主論文の要旨

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

（高脂肪・コレステロール食は線維性脂肪性肝炎ラットにおいて、カスパーゼ活性を抑制することにより主に壊死を誘導した）

名古屋大学大学院医学系研究科　健康社会医学専攻
社会生命科学講座　環境労働衛生学分野
（指導 ： 加藤 昌志　教授）
HUSNA YETTI

【Introduction】

The pathogenesis progression of nonalcoholic steatohepatitis (NASH) remains obscure. Apoptosis is considered as one of NASH pathogenesis, and is correlated with fibrosis progression. Yet, necrosis, another type of hepatocyte death, may also present concurrently with apoptosis, and may induce fibrogenesis.

The utilization of an apoptosis marker, serum keratin 18 fragment (K18Asp396) levels measured by M30 antibody, has become the focus of many studies. By using a rat model with steatohepatitis and severe fibrosis, this study aimed to: 1) reveal the vulnerability of stroke-prone spontaneously hypertensive (SHRSP5/Dmcr) rat livers to apoptosis and necrosis and to understand their molecular signaling pathway, induced by high-fat-cholesterol (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).

【Methods】

Male rats were assigned to six groups, and fed with control and HFC diets for 2, 8 and 14 weeks. Liver sections were stained by hematoxylin and eosin (H&E) to determine necrosis scores using Knodell HAI scores with minor modifications, apoptotic cell numbers were measured by counting TUNEL-positive cells, both in 20 randomly selected microscopic fields (×200). Protein involved in molecular mechanism and K18Asp396 levels were investigated.

【Results】

1. Histological liver damage

The appearances of TUNEL-positive cells were rarely detected in all liver sections (Fig. 1A). However, HFC diet significantly induced 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 (Fig. 1C). The necrosis area was found predominantly in HFC diet groups compared to those in control group. HFC diet significantly increased necrotic scores at 2 weeks, which were further increased at 8 weeks, but decreased thereafter (Fig. 1D).

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

HFC diet significantly increased serum TNF-α levels at 2, 8, and 14 weeks (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).

3. Pro- and anti-apoptosis proteins, Hsp90α/ß and cytochrome c

In rats fed HFC diet, the expression of anti-apoptosis protein Bcl-2 and Bcl-xl were significantly lower, inversely, pro-apoptosis protein Bak and Bax were significantly increased

compared to those on the control diet at 2, 8 and/or 14 weeks, respectively (Fig. 2B). Furthermore, the diet increased expression of heat shock protein (Hsp) 90α/ß at 8 weeks. HFC diet significantly decreased cytochrome c expressions in mitochondria at each period and in cytosol or liver homogenate at 14 weeks (Fig. 2C−D).

4. Caspase activities

HFC diet significantly increased caspase 8, 9, and 3/7 activities at 2 weeks, whereas it dramatically decreased thereafter (Fig. 3A−C).

5. Caspase cleave fragmented K18 in liver and serum

HFC-diet-feeding significantly decreased K18 fragment (45 kDa) in the liver and tended to increase it in the serum at 8 and 14 weeks (Fig. 3D−E). In the immunoassay result, HFC diet significantly increased K18Asp396 levels in the liver at 2 weeks, but it significantly decreased them at 8 and 14 weeks (Fig. 3F). In contrast, HFC diet clearly increased serum K18Asp396 levels, especially at 8 and 14 weeks (Fig. 3G).

6. Correlations among histopathological findings, caspase activities and apoptosis markers

Serum K18Asp396 levels were moderately and strongly correlated with TUNEL-positive cells and the severity of liver necrosis or fibrosis, respectively (Table 2). 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.

In the extrinsic apoptosis pathway, 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 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. In line with previous reports, 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 Bax/Bak could provoke mitochondrial permeability and cytochrome c release. 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 cytochome c in mitochondria at each period, suggesting mitochondria may be damaged by 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.

The insufficient energy supply as reported in our previous study and the presence of a caspase inhibitor such as Hsp 90α/ß resulted in the decrease of caspase activity, consequently increasing cell sensitization to necrosis under TNF-α upregulation. Indeed, HFC-diet feeding increased the Hsp 90α/ß protein level at 8 weeks, which may have indirectly inhibited caspase 8, and directly inhibited caspase 9 and 3/7. 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. Remarkably, necrotic cells further increased at 8 and 14 weeks compared with those at 2 weeks.

The severe increase of necrosis in HFC diet-fed rats rendered a strong correlation between liver fibrosis and necrosis scores. These findings indicated that necrosis played a major role in inducing fibrosis in the present rat model.

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. Therefore, serum K18Asp396 levels may be a good biomarker for evaluating both liver necrosis and fibrosis progression besides apoptosis. Even though M30 (detect K18 fragment) is not a biomarker for necrosis, K18 fragment was detected in secondary necrosis, a process different from primary necrosis, which is preceded by apoptosis. Nevertheless, they have similar morphological features. When membrane integrity is lost during secondary necrosis, K18 fragments are released from the cells; concomitantly, serum K18Asp396 levels also start to increase. 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.

【Conclusions】

The HFC diet induced an early apoptosis process and then deteriorated into necrosis due to the suppression of caspase 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.