: Male rats were fed with control and HFC diets for 2, 8 and 14 weeks. Liver apoptosis cells, necrosis score, and the molecular mechanism and K18Asp396 levels were investigated.

Key findings: HFC diet increased TUNEL-positive cells only at 2 weeks and necrosis scores strongly in the livers of rats during the entire period. This diet increased hepatic Bax/Bak but decreased Bcl-2/Bcl-xl expression during the entire period; however, it upregulated caspase 8, 9, and 3/7 activities only at 2 weeks, but downregulated them at 14 weeks. Additionally, this diet did not increase hepatic cytochrome c expression. Serum K18Asp396 levels have a positive correlation with necrosis score.

Significance: In SHRSP5/Dmcr rats, HFC diet caused hepatocyte necrosis rather than apoptosis by the downregulation of all caspase activity. Serum K18Asp396 levels may be a good biomarker of hepatocyte necrosis.

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Introduction

Nonalcoholic steatohepatitis (NASH), an advanced stage of nonalcoholic fatty liver disease (NAFLD), is a major concern because of its capacity to evolve into cirrhosis and hepatocellular carcinoma, but its pathogenesis progression remains obscure (Feldstein et al., 2003; Tiniakos et al., 2010). Apoptosis is considered as one of NASH pathogenesis, and is correlated with fibrosis progression (Canbay et al., 2002; Feldstein et al., 2003). Yet, necrosis, another type of hepatocyte death, may also present concurrently with apoptosis (Tiniakos et al., 2010). When necrotic hepatocytes were broken, their constituents stimulated an inflammatory response, which may have induced fibrogenesis (Sokol, 2002).

Meanwhile, there is an urgent need to ascertain a non-invasive biomarker to replace or reduce the application of liver biopsy, which has several limitations even as the gold standard for diagnosis (Wieckowska and Feldstein, 2008). Considering that apoptosis is the prominent feature of NASH, the utilization of an apoptosis marker has become the focus of many studies (Tsutsui et al., 2010; Wieckowska et al., 2006). Recently, the measurement of serum keratin 18 fragment (K18Asp396) levels by M30 antibody proved to be a useful candidate as a surrogate biomarker of apoptosis (Lavallard et al., 2011; Wieckowska et al., 2006). M65 antibody, on the other hand, measures total (intact and fragmented) keratin 18 released from necrotic cells (Lavallard et al., 2011). A combination of both M30 and M65 is considered useful for examining the predominant occurrence of apoptosis or necrosis (Lavallard et al., 2011; Tsutsui et al., 2010).

We recently developed a novel stroke-prone spontaneously hypertensive (SHRSP5/Dmcr) rat model, formerly called arteriolipidosis-prone rats, for the study of high-fat and high-cholesterol (HFC) diet-induced hepatosteatosis and liver fibrosis (Jia et al., 2012; Kitamori et al., 2012). SHRSP5/Dmcr substrain is produced as the fifth SHRSP by selective brother–sister inbreeding of SHRSP with stronger hypercholesterolemic

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responses to the HFC diet (Kitamori et al., 2012). Therefore, although this strain has not been genetically modified, the extensive enlargement and a whitish color of the liver of SHRSP5/Dmcr rat have fortunately been found and the hepatosteatosis and fibrosis progression has been analyzed (Kitamori et al., 2012; Moriya et al., 2012). Even though this diet did not cause obesity and insulin resistance, it did result in liver damage biochemically and histopathologically similar to human NASH, i.e., hepatic steatohepatitis and perivenular fibrosis with a chicken-wire network. We also clarified the depletion of hepatic adenosine triphosphate (ATP) using SHRSP5/Dmcr rats (Jia et al., 2012).

Therefore, our study aimed to: 1) reveal the vulnerability of SHRSP5/Dmcr rat livers to apoptosis and necrosis and to understand their molecular signaling pathway, induced by HFC-diet feeding in a time-dependent manner; 2) clarify the relationship between apoptosis or necrosis and fibrosis; and 3) investigate the progression of liver damage using a serum biomarker of hepatocyte cell deaths (K18).

Materials and methods

Diets

Stroke-prone control chow diet (20.8% crude protein, 4.8% crude lipid, 3.2% crude fiber, 5.0% crude ash, 8.0% moisture, and 58.2% carbohydrate) was used as a control diet. HFC diet was a mixture of 68% control diet, 25% palm oil, 5% cholesterol and 2% cholic acid. Both diets were obtained from Funabashi Farm (Chiba, Japan).

Animal experiments

This study was performed according to the Guidelines for Animal Experiments of the Kinjo Gakuin University Animal Centre. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Kinjo Gakuin University Animal Centre (Approval No. 27). Male SHRSP5/Dmcr rats at 10 weeks old were housed in a temperature- and light-controlled environment (23–25 °C and 55–60% relative humidity, 12-h light/dark cycle), and were assigned to 6 groups ($n = 6$ /group, except for control at 14 weeks $n = 7$). Three groups were fed control diet, and another three groups were fed HFC diet for 2, 8 and 14 weeks. After an 18–20 h fast from the last feeding, all rats were sacrificed under anesthesia by pentobarbital (70 mg/kg), and the blood and livers were removed. A part of each liver was fixed by 4% buffered paraformaldehyde for histological examination; the remaining samples were stored at -80 °C for subsequent analysis. Serum was collected after centrifuging the blood at $3,500 \times g$ for 10 min, and stored at -80 °C until use.

Histopathological detection of liver tissues

Liver tissues were embedded in paraffin, and 4- μ m sections were cut. Tissue sections were stained by hematoxylin and eosin (H&E) to determine necrosis, and a DeathEnd™ Colorimetric TUNEL System kit (Promega, Madison, USA) was used for apoptotic cell analysis. Necrosis scores were determined using Knodell HAI scores with minor modifications (Brunt, 2000), and apoptotic cell numbers were measured by counting TUNEL-positive cells, both in 20 randomly selected microscopic fields ($\times 200$). Specimens were examined under a light microscope using an Olympus BX50 (Olympus Corporation, Japan), and captured by DMD108 (Leica, Wetzlar, Germany). The fibrosis area has been reported elsewhere (Jia et al., 2012).

Serum TNF- α , TNF-R1 and TNF-R2

Serum TNF- α , TNF-R1 and TNF-R2 were measured by commercial kits (R&D Systems Inc., Minneapolis, MN) according to the manufacturer's instructions; the result of TNF- α measurement was also used elsewhere (Jia et al., 2012).

Isolation of cytosolic and mitochondrial fractions

Liver homogenates were prepared with 3 volumes of 0.25 M sucrose–10 mM phosphate buffer (pH 7.4). Homogenates were centrifuged at $10,000 \times g$ for 10 min. The supernatants were further centrifuged at $105,000 \times g$ for 1 h, and the resulting supernatants were used as the cytosolic fractions.

Mitochondrial fractions were isolated according to instructions from Mitochondria Isolation Kit for Tissue (Thermo Scientific, Rockford, USA). All steps were carried out at 4 °C. Briefly, livers were homogenized in phosphate buffered saline (PBS) with a Dounce homogenizer, then centrifuged at $1,000 \times g$ for 3 min. The resultant pellet was resuspended in BSA/Reagent A solution. After centrifugation at $700 \times g$ for 10 min, the supernatant was further centrifuged at $3,000 \times g$ for 15 min. The mitochondrial fraction was obtained in the pellet after centrifugation at $12,000 \times g$ for 5 min.

Western blot analysis

Homogenate, cytosolic or mitochondrial fractions were subjected to 10% or 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane as described elsewhere (Ramdhan et al., 2008). The membranes were incubated overnight at 4 °C with their respective primary polyclonal antibodies against TNF- α , TNF-R1, Bcl-2, Bcl-xl, Bax, Heat shock protein (Hsp) 90 α / β , keratin 18 (K18), and cytochrome c (Santa Cruz Biotechnology, Inc., CA, USA); Bak (Cell Signaling Technology, Danvers, MA), and Mcl-1 (Epitomic, Inc., California). As an internal standard, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, Inc., CA, USA) was used for respective protein in liver homogenate and cytosolic fraction, and cytochrome c oxidase subunit IV (COX IV) (Cell Signaling Technology, Danvers, MA) for that in mitochondria fraction. The specific proteins were detected by 1-Step™ NBT/BCIP (Pierce Biotechnology, Rockford, IL, USA) or ECL Western Blotting Detection Reagent (GE Healthcare, Buckinghamshire, UK).

Caspase 8, 9, and 3/7 activities assay

Caspase 8, 9, and 3/7 activities in liver were measured using a Caspase-Glo assay kit (Promega, Madison, USA) according to the manufacturer's instructions.

Measurement of K18Asp396 levels in liver tissue and serum

When the K18 was cleaved by activated caspases, a neoepitope Asp396 was exposed and specifically recognized by the M30 antibody (Leers et al., 1999). The K18Asp396 levels in the liver homogenate and serum were analyzed in duplicate using a commercially available immunoassay M30-Apoptosense ELISA kit (PEVIVA AB, Bromma, Sweden) according to the manufacturer's instructions. Reference concentrations of M30 antibody were used to prepare assay calibration. The absorbance level was determined with a microplate reader at 450 nm, using a Power scan 4 Multi-Mode Microplate Reader (DS Pharma Biomedical, Osaka, Japan).

Statistical analysis

Data were expressed as mean \pm standard deviation (SD). Student's *t*-test was used for comparing the two groups, and Tukey–Kramer HSD post hoc test and Steel–Dwass test were used for apoptosis cell count and the necrosis score, respectively. Correlation coefficients were calculated by Spearman rank correlation analysis. All analyses were performed by SPSS v. 19.0 (Chicago, IL), except for the Steel–Dwass test by R for Windows. $P < 0.05$ was considered statistically significant.

Results

Histological liver damage

The H&E staining showed that HFC diet induced mild steatosis with foci of inflammatory infiltration at 2 weeks, which progressed to

extensive macrovesicular steatosis, inflammatory cell infiltrations and ballooning degeneration at 8 and 14 weeks (Jia et al., 2012; Kitamori et al., 2012). Severe fibrosis was evidenced in HFC diet-fed rats by Elastic Van Gieson staining at 8 and 14 weeks (Jia et al., 2012).

The appearances of TUNEL-positive cells were rarely detected in all liver sections (Fig. 1A). However, the HFC diet significantly induced

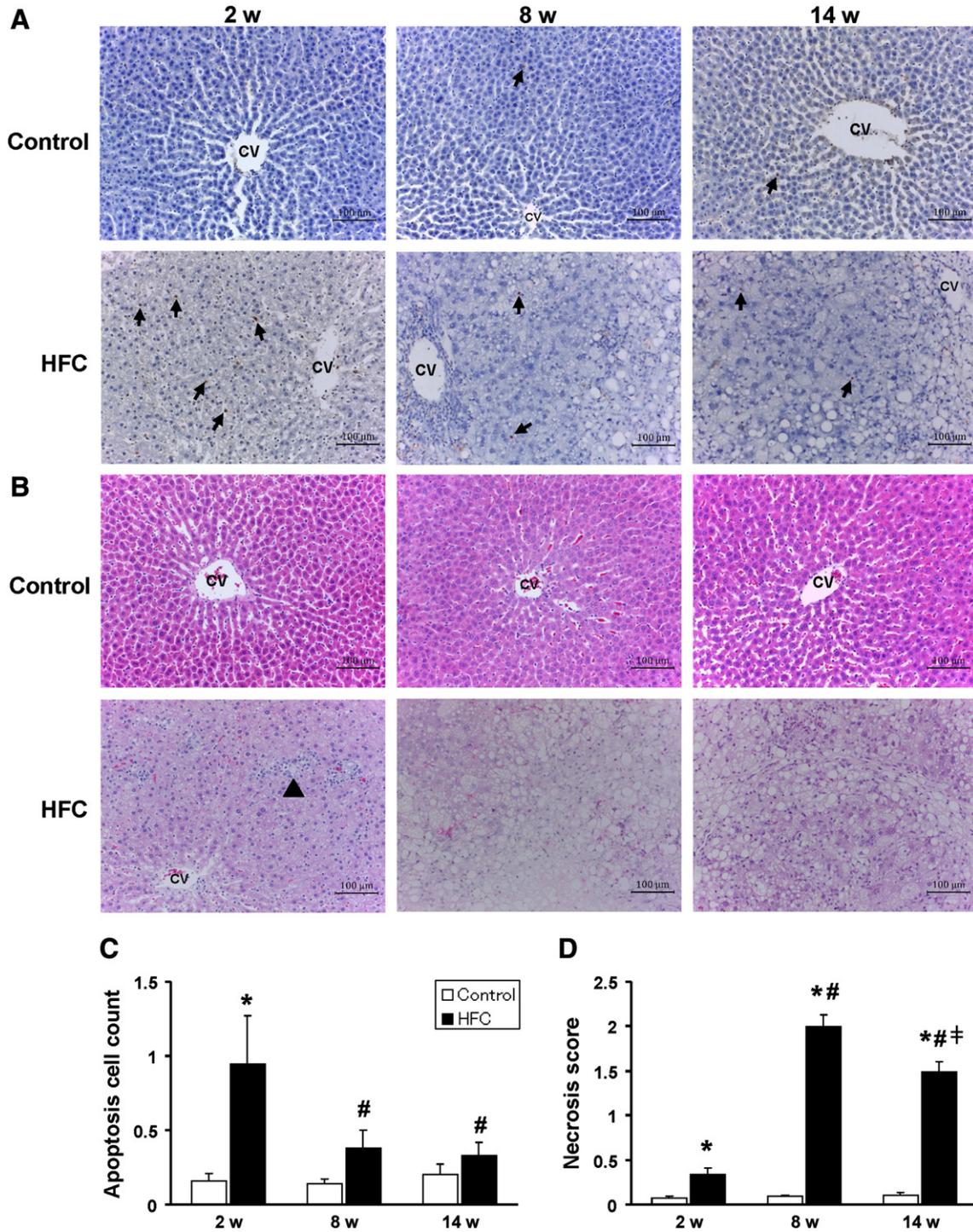


Fig. 1. Representative liver histology image (original magnification: $\times 200$) from control and HFC diet. (A) TUNEL staining. TUNEL-positive cells (arrow) were rarely detected, however, the HFC diet significantly induced apoptosis only at 2 weeks compared to the control group and did not significantly increase compared to control diet at 8 weeks and 14 weeks. (B) Hematoxylin and eosin staining. Minimal or no evidence of injury was found in control diet, focal necrosis (arrowhead) was observed in the liver of rat at 2 weeks HFC diet feeding (score 1), and most prominent marked necrosis which involved more than 2/3 lobules (score 4) were observed at 8 weeks HFC diet feeding. Total TUNEL-positive cell count and total of necrosis score are provided in (C) and (D). A total of 20 randomly selected fields were analyzed as described in Material and methods. Data were averaged per group and presented as mean \pm SD, $n =$ six rats in each group (exception for control at 14 weeks, $n = 7$). * $P < 0.05$ vs. control; # $P < 0.05$ vs. corresponding values at 2 weeks; ** $P < 0.05$ vs. corresponding values at 8 weeks, using Tukey–Kramer HSD post hoc test and Steel–Dwass test for apoptosis cell count and necrosis score, respectively. Abbreviations: HFC, high-fat and high-cholesterol; CV, central vein.

Table 1
Serum levels of TNF- α , TNF-R1 and TNF-R2.

Duration	Diet	TNF- α (pg/ml)	TNF-R1 (pg/ml)	TNF-R2 (pg/ml)
2 weeks	Control	1.3 \pm 1.9	73 \pm 27	1315 \pm 699
	HFC	7.2 \pm 3.3 (5.5)*	115 \pm 51 (1.6)	2113 \pm 493 (1.6)*
8 weeks	Control	0.5 \pm 0.5	82 \pm 17	968 \pm 112
	HFC	13.0 \pm 3.3 (26)*	199 \pm 41 (2.4)*	4623 \pm 1146 (4.8)*
14 weeks	Control	2.1 \pm 2.2	78 \pm 24	974 \pm 81
	HFC	15.8 \pm 6.2 (7.5)*	209 \pm 48 (2.7)*	5190 \pm 1184 (5.3)*

Values are mean \pm SD; numbers in parentheses represent mean fold change compared with control and HFC group, respectively. $n =$ six rats in each group. * $P < 0.05$ vs. control. TNF- α results were also used in a previous manuscript (Jia et al., 2012). Abbreviations: HFC, high-fat and high-cholesterol; TNF- α , tumor necrosis factor- α ; TNF-R1, tumor necrosis factor- α receptor 1; TNF-R2, tumor necrosis factor- α receptor 2.

apoptosis only at 2 weeks compared to the control group. At the following feeding period, the number of TUNEL-positive cells was reduced compared to those of 2-week feeding.

Unlike the occurrence of apoptotic cells, the necrosis area, as indicated by increased eosinophilia, vacuolization, cell disruption, karyolysis and loss of hepatic architecture, was found predominantly in HFC diet groups compared to those in control group. The HFC diet significantly increased necrotic scores at 2 weeks, which were further increased at 8 weeks, but decreased thereafter (Fig. 1D).

Serum and liver concentration of TNF- α and TNF-R1

TNF- α could evoke multiple cellular responses, such as apoptosis (Ding and Yin, 2004) and necrosis (Morgan et al., 2008). In the signaling process TNF- α are mediated by TNF-receptor 1 (TNF-R1) (Ding and Yin, 2004) and potentiated by TNF-receptor 2 (TNF-R2) (Chan et al., 2003). The HFC diet significantly increased serum TNF- α levels at 2, 8, and 14 weeks, compared with levels in the respective control diet (Table 1). Serum TNF-R1 and TNF-R2 also significantly increased gradually at each period of HFC-diet feeding, except at 2 weeks for TNF-R1. HFC diet did not influence TNF- α protein expression in the liver (data not shown), while it significantly decreased the receptor TNF-R1 and TNF-R2 (data not shown) at 14 weeks (Fig. 2B).

Pro- and anti-apoptosis proteins, Hsp90 α/β and cytochrome c

The expression of anti-apoptosis protein Bcl-2 and Bcl-xl were significantly lower in rats fed the HFC diet, compared to those on the control diet at 2, 8 and/or 14 weeks, respectively (Fig. 2B). The decreasing trend of Bcl-2 protein toward prolonging the duration of the experiment by control may suggest the ageing effect (Molpeceres et al., 2007; Sasaki et al., 2001). Nevertheless, HFC diet further decreased Bcl-2 protein expression. Inversely, although HFC-diet feeding did not influence Bax protein at 2 weeks as with the control diet, it did increase it in later periods and Bak protein at all periods. HFC-diet feeding, however, did not influence Mcl-1 protein expression (data not shown). Furthermore, the diet predominantly increased expression of Hsp90 α/β at 8 weeks compared to those on the control diet.

In order to determine the effect of Bax and Bak on cytochrome c release from the mitochondria, we analyzed the expression of cytochrome c in the mitochondria, cytosol and liver homogenate. HFC diet significantly decreased cytochrome c expressions in mitochondria at each

period (Fig. 2C and D). The diet also decreased those levels to 0.4-fold at 14 weeks, as opposed to those with the control diet in cytosol and liver homogenate.

Caspase activities

To assess the functions of aspartic acid-specific protease families which are involved in apoptosis, we examined caspase 8 and 9 as initiator caspases, and caspase 3/7 as an effector caspase (Logue and Martin, 2008). The HFC diet significantly increased caspase 8, 9, and 3/7 activities at 2 weeks (2.3-, 1.9- and 1.2-fold, respectively), whereas it dramatically decreased thereafter (Fig. 3A–C).

Caspase cleave fragmented K18 in liver and serum

HFC-diet-feeding appeared to decrease liver-fragmented K18 (45 kDa) at 2 weeks, and significantly decreased it at 8 and 14 weeks (Fig. 3D). Conversely, the HFC diet tended to increase serum-fragmented K18 protein at 8 and 14 weeks (Fig. 3E). Meanwhile, in the immunoassay result, the HFC diet significantly increased K18Asp396 levels in the liver at 2 weeks, but it significantly decreased them at 8 and 14 weeks (Fig. 3F), as caspase 8, 9, and 3/7 activities also decreased. In contrast, the HFC diet clearly increased serum K18Asp396 levels, especially at 8 and 14 weeks, when they were 8.1- and 6.4-fold higher than the respective control values (Fig. 3G).

Correlations among histopathological findings, caspase activities and apoptosis markers

The TUNEL-positive cells were moderately correlated with serum K18Asp396 levels, but not with the liver levels (Table 2). Interestingly, the severity of liver necrosis and fibrosis was strongly correlated with serum K18Asp396 levels and negatively correlated with the liver levels. Caspase 8, 9 and 3/7 activities positively correlated with liver K18Asp396 levels, and negatively correlated with serum levels. Furthermore, the fibrosis area showed a strong correlation with the necrosis score ($r = 0.813$, $P < 0.001$), and a weak correlation with the TUNEL-positive cells ($r = 0.559$, $P < 0.001$).

Discussion

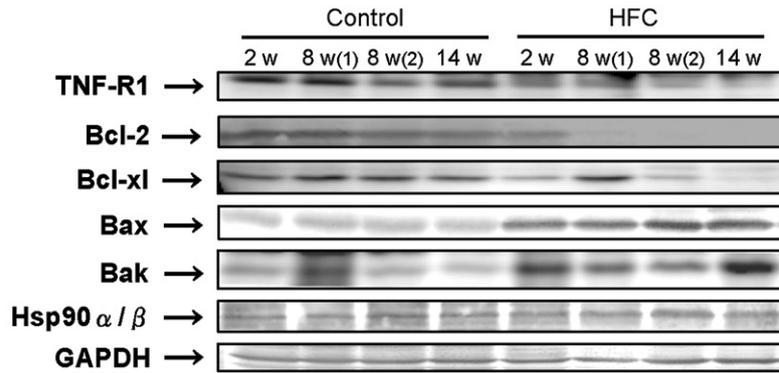
In the current study, we demonstrated: the vulnerability of SHRSP5/Dmcr rat livers to necrosis compared to apoptosis, and their molecular signaling pathway, induced by HFC-diet feeding; fibrosis is predominantly induced by necrosis; and serum K18Asp396 as a possibly useful biomarker for necrosis and fibrosis, besides apoptosis.

Molecular signaling pathway of apoptosis alteration into necrosis induced by prolonged HFC-diet feeding

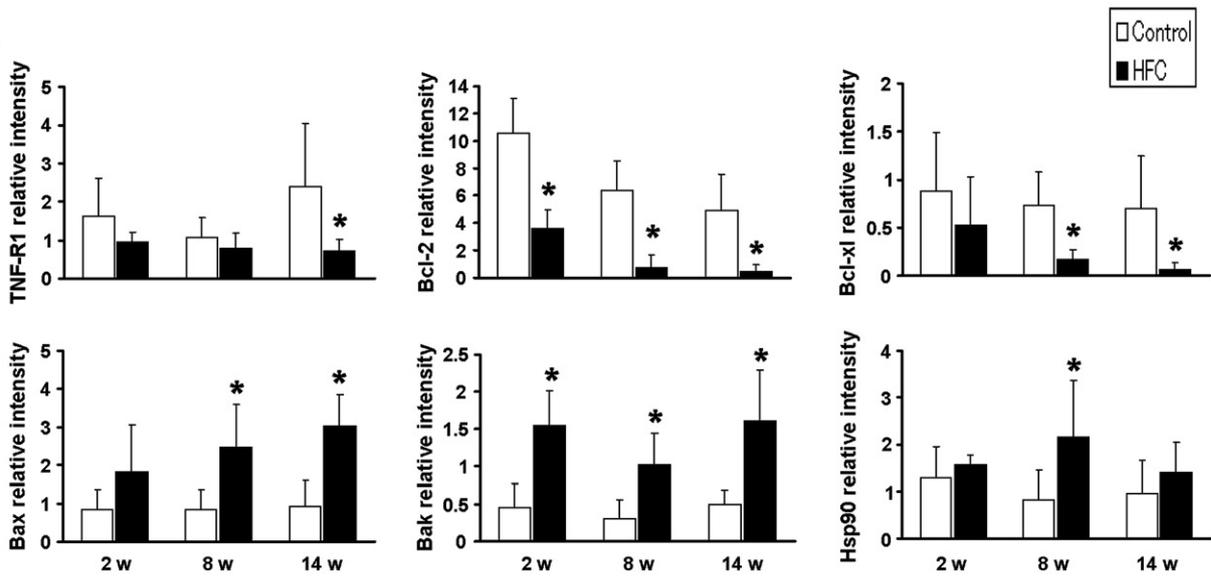
In the extrinsic apoptosis pathway, TNF- α binding to TNF-R1 leads to the trimerization and recruitment of an adaptor protein, TNF-R1-associated Death Domain, or Fas-Associated Death Domain (FADD). FADD recruits and activates caspase 8, followed by translocating Bax/Bak to the mitochondria (Ding and Yin, 2004). The HFC diet increased serum levels of TNF- α and TNF-R1 at each period of time, but did not increase hepatic TNF-R1 levels, suggesting that apoptosis signaling

Fig. 2. Western blot analysis. (A) Representative Western blot images of TNF-R1, Bcl-2 family protein and Hsp90 α/β . GAPDH was used as loading control for respective protein in cytosol and liver homogenate. The two bands at 8 weeks represented the results from different rat. (B) Relative intensity of TNF-R1, Bcl-2 family protein and Hsp 90 α/β in liver. HFC diet decreased TNF-R1 and anti-apoptosis protein (Bcl-2 and Bcl-xl), and increased pro-apoptosis protein (Bax and Bak) compared to control diet. (C) Representative Western blot images of cytochrome c. COX IV was used as loading control for respective protein in mitochondria. The two bands at 8 weeks represented the results from different rat. (D) HFC diet decreased cytochrome c in mitochondria at each period and in cytosol and liver homogenate at 14 weeks. Values are mean \pm SD of six rats per group (exception for control at 14 weeks, $n = 7$). * $P < 0.05$ vs. control. Abbreviations: HFC, high-fat and high-cholesterol; TNF-R1, tumor necrosis factor- α receptor 1; Hsp90 α/β , heat shock protein 90 α/β ; Cyt. c, cytochrome c; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; COX IV, cytochrome c oxidase subunit IV.

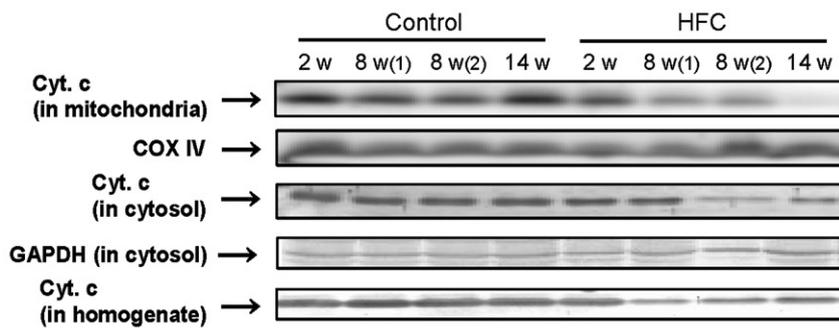
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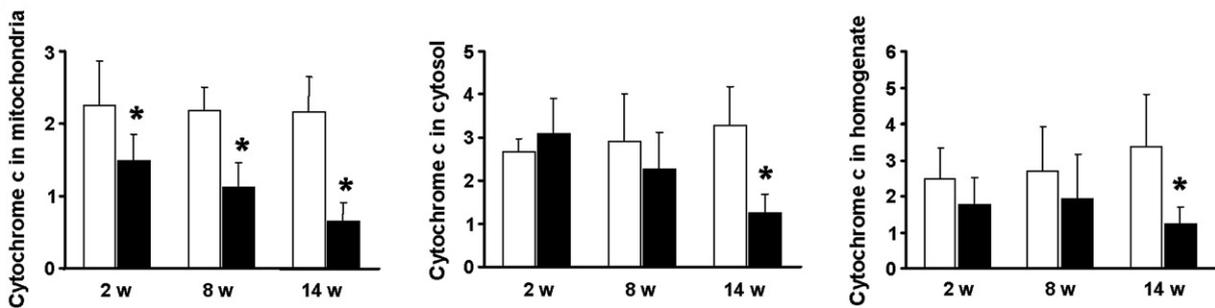
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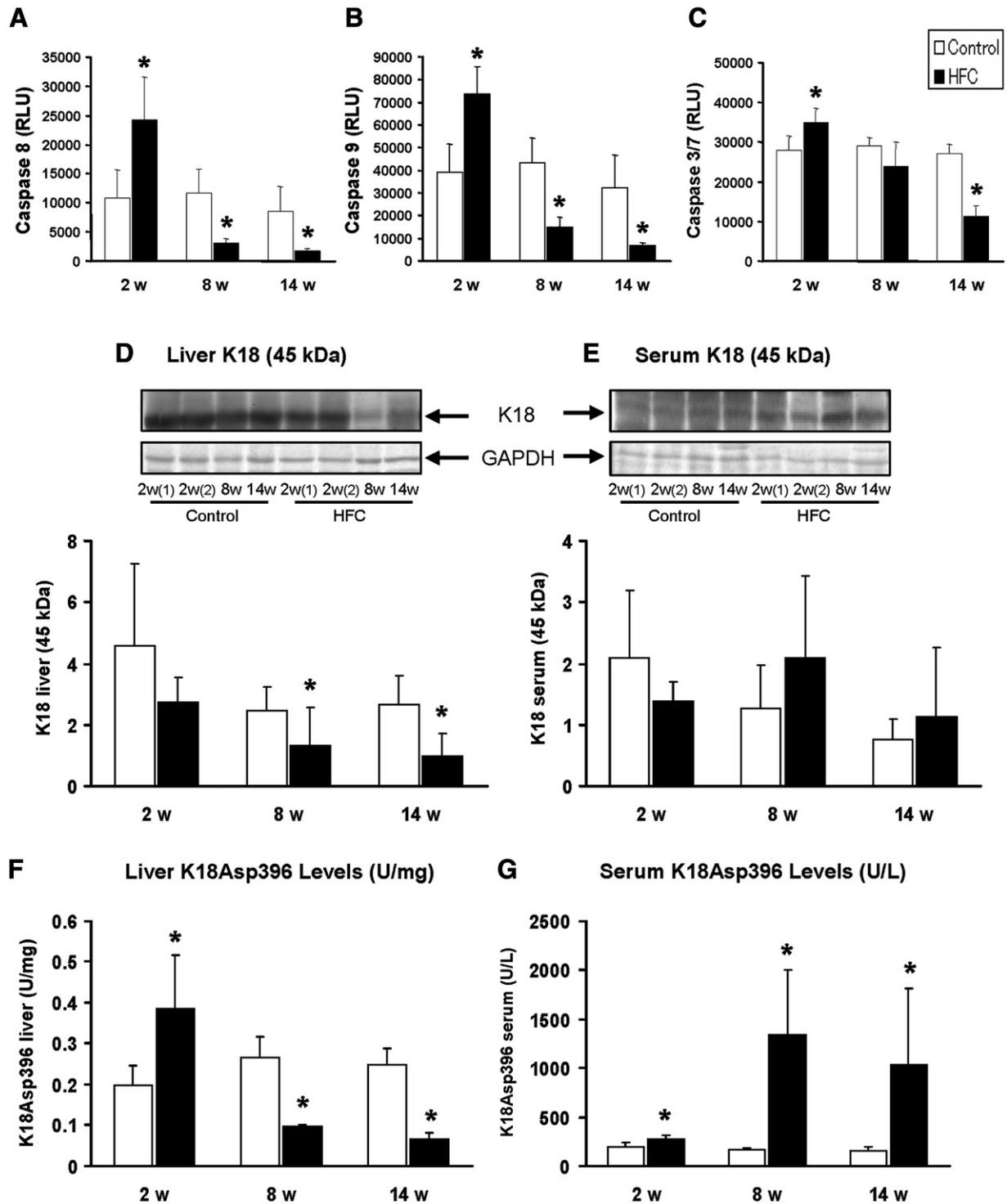


Fig. 3. Activities (RLU) of caspase 8, 9, and 3/7 in the liver of control and HFC diet. Caspase 8 (A), 9 (B) and 3/7 (C) activities were significantly increased at 2 weeks on HFC diet compared to control diet, and then decreased as feeding duration was prolonged. Western blot analysis of fragmented K18 in liver tissue (D) and serum sample (E) on 45 kDa (representative images on the above panel, the two bands at 2 weeks represented the results from different rat). K18Asp396 levels in liver (F) and serum (G). Values are mean \pm SD of six rats per group (exception for control at 14 weeks, $n = 7$). * $P < 0.05$ vs. control. Abbreviations: HFC, high-fat and high-cholesterol; RLU, relative light unit.

may not be induced throughout the observed period, since caspase 8 activity was only increased at 2 weeks. Fas signaling may be involved in the apoptosis signaling at the early stage.

In the intrinsic apoptosis pathway, the imbalance of pro- and anti-apoptosis proteins (Bcl-2 family) induces hepatocyte apoptosis (Logue and Martin, 2008; Wang et al., 2006; Wang et al., 2008). In line with these reports, the HFC diet upregulated Bax/Bak after 2 weeks along

with an exclusive downregulation of Bcl-2/Bcl-xl in the liver, suggesting an increase in apoptosis signaling throughout the feeding. Increasing the pro-apoptotic protein Bax/Bak could provoke mitochondrial permeability and cytochrome c release (Logue and Martin, 2008). Unexpectedly, the expression of cytochrome c did not increase and was lower in the HFC-diet group than in the control. However, HFC diet decreased the expressions of cytochrome c in mitochondria at each period, suggesting

Table 2
Correlations among histopathological findings, caspase activities (RLU) and apoptosis markers.

	Serum K18Asp396 (U/L)		Liver K18Asp396 (U/mg)	
	r	P value	r	P value
Apoptosis cell count	0.599	<0.001	−0.131	0.439
Necrosis score	0.796	<0.001	−0.51	0.001
Fibrosis area (%)	0.711	<0.001	−0.448	0.002
Caspase 8	−0.49	0.002	0.792	<0.001
Caspase 9	−0.461	0.004	0.792	<0.001
Caspase 3/7	−0.389	0.017	0.718	<0.001

r, Spearman's correlation coefficient, using data of control and HFC group. Fibrosis area was quantified with an image analysis system (BZ-9000, KEYENCE, Dynamic Cell Count, Keyence), and expressed as a percentage of the total area, of which results were also used in a previous manuscript (Jia et al., 2012).

mitochondria may be damaged by the HFC-diet feeding at early stage. Additionally, and similar to those of caspase 8, elevated activities of caspase 9 on the HFC group abruptly decreased after 2 weeks, and at 14 weeks for caspase 3/7. Taken together, although the HFC diet partially upregulated the extrinsic and intrinsic apoptosis pathways throughout the feeding, downregulation of all caspase activities did not always support the increase of the apoptosis process especially at the later stage, as shown in the findings of a decreased number of TUNEL-positive cells. Likewise, our results suggest that only a small minority of hepatocytes undergo apoptosis after 2 to 14 weeks of HFC diet feeding.

The insufficient energy supply (Miyoshi et al., 2006) and the presence of a caspase inhibitor such as Hsp 90 α / β resulted in the decrease of caspase activity, consequently increasing cell sensitization to necrosis under TNF- α upregulation (Berghe et al., 2003; Morgan et al., 2008). Our previous study demonstrated the time-dependent depletion of ATP by HFC diet-feeding at 2, 8, and 14 weeks (Jia et al., 2012) parallel with significantly decreased anti-oxidative stress protein, Cu²⁺/Zn²⁺-superoxide dismutase (SOD1), compared to control throughout the study (Moriya et al., 2012). Decreasing of anti-oxidative stress protein will increase oxidative stress which causes mitochondrial damage and reduction of ATP content (Rolo AP et al., 2012) leading to the necrosis process. Indeed, reduced hepatic ATP levels were reported in rat fatty liver (Vendemiale et al., 2001) and NASH animal model fed by methionine and choline-deficient (MCD) diet (Serviddio et al., 2008) due to mitochondrial dysfunction. Additionally, impaired energy homeostasis was also found in patients with nonalcoholic steatohepatitis (Cortez-Pinto et al., 1999).

Furthermore, HFC-diet feeding increased the Hsp 90 α / β protein level at 8 weeks, which may have indirectly inhibited caspase 8 (Berghe et al., 2003), and directly inhibited caspase 9 and 3/7 (Pandey et al., 2000). Therefore, taken together, even though liver TNF- α and TNF-R1 were not increased during the prolonged HFC-diet feeding in the current study, their increase in serum may have triggered the necrosis process predominantly. Indeed, cytochrome c release (Knight and Jaeschke, 2002) and translocation Bax to mitochondria also occur in the necrosis process (Adams et al., 2001). Remarkably, necrotic cells further increased at 8 and 14 weeks compared with those at 2 weeks. These results correlated with the increase in the serum alanine aminotransferase (ALT) levels (Kitamori et al., 2012).

The alteration in cell-death phenotype from apoptosis to necrosis in our study is similar to the previous study described in an ischemia-reperfusion model using Sprague–Dawley rats (Gujral et al., 2001). Moreover, necrosis was significantly further increased after 65-day chronic studies in dietary NASH rat model (Baumgardner et al., 2008).

The severe increase of necrosis in HFC diet-fed rats rendered a strong correlation between liver fibrosis and necrosis scores, while only a slight increase of apoptosis in this model led to a weak correlation between fibrosis and TUNEL-positive cells. These findings indicated that

necrosis played a major role in inducing fibrosis in the present rat model.

Serum K18Asp396 level possibly a good biomarker for evaluating liver necrosis and fibrosis, besides apoptosis

Since the K18 fragment on 45 kDa detected by Western blot did not entirely reflect the K18 fragment measured by M30 antibody (K18Asp396), especially at 2 weeks of feeding, the latter levels may be a better biomarker than the detection of only 45 kDa of K18 fragments by Western blot. This could be because many portions of this K18 fragment cleaved into a smaller molecular weight, all of which could be detected by M30 antibody (Bantel et al., 2001; Leers et al., 1999). Our focus was directed to whether the serum or hepatic K18Asp396 level, a recently developed biomarker of hepatocyte apoptosis (Lavallard et al., 2011; Tsutsui et al., 2010), was an appropriate biomarker for necrosis. Surprisingly, we observed a strong positive correlation between serum K18Asp396 levels and necrosis scores or fibrosis areas, but a moderate positive correlation with apoptosis. This may be due to the minor expression of apoptosis cells in the current model. Previous studies also found a significant correlation between serum K18 levels and fibrosis (Lavallard et al., 2011; Wieckowska et al., 2006). Therefore, serum K18Asp396 levels may be a good biomarker for evaluating both liver necrosis and fibrosis progression besides apoptosis. The negative correlations between liver K18Asp396 levels and necrosis and fibrosis suggest that this epitope elutes into blood during necrotic or fibrotic changes.

One might be skeptical about the correlation between K18Asp396 levels measured by M30 antibody and necrosis, because M30 antibody is known as an apoptosis biomarker, not a biomarker for necrosis (Lavallard et al., 2011). We examined the total K18 (M65 antibody) level which specifies the necrosis process (Tsutsui et al., 2010), but unfortunately, the value was lower than the M30 antibody level. This antibody probably could not bind well with the epitope of total K18 fragments in serum or the liver homogenate of the present model. However, K18 fragment was detected in secondary necrosis (Bantel et al., 2001), a process different from primary necrosis, which is preceded by apoptosis (Silva et al., 2008). Nevertheless, they have similar morphological features (Krysko et al., 2008). When membrane integrity is lost during secondary necrosis, K18 fragments are released from the cells (Bantel et al., 2001; Schutte et al., 2004); concomitantly, serum K18Asp396 levels also start to increase. Thus, its levels were greater in the serum than in the liver. The difficulty in distinguishing between these two modes of necrosis cells could be a limitation of this study. Hence, we used the word “necrosis” for both primary and secondary necrosis. Furthermore, the exact half-life of the K18 fragment is unknown. However, a previous study reported the half-life of keratin fragments to be about 10–15 h, depending on the size of the fragment (Barak et al., 2004), while Denk et al. (1987) reported the half-life of K18 in hepatocytes to be about 4 days. These studies suggest that the half-life of K18 is short, and in addition to a strong correlation between serum K18Asp396 levels and necrosis scores in our result, supports the conclusion that M30 could be a biomarker of necrosis.

In order to confirm our results, we additionally measured serum M30 levels in the liver with a different extent of damages induced by different proportions of HFC diet intake: 10% HFC (a mixture diet, control: HFC = 1:9), 50% HFC (control: HFC = 1:1) and 100% HFC diet (HFC diet in the current study). These diet feedings for 8 weeks caused minimal or no, mild and severe necrosis and fibrosis, respectively; serum M30 levels increased dose dependently, 211 \pm 51, 758 \pm 346, and 1200 \pm 388 U/L, respectively.

A strong correlation found in our study between caspase 9, and a moderate correlation between caspase 3/7 and liver K18Asp396 levels, may corroborate the theory that K18 is mainly cleaved by caspase 9 and also by caspase 3/7 (Schutte et al., 2004). Even though it is not cleaved by caspase 8 (Schutte et al., 2004), a strong correlation between

caspace 8 and liver K18Asp396 levels was also found. Perhaps caspace 8 only showed the upstream apoptosis signal in accordance with the downstream one shown by caspace 9. The negative correlation between all caspace activity with serum K18Asp396 levels suggests that K18Asp396 is released in the secondary necrosis process.

Conclusions

In summary, the HFC diet induced an early apoptosis process and then deteriorated into necrosis due to the suppression of caspace activities and energy supply. Necrosis is suggested to play a major role in inducing fibrosis progression. Furthermore, serum K18Asp396 levels may be a candidate as a non-invasive biomarker for detecting necrosis.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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