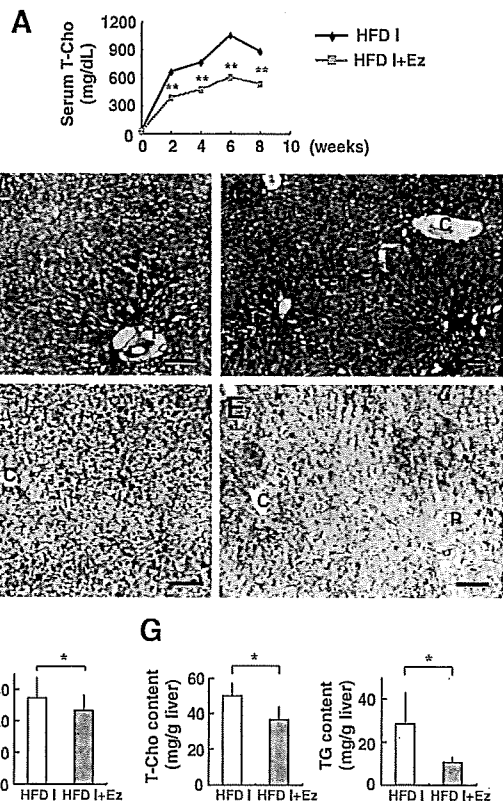


**Figure 7.** Two-dimensional SDS-PAGE analysis of hepatic proteins. Rabbits were fed SD or HFD II for nine months. Proteins were extracted from the livers of SD- and HFD II-fed rabbits and were separated by two-dimensional SDS-PAGE. After the gels were stained with SYPRO Ruby, the protein spots were cut out, digested, and analyzed by quadrupole time-of-flight. Then, the proteins were identified from the obtained amino acid sequences using protein BLAST or FASTA databases. 1 and 1', glutathione transferase; 2 and 2', glutathione *S*-transferase Yc ( $\alpha$  ID); 3 and 3', MAT1; 4 and 4', ST3A1; 5 and 5', GNMT; 6 and 6', aldehyde dehydrogenase, mitochondrial (ALDH class 2); 7 and 7', keratin, type II or cytoskeletal 2 epidermal; 8 and 8',  $\alpha$ -tubulin, actin,  $\beta$ -like 2, or similar to actin, cytoplasmic 1; 9 and 9', actin,  $\beta$ -like 2; 10 and 10', keratin 10 or CYP2B3; 11 and 11', similar to actin, cytoplasmic 1. Blue circles, proteins down-regulated in HFD II-fed rabbits; red circles, proteins up-regulated in HFD II-fed rabbits.

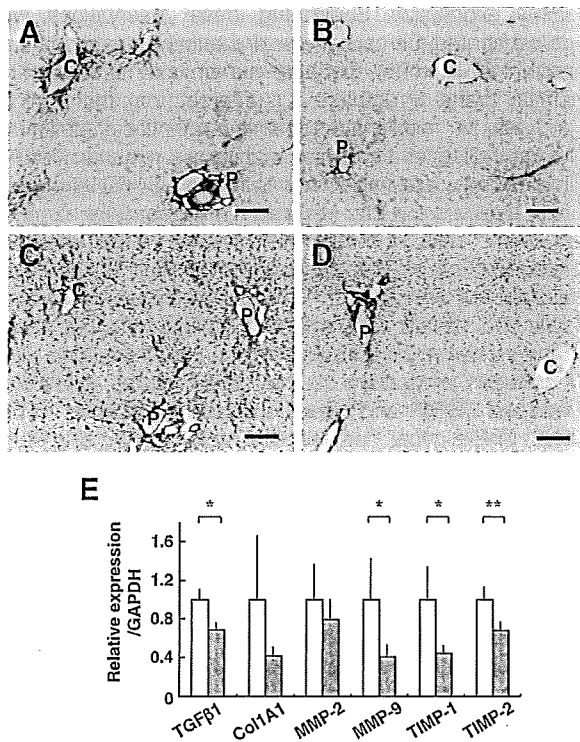
Because a marked increase in serum and hepatic T-Chol levels was evident in our HFD-fed rabbits, we tested ezetimibe, a relatively new compound that inhibits NPC1L1 in hepatocytes and the intestine. Rabbits were fed HFD I (the original diet) with or without ezetimibe (0.6 mg/kg/day) for 2 months. Both control and experimental rabbits showed normal increases in body weight (data not shown). Levels of AST, ALT, and TG remained within the normal range in rabbits fed HFD I with or without ezetimibe (AST,  $38.0 \pm 18.4$  versus  $20.8 \pm 7.4$  IU/L; ALT,  $11.0 \pm 1.0$  versus  $7.8 \pm 3.0$  IU/L; and TG,  $31.0 \pm 9.5$  versus  $27.4 \pm 16.8$  mg/dl). Serum T-Chol was significantly lower in HFD I-fed rabbits treated with ezetimibe than in untreated rabbits during the course of ezetimibe-treatment for up to 8 weeks (Figure 8A). A histological examination showed that ezetimibe suppressed fat deposition (Figure 8, B–F) and reduced the hepatic content of total T-Chol and TG (Figure 8G). Furthermore, liver fibrosis, mildly induced in this 2-month HFD I model, was suppressed by the ezetimibe treatment (Figure 9, A–D). Moreover, ezetimibe significantly decreased the expression of genes associated with liver fibrosis, such as TGF $\beta$ 1, MMP-9, and TIMP-1 and -2. The drug also suppressed expression of Col1A1 and MMP-2, although the difference was not significant (Figure 9E).



**Figure 8.** Effect of ezetimibe (Ez) on hepatic steatosis in HFD I-fed rabbits. Rabbits were fed HFD I (the original diet) with or without ezetimibe (0.6 mg/kg/day) (HFD I or HFD I + Ez) for two months. Histological sections were prepared from HFD I-fed (B and D) and HFD I + Ez-fed rabbits (C and E). A: Changes in serum T-Chol levels. B and C: H&E staining. D and E: Oil red O staining. Scale bars = 100  $\mu$ m. F: Quantification of the lipid droplet area in the liver. Oil red O-stained areas were measured in the livers of HFD I- and HFD I + Ez-fed rabbits. The stained area was significantly smaller in HFD I + Ez-fed rabbits. G: Hepatic total cholesterol and triglyceride contents. P, portal vein; C, central vein. HFD I,  $n = 5$ . HFD I + Ez,  $n = 5$ . \* $P < 0.05$ ; \*\* $P < 0.01$ .

## Discussion

There are several important differences in characteristics of NASH between this rabbit model and humans. Hepatic levels of cholesterol, but not of TG and FFA, exhibited a marked increase in HFD II-fed rabbits compared with those in SD-fed rabbits, and the PPAR $\gamma$  and aP2 mRNA levels were induced in the HFD II model in a manner similar to that observed in human NASH (Figure 3, C and D).<sup>26,27</sup> NAFLD shows various patterns of lipid deposition in the liver,<sup>28</sup> which may be influenced by the diet composition. In this context, increased PPAR $\gamma$  and aP2 may contribute to a decrease in cholesterol level in hepatocytes of this rabbit model induced by high-cholesterol diets. Mitochondrial free cholesterol, but not TG and FFA, sensitizes hepatocytes to TNF $\alpha$ - and Fas-induced apoptosis through mitochondrial glutathione exhaustion.<sup>29</sup> We also observed a marked reduction in glutathione and glutathione-metabolic enzymes in the HFD II-fed rabbit livers (Figure 6, E and F). In this context, it is likely that cholesterol overload, together with a dysregulated antioxidative system in hepatocytes, may trigger liver injury.<sup>30,31</sup> Furthermore, this rabbit model showed increased levels



**Figure 9.** Effect of ezetimibe (Ez) on liver fibrosis in HFD I-fed rabbits. Rabbits were fed HFD I (the original diet) with or without ezetimibe (0.6 mg/kg/day) (HFD I or HFD I + Ez) for two months as mentioned in Figure 8. Histological sections were prepared from HFD I-fed (A and C) and HFD I + Ez-fed rabbits (B and D). A and B: Liver sections were stained with Sirius red. Collagen depositions were reduced by ezetimibe administration. C and D: Liver sections were immunostained for  $\alpha$ SMA. Note that the  $\alpha$ SMA<sup>+</sup> cells were reduced by ezetimibe administration. Scale bars = 100  $\mu$ m. E: Expression of fibrosis-related genes in the liver of HFD I-fed rabbits ( $\square$ ) and HFD I + Ez-fed rabbits ( $\blacksquare$ ). P, portal vein; C, central vein. HFD I,  $n = 5$ . HFD I + Ez,  $n = 5$ . Ez, ezetimibe. \* $P < 0.05$ ; \*\* $P < 0.01$ .

of serum bile acids (Table 2), which are presumably induced by cholesterol overload and down-regulation of the bile acid export-related genes, such as the bile salt export pump and multidrug resistance-associated protein 2 (data not shown).

Although it has been reported that FFA and leptin increase and adiponectin decreases in the serum of NASH patients,<sup>32</sup> serum and hepatic levels of FFA did not change in this rabbit model, possibly owing to the lack of obesity. A recent study reported the reduction of serum adiponectin in a rabbit model fed 10% lard and 2% cholesterol-containing HFD for 8 and 12 weeks.<sup>33</sup> Unfortunately, we were unable to determine the adiponectin and leptin levels in our rabbit model for unknown reasons (data not shown).

We also observed no increase in fasting glucose, fasting insulin, or HOMA-IR in this rabbit model. However, as stated above, this rabbit model showed no obesity and failed to induce high FFA levels in the liver and serum, which may be reasons for its failure to induce type II diabetes and insulin resistance. Furthermore, as shown in Figure 3D, G6Pase and PEPCK mRNA expression levels were markedly suppressed in the HFD II model, similar to a report on cirrhotic NASH patients.<sup>34</sup> Cholesterol overload and the resulting hepatocyte dysfunction in cirrhosis

are assumed to be the reasons for this down-regulation, indicating the actual impairment of glucose metabolism in the HFD II liver.

The serum ALT level has long been used as a surrogate marker for liver injury. However, ALT values do not correlate well with the severity of liver injury in human NAFLD.<sup>35,36</sup> Similar to that in patients with NAFLD but that unlike viral or drug-induced hepatitis, the ALT level in this rabbit model remained unchanged, but the mechanism remains unknown and should be clarified in future research.

Gut-derived lipopolysaccharide activates Kupffer cells by activating lipopolysaccharide receptors,<sup>37</sup> leading to increased production of inflammatory cytokines such as TNF $\alpha$ , IL-8, and IL-18.<sup>38,39</sup> The development of steatohepatitis in a NASH model, induced by a methionine- and choline-deficient diet, was partly inhibited in TLR4 mutant mice,<sup>40,41</sup> suggesting a role for TLR4-dependent signaling in the occurrence of this type of liver damage. In addition, the lipoprotein component of endotoxin from Gram-negative and -positive bacteria activates TLR2 and/or TLR4, which leads to common downstream activation of TRAF6 via the adapter molecule MyD88.<sup>42</sup> This cascade of events culminates in nuclear factor- $\kappa$ B activation, leading to the induction of TNF $\alpha$  and other proinflammatory cytokines.<sup>42</sup> Although TNF $\alpha$  has been identified as a central mediator contributing to insulin resistance and liver damage in NASH, little is known about the role of TLR2 or TLR4 in the induction of TNF $\alpha$ . In the present study, we observed an increase in the expression of TLR4/CD14/MD2 and TLR2 and cytokine induction in HFD II-fed rabbits. Persistent hepatic inflammation also triggers the activation of stellate cells and excess collagen production, resulting in the development of liver fibrosis.<sup>43,44</sup> Stellate cells are activated by LPS through TLR4/CD14/MD2 signaling.<sup>45,46</sup> Thus, the role of LPS in triggering steatohepatitis in this HFD II model deserves to be studied further in relation to hepatic fibrogenesis.

In the present study, we showed a reduction in hepatic glutathione content and decreased GST and SOD mRNA expression. Serum levels of antioxidants such as vitamin E, copper, and coenzyme Q10 increased significantly, but the levels of vitamin A and folic acid decreased significantly. As a result, there was an imbalance between oxidative stress and antioxidant protection systems in the present steatohepatitis model, as in human NASH patients.<sup>47-52</sup> The molecular mechanisms leading to the dysregulation of small antioxidant molecules are currently unknown. Most vitamin A in the body (approximately 70%) is usually stored in quiescent hepatic stellate cells,<sup>53</sup> but vitamin A storage is impeded when stellate cells are activated, under which activation they express  $\alpha$ SMA and produce extracellular matrix materials including type I collagen.<sup>43,44</sup> Thus, the reduction of serum vitamin A levels may reflect activation of stellate cells and the progression of fibrosis in our rabbits with HFD II-induced NASH. Folic acid is reportedly involved in the maintenance of normal concentrations of homocysteine, methionine, and S-adenosylmethionine.<sup>54</sup> Folic acid deficiency and abnormal hepatic methionine metabolism

are characteristics of alcoholic liver disease.<sup>54</sup> Thus, the reduction in serum folic acid observed in our rabbit NASH model might be a common feature of both alcoholic and nonalcoholic liver disease. Furthermore, the proteome analysis identified a reduction in GNMT and MAT1, which are associated with liver steatosis and fibrosis, in HFD II-fed rabbits.<sup>55–57</sup> Martínez-Chantar et al<sup>55</sup> showed that GNMT-knockout mice exhibited an elevation in serum aminotransferase, methionine, and S-adenosylmethionine and developed hepatic steatosis, fibrosis, and hepatocellular carcinoma. MAT1A-knockout mice were also reported to show liver steatosis.<sup>57</sup> These enzymes play an important role in the synthesis and degradation of S-adenosylmethionine.<sup>58</sup> Thus, an imbalance in S-adenosylmethionine and methionine metabolism may play a role in the development of steatohepatitis in our model.<sup>58</sup>

Phagocytic NADPH oxidases, such as gp91-phox, p40-phox, p67-phox, and p22-phox, increased in the present rabbit model (Supplemental Table S2, see <http://ajp.amjpathol.org>); however, the role of NADPH oxidase, a key molecule in the development of atherosclerosis,<sup>59</sup> in NASH is poorly understood. In alcoholic liver injury, NADPH oxidase is important for reactive oxygen species production in Kupffer cells and in hepatic stellate cells that initiate and promote liver injury.<sup>60,61</sup> We previously reported that the phagocytic activity of Kupffer cells promotes oxidative stress, inflammation, and fibrosis in steatohepatitis.<sup>7</sup> In this context, NADPH oxidase could play a prominent role in the pathogenesis of human NASH.

In the present study, we created a rabbit model of steatohepatitis, in which advanced fibrosis (close to cirrhosis) was produced by feeding a HFD. Hypercholesterolemia is a risk factor for liver injury as well as for atherosclerosis,<sup>2–5</sup> therefore, lowering the serum T-Chol level by dieting or by medications that reduce the synthesis and absorption of cholesterol could be a promising therapy for NAFLD including NASH. In this context, statins, which are HMG-CoA reductase inhibitors, have been reported to improve NASH.<sup>62,63</sup> Rallidis et al<sup>62</sup> showed that pravastatin treatment lowered serum ALT and improved histological steatosis in 5 NASH patients. Hyogo et al<sup>63</sup> treated patients with atorvastatin and found that 23 patients (74.2%) exhibited normalized transaminases and histological improvement of liver steatosis and the NAFLD activity score, and these changes were accompanied by a significant increase in serum adiponectin and a significant decrease in serum TNF $\alpha$ . In the present study, we studied the effect of ezetimibe, which inhibits NPC1L1 and therefore blocks intestinal absorption of cholesterol from the diet or that excreted in the bile.<sup>11–15</sup> The effect of ezetimibe on NASH has already been reported in rat models. Assy et al<sup>19</sup> studied methionine and choline deficiency-induced steatohepatitis in rats, in which ezetimibe administration alone or together with rosiglitazone, metformin, and valsartan reduced the hepatic levels of TG, T-Chol, and malondialdehyde and also significantly attenuated histological steatosis. In Zucker obese fatty rats, Deushi et al<sup>18</sup> reported that ezetimibe administration reduced the serum and hepatic levels of T-Chol and TG, the number of Oil red O-positive hepatocytes, Sirius red-stained collagen deposition, and

$\alpha$ SMA expression. Supporting these observations, we demonstrated the usefulness of ezetimibe treatment in a rabbit NASH model. Because human lipid metabolism is similar to that of rabbits but is different from that of mice and rats, our results strengthen the potential of ezetimibe for controlling fat deposition and fibrosis in human NASH. Interestingly, ezetimibe not only improved HFD I-induced liver steatosis but also reduced fibrosis and the number of  $\alpha$ SMA<sup>+</sup> cells. In chronic liver disease, TGF $\beta$ 1 plays an important role in the progression of liver fibrosis and stellate cell activation.<sup>43,44</sup> TGF $\beta$ 1 produced by Kupffer cells and stellate cells activates stellate cells in a paracrine as well as an autocrine manner and stimulates type I collagen production. In our rabbit model, ezetimibe reduced TGF $\beta$ 1 and type I collagen expression in the liver. Unlike mice, humans and rabbits show abundant NPC1L1 expression in the liver.<sup>15</sup> Further studies are required to clarify the effect of ezetimibe prophylactically after the HFD-fed rabbits have already developed steatohepatitis and to examine whether ezetimibe directly affects cholesterol metabolism in hepatocytes, thereby participating in the local prevention of fibrosis caused by abnormal cholesterol metabolism.<sup>14</sup>

In conclusion, rabbits fed HFD II for 9 months developed steatohepatitis with advanced fibrosis accompanied by the augmented expression of relevant genes. We demonstrated the presence of an imbalance between oxidative stress and antioxidant levels in HFD II-fed rabbits. Ezetimibe therapy was promising for alleviating the pathological changes in this model, suggesting the potential usefulness of this compound for human liver diseases caused by cholesterol overload.

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### References

1. Ford ES, Giles WH, Dietz WH: Prevalence of the metabolic syndrome among US adults: findings from the Third National Health and Nutrition Examination Survey. *JAMA* 2002, 287:356–359
2. Angulo P: Nonalcoholic fatty liver disease. *N Engl J Med* 2002, 346:1221–1231
3. Ludwig J, Viggiano TR, McGill DB, Oh BJ: Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. *Mayo Clin Proc* 1980, 55:434–438
4. Matteoni CA, Younossi ZM, Gramlich T, Boparai N, Liu YC, McCullough AJ: Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. *Gastroenterology* 1999, 116:1413–1419
5. Brunt EM, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR: Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. *Am J Gastroenterol* 1999, 94:2467–2474
6. Neuschwander-Tetri BA, Caldwell SH: Nonalcoholic steatohepatitis: summary of an AASLD Single Topic Conference. *Hepatology* 2003, 37:1202–1219
7. Otagawa K, Kinoshita K, Fujii H, Sakabe M, Shiga R, Nakatani K, Ikeda K, Nakajima Y, Ikura Y, Ueda M, Arakawa T, Hato F, Kawada N: Erythrophagocytosis by liver macrophages (Kupffer cells) promotes oxidative stress, inflammation, and fibrosis in a rabbit model of

- steatohepatitis: implications for the pathogenesis of human nonalcoholic steatohepatitis. *Am J Pathol* 2007, 170:967-980
8. Buja LM, Kita T, Goldstein JL, Watanabe Y, Brown MS: Cellular pathology of progressive atherosclerosis in the WHHL rabbit. An animal model of familial hypercholesterolemia. *Arteriosclerosis* 1983, 3:87-101
  9. Okita M, Hayashi M, Sasagawa T, Takagi K, Suzuki K, Kinoyama S, Ito T, Yamada G: Effect of a moderately energy-restricted diet on obese patients with fatty liver. *Nutrition* 2001, 17:542-547
  10. Huang MA, Greenon JK, Chao C, Anderson L, Peterman D, Jacobson J, Emick D, Lok AS, Conjeevaram HS: One-year intense nutritional counseling results in histological improvement in patients with non-alcoholic steatohepatitis: a pilot study. *Am J Gastroenterol* 2005, 100:1072-1081
  11. Altmann SW, Davis HR Jr, Zhu LJ, Yao X, Hoos LM, Tetzloff G, Iyer SP, Maguire M, Golovko A, Zeng M, Wang L, Murgolo N, Graziano MP: Niemann-Pick C1 like 1 protein is critical for intestinal cholesterol absorption. *Science* 2004, 303:1201-1204
  12. Davis HR Jr, Zhu LJ, Hoos LM, Tetzloff G, Maguire M, Liu J, Yao X, Iyer SP, Lam MH, Lund EG, Detmers PA, Graziano MP, Altmann SW: Niemann-Pick C1 like 1 (NPC1L1) is the intestinal phytosterol and cholesterol transporter and a key modulator of whole-body cholesterol homeostasis. *J Biol Chem* 2004, 279:33586-33592
  13. Temel RE, Tang W, Ma Y, Rudel LL, Willingham MC, Ioannou YA, Davies JP, Nilsson LM, Yu L: Hepatic Niemann-Pick C1-like 1 regulates biliary cholesterol concentration and is a target of ezetimibe. *J Clin Invest* 2007, 117:1968-1978
  14. Ge L, Wang J, Qi W, Miao HH, Cao J, Qu YX, Li BL, Song BL: The cholesterol absorption inhibitor ezetimibe acts by blocking the sterol-induced internalization of NPC1L1. *Cell Metab* 2008, 7:508-519
  15. Hawes BE, O'Neill KA, Yao X, Crona JH, Davis HR Jr, Graziano MP, Altmann SW: In vivo responsiveness to ezetimibe correlates with Niemann-Pick C1 like-1 (NPC1L1) binding affinity: comparison of multiple species NPC1L1 orthologs. *Mol Pharmacol* 2007, 71:19-29
  16. Knopp RH, Dujovne CA, Le Beau A, Lipka LJ, Suresh R, Veltri EP: Evaluation of the efficacy, safety, and tolerability of ezetimibe in primary hypercholesterolemia: a pooled analysis from two controlled phase III clinical studies. *Int J Clin Pract* 2003, 57:363-368
  17. Gómez-Garre D, Muñoz-Pacheco P, Gonzalez-Rubio ML, Aragoncillo P, Granados R, Fernandez-Cruz A: Ezetimibe reduces plaque inflammation in a rabbit model of atherosclerosis and inhibits monocyte migration in addition to its lipid-lowering effect. *Br J Pharmacol* 2009, 156:1218-1227
  18. Deushi M, Nomura M, Kawakami A, Haraguchi M, Ito M, Okazaki M, Ishii H, Yoshida M: Ezetimibe improves liver steatosis and insulin resistance in obese rat model of metabolic syndrome. *FEBS Lett* 2007, 581:5664-5670
  19. Assy N, Grozovski M, Bersudsky I, Szvalb S, Hussein O: Effect of insulin-sensitizing agents in combination with ezetimibe, and valsartan in rats with non-alcoholic fatty liver disease. *World J Gastroenterol* 2006, 12:4369-4376
  20. Yamagishi S, Nakamura K, Matsui T, Sato T, Takeuchi M: Inhibition of intestinal cholesterol absorption by ezetimibe is a novel therapeutic target for fatty liver. *Med Hypotheses* 2006, 66:844-846
  21. Buysens N, Kockx MM, Herman AG, Lazou JM, Van den Berg K, Wisse E, Geerts A: Centrolubular liver fibrosis in the hypercholesterolemic rabbit. *Hepatology* 1996, 24:939-946
  22. Seki S, Kitada T, Yamada T, Sakaguchi H, Nakatani K, Wakasa K: In situ detection of lipid peroxidation and oxidative DNA damage in non-alcoholic fatty liver diseases. *J Hepatol* 2002, 37:56-62
  23. Otogawa K, Ogawa T, Shiga R, Nakatani K, Ikeda K, Nakajima Y, Kawada N: Attenuation of acute and chronic liver injury in rats by iron-deficient diet. *Am J Physiol Regul Integr Comp Physiol* 2008, 294:R311-R320
  24. Kawada N, Kristensen DB, Asahina K, Nakatani K, Minamiyama Y, Seki S, Yoshizato K: Characterization of a stellate cell activation-associated protein (STAP) with peroxidase activity found in rat hepatic stellate cells. *J Biol Chem* 2001, 276:25318-25323
  25. Kristensen DB, Kawada N, Imamura K, Miyamoto Y, Tateno C, Seki S, Kuroki T, Yoshizato K: Proteome analysis of rat hepatic stellate cells. *Hepatology* 2000, 32:268-277
  26. Greco D, Kotronen A, Westerbacka J, Puig O, Arkkila P, Kiviluoto T, Laitinen S, Kolak M, Fisher RM, Hamsten A, Auvinen P, Yki-Jarvinen H: Gene expression in human NAFLD. *Am J Physiol Gastrointest Liver Physiol* 2008, 294:G1281-G1287
  27. Nakamura M, Kohjima M, Morizono S, Kotoh K, Yoshimoto T, Miyagi I, Enjoji M: Evaluation of fatty acid metabolism-related gene expression in nonalcoholic fatty liver disease. *Int J Mol Med* 2005, 16:631-635
  28. Puri P, Baillie RA, Wiest MM, Mirshahi F, Choudhury J, Cheung O, Sargeant C, Contos MJ, Sanyal AJ: A lipidomic analysis of nonalcoholic fatty liver disease. *Hepatology* 2007, 46:1081-1090
  29. Marí M, Caballero F, Colell A, Morales A, Caballeria J, Fernandez A, Enrich C, Fernandez-Checa JC, Garcia-Ruiz C: Mitochondrial free cholesterol loading sensitizes to TNF- and Fas-mediated steatohepatitis. *Cell Metab* 2006, 4:185-198
  30. Begeriche K, Igoudjil A, Pessayre D, Fromenty B: Mitochondrial dysfunction in NASH: causes, consequences and possible means to prevent it. *Mitochondrion* 2006, 6:1-28
  31. Parola M, Robino G: Oxidative stress-related molecules and liver fibrosis. *J Hepatol* 2001, 35:297-306
  32. Musso G, Gambino R, Durazzo M, Biroli G, Carello M, Faga E, Pacini G, De Michieli F, Rabbione L, Premoli A, Cassader M, Pagano G: Adipokines in NASH: postprandial lipid metabolism as a link between adiponectin and liver disease. *Hepatology* 2005, 42:1175-1183
  33. Fu JF, Fang YL, Liang L, Wang CL, Hong F, Dong GP: A rabbit model of pediatric nonalcoholic steatohepatitis: the role of adiponectin. *World J Gastroenterol* 2009, 15:912-918
  34. Sreekumar R, Rosado B, Rasmussen D, Charlton M: Hepatic gene expression in histologically progressive nonalcoholic steatohepatitis. *Hepatology* 2003, 38:244-251
  35. Mofrad P, Contos MJ, Haque M, Sargeant C, Fisher RA, Luketic VA, Sterling RK, Shiffman ML, Stravitz RT, Sanyal AJ: Clinical and histologic spectrum of nonalcoholic fatty liver disease associated with normal ALT values. *Hepatology* 2003, 37:1286-1292
  36. Wong VW, Wong GL, Tsang SW, Hui AY, Chan AW, Choi PC, Chim AM, Chu S, Chan FK, Sung JJ, Chan HL: Metabolic and histological features of non-alcoholic fatty liver disease patients with different serum alanine aminotransferase levels. *Aliment Pharmacol Ther* 2009, 29:387-396
  37. Enomoto N, Yamashina S, Kono H, Schemmer P, Rivera CA, Enomoto A, Nishiura T, Nishimura T, Brenner DA, Thurman RG: Development of a new, simple rat model of early alcohol-induced liver injury based on sensitization of Kupffer cells. *Hepatology* 1999, 29:1680-1689
  38. Tomita K, Tamiya G, Ando S, Ohsumi K, Chiyo T, Mizutani A, Kitamura N, Toda K, Kaneko T, Horie Y, Han JY, Kato S, Shimoda M, Oike Y, Tomizawa M, Makino S, Ohkura T, Saito H, Kumagai N, Nagata H, Ishii H, Hibi T: Tumour necrosis factor  $\alpha$  signalling through activation of Kupffer cells plays an essential role in liver fibrosis of non-alcoholic steatohepatitis in mice. *Gut* 2006, 55:415-424
  39. Wang HN, Wang YR, Liu GQ, Liu Z, Wu PX, Wei XL, Hong TP: Inhibition of hepatic interleukin-18 production by rosiglitazone in a rat model of nonalcoholic fatty liver disease. *World J Gastroenterol* 2008, 14:7240-7246
  40. Szabo G, Velayudham A, Romics L Jr, Mandrekar P: Modulation of non-alcoholic steatohepatitis by pattern recognition receptors in mice: the role of Toll-like receptors 2 and 4. *Alcohol Clin Exp Res* 2005, 29:140S-145S
  41. Rivera CA, Adegboyega P, van Rooijen N, Tagalicud A, Allman M, Wallace M: Toll-like receptor-4 signaling and Kupffer cells play pivotal roles in the pathogenesis of non-alcoholic steatohepatitis. *J Hepatol* 2007, 47:571-579
  42. Akira S, Takeda K: Toll-like receptor signalling. *Nat Rev Immunol* 2004, 4:499-511
  43. Kawada N: The hepatic perisinusoidal stellate cell. *Histol Histopathol* 1997, 12:1069-1080
  44. Friedman SL: Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. *J Biol Chem* 2000, 275:2247-2250
  45. Paik YH, Schwabe RF, Batailler R, Russo MP, Jobin C, Brenner DA: Toll-like receptor 4 mediates inflammatory signaling by bacterial lipopolysaccharide in human hepatic stellate cells. *Hepatology* 2003, 37:1043-1055
  46. Seki E, De Minicis S, Osterreicher CH, Kluwe J, Osawa Y, Brenner DA, Schwabe RF: TLR4 enhances TGF- $\beta$  signaling and hepatic fibrosis. *Nat Med* 2007, 13:1324-1332
  47. Koruk M, Taysi S, Savas MC, Yilmaz O, Akcay F, Karakok M: Oxidative stress and enzymatic antioxidant status in patients with nonalcoholic steatohepatitis. *Ann Clin Lab Sci* 2004, 34:57-62
  48. Nobili V, Pastore A, Gaeta LM, Tozzi G, Comparcola D, Sartorelli MR, Marcellini M, Bertini E, Piemonte F: Glutathione metabolism and an-

- tioxidant enzymes in patients affected by nonalcoholic steatohepatitis. *Clin Chim Acta* 2005, 355:105-111
49. Yesilova Z, Yaman H, Oktenli C, Ozcan A, Uygun A, Cakir E, Sanisoglu SY, Erdil A, Ates Y, Aslan M, Musabak U, Erbil MK, Karaeren N, Dagalp K: Systemic markers of lipid peroxidation and antioxidants in patients with nonalcoholic fatty liver disease. *Am J Gastroenterol* 2005, 100:850-855
  50. Chalasani N, Deeg MA, Crabb DW: Systemic levels of lipid peroxidation and its metabolic and dietary correlates in patients with non-alcoholic steatohepatitis. *Am J Gastroenterol* 2004, 99:1497-1502
  51. Akin K, Beyler AR, Kaya M, Erden E: The importance of iron and copper accumulation in the pathogenesis of non-alcoholic steatohepatitis. *Turk J Gastroenterol* 2003, 14:228-233
  52. Machado MV, Ravasco P, Jesus L, Marques-Vidal P, Oliveira CR, Proenca T, Baldeiras I, Camilo ME, Cortez-Pinto H: Blood oxidative stress markers in non-alcoholic steatohepatitis and how it correlates with diet. *Scand J Gastroenterol* 2008, 43:95-102
  53. Wake K: "Sternzellen" in the liver: perisinusoidal cells with special reference to storage of vitamin A. *Am J Anat* 1971, 132:429-462
  54. Purohit V, Abdelmalek MF, Barve S, Benevenga NJ, Halsted CH, Kaplowitz N, Kharbanda KK, Liu QY, Lu SC, McClain CJ, Swanson C, Zakhari S: Role of S-adenosylmethionine, folate, and betaine in the treatment of alcoholic liver disease: summary of a symposium. *Am J Clin Nutr* 2007, 86:14-24
  55. Martínez-Chantar ML, Vazquez-Chantada M, Ariz U, Martínez N, Varela M, Luka Z, Capdevila A, Rodríguez J, Aransay AM, Matthiesen R, Yang H, Calvisi DF, Esteller M, Fraga M, Lu SC, Wagner C, Mato JM: Loss of the glycine N-methyltransferase gene leads to steatosis and hepatocellular carcinoma in mice. *Hepatology* 2008, 47:1191-1199
  56. Wortham M, He L, Gyamfi M, Copple BL, Wan YJ: The transition from fatty liver to NASH associates with SAMe depletion in db/db mice fed a methionine choline-deficient diet. *Dig Dis Sci* 2008, 53:2761-2774
  57. Lu SC, Alvarez L, Huang ZZ, Chen L, An W, Corrales FJ, Avila MA, Kanel G, Mato JM: Methionine adenosyltransferase 1A knockout mice are predisposed to liver injury and exhibit increased expression of genes involved in proliferation. *Proc Natl Acad Sci USA* 2001, 98:5560-5565
  58. Mato JM, Lu SC: Role of S-adenosyl-L-methionine in liver health and injury. *Hepatology* 2007, 45:1306-1312
  59. Park YM, Febbraio M, Silverstein RL: CD36 modulates migration of mouse and human macrophages in response to oxidized LDL and may contribute to macrophage trapping in the arterial intima. *J Clin Invest* 2009, 119:136-145
  60. Batailler R, Schwabe RF, Choi YH, Yang L, Paik YH, Lindquist J, Qian T, Schoonhoven R, Hagedorn CH, Lemasters JJ, Brenner DA: NADPH oxidase signal transduces angiotensin II in hepatic stellate cells and is critical in hepatic fibrosis. *J Clin Invest* 2003, 112:1383-1394
  61. Wheeler MD, Kono H, Yin M, Nakagami M, Uesugi T, Arteel GE, Gabele E, Rusyn I, Yamashina S, Froh M, Adachi Y, Jimuro Y, Bradford BU, Smutney OM, Connor HD, Mason RP, Goyert SM, Peters JM, Gonzalez FJ, Samulski RJ, Thurman RG: The role of Kupffer cell oxidant production in early ethanol-induced liver disease. *Free Radic Biol Med* 2001, 31:1544-1549
  62. Rallidis LS, Drakoulis CK, Parasi AS: Pravastatin in patients with nonalcoholic steatohepatitis: results of a pilot study. *Atherosclerosis* 2004, 174:193-196
  63. Hyogo H, Tazuma S, Arihiro K, Iwamoto K, Nabeshima Y, Inoue M, Ishitobi T, Nonaka M, Chayama K: Efficacy of atorvastatin for the treatment of nonalcoholic steatohepatitis with dyslipidemia. *Metabolism* 2008, 57:1711-1718

# Reversibility of fibrosis, inflammation, and endoplasmic reticulum stress in the liver of rats fed a methionine–choline-deficient diet

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Fatty liver disease has become a health problem related to metabolic syndrome worldwide, although its molecular pathogenesis requires further study. It is also unclear whether advanced fibrosis of steatohepatitis will regress when diet is controlled. The aim of this study was to investigate whether the resolution of fibrosis occurs in steatohepatitis induced by a methionine–choline-deficient diet (MCDD). Manifestation of endoplasmic reticulum (ER) stress in this model was also studied. Nonalcoholic steatohepatitis with advanced fibrosis was induced in rats by feeding them an MCDD for 10 weeks. Instead of MCDD, a methionine–choline control diet (CD) was given for the last 2 weeks to the experimental group. Fibrosis and inflammation were determined by tissue staining. Protein and gene expressions were determined by immunoblotting and quantitative reverse transcription-PCR (RT-PCR), respectively. Expressions of caspase-7, caspase-12, glucose-regulated protein 78 (GRP78), and protein disulfide isomerase were evaluated to clarify the presence of ER stress. Changing the diet from MCDD to CD triggered the reduction of fat in hepatocytes, a decrease in inflammatory gene expression and oxidative stress, and regression of fibrosis accompanied by the disappearance of activated stellate cells and macrophages. Immunohistochemistry, immunoblotting, and RT-PCR analysis all indicated the occurrence of ER stress in steatohepatitis, while it recovered immediately after changing the diet from MCDD to CD. The ratio of hepatocyte proliferation/apoptosis increased significantly during the recovery stage. This simple experiment clearly shows that changing the diet from MCDD to a normal diet (CD) triggers the resolution of hepatic inflammatory and fibrotic reactions and hepatocyte apoptosis, suggesting that MCDD-induced steatohepatitis is also reversible. ER stress appears and disappears in association with the generation and regression of steatohepatitis, respectively, with fibrosis.

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**KEYWORDS:** caspase; cytoglobin; hepatic stellate cells; hepatocytes; Kupffer cells; oxidative stress

Nonalcoholic fatty liver disease (NAFLD) is a relatively newly defined hepatic sequela of obesity and type II diabetes mellitus,<sup>1–3</sup> and it is one of the most common causes of chronic liver disease in many countries.<sup>4</sup> A number of studies have identified a significant correlation between hepatic steatosis and fibrosis.<sup>5–7</sup> NAFLD covers a progressive spectrum of liver pathologies from simple steatosis to nonalcoholic steatohepatitis (NASH), which is characterized by necroinflammation and fibrosis, and, subsequently, by cirrhosis.<sup>8</sup> It is also speculated that NASH may progress to hepatocellular carcinoma.<sup>9–11</sup>

Although adipocytokines, cytokines, and free fatty acids (FFAs) derived from peripheral and visceral fat tissues, which are known as mediators of metabolic syndrome, are assumed

to contribute to the initiation of inflammatory reactions in the liver, the link between the development of hepatic steatosis and fibrosis is still poorly understood.<sup>12</sup> Furthermore, whether the regression of liver fibrosis caused by NASH occurs after controlling diet has not been proven, but the reversibility of liver fibrosis has been evidenced in patients who have successfully achieved the eradication of hepatitis C virus after interferon therapy.<sup>13,14</sup>

Over the past decade, it has become clear that obesity is associated with the activation of cellular stress signaling and inflammatory pathways.<sup>15–17</sup> A key cell organelle in cellular stress response is the endoplasmic reticulum (ER), a membranous network that functions in the synthesis and processing of secreted and membranous proteins. Certain

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pathological stress conditions disrupt ER homeostasis and lead to the accumulation of unfolded or misfolded proteins in the ER lumen, known as ER stress, resulting in the activation of signal transduction systems.<sup>18–20</sup> ER stress is caused by glucose or nutrient deprivation, viral infections, lipids, increased synthesis of secretory proteins, and expression of mutant or misfolded proteins, and has been recently implicated in human diseases, such as Alzheimer's disease, Parkinson's disease, diabetes mellitus, and liver disease.<sup>21–24</sup>

In this study, a rat steatohepatitis model induced by a methionine–choline-deficient diet (MCDD) for 10 weeks was used to obtain evidence for the resolution of fibrosis in fatty liver after changing the diet to a methionine–choline control diet (CD). The manifestation of ER stress was also studied. The results of this study clearly indicated that a dietary change from MCDD to CD immediately initiates tissue remodeling, which is associated with the cessation of cellular apoptosis and ER stress.

## MATERIALS AND METHODS

### Materials

Mouse monoclonal antibody against  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA, Clone 1A4) and mouse monoclonal antibody against 5-bromo-2-deoxyuridine (BrdU, Clone 1BU33, 1:1000) were obtained from Sigma Chemical (St Louis, MO, USA). Rat monoclonal antibody against caspase-12 and goat polyclonal antibodies against glucose-regulated protein 78 (GRP78) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibodies against caspase-7, rabbit polyclonal antibodies against cleaved caspase-7 (Asp198), and rabbit polyclonal antibodies against protein disulfide isomerase (PDI) were obtained from Cell Signaling Technology (Danvers, MA, USA). Mouse monoclonal antibody against CD68 (Clone, KP1) was purchased from Dako Denmark A/S (Glostrup, Denmark). Mouse monoclonal antibody against heme oxygenase-1 (HO-1, Hsp32) was obtained from Assay Designs (Ann Arbor, MI, USA). Mouse monoclonal antibody against 4-hydroxy-2-nonenal (4-HNE) was purchased from the Japan Institute for the Control of Aging (Shizuoka, Japan). Mouse monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was obtained from Chemicon International (Temecula, CA, USA). Rabbit anti-cytoglobin antibodies were produced in our laboratory as described previously.<sup>25</sup> Horseradish peroxidase (HRP)-conjugated polyclonal rabbit anti-goat immunoglobulins, HRP-conjugated polyclonal rabbit anti-mouse immunoglobulins, HRP-conjugated polyclonal rabbit anti-rat immunoglobulins, and HRP-conjugated polyclonal swine anti-rabbit immunoglobulins were obtained from Dako Denmark A/S. Hybond-ECL nitrocellulose membranes and ECL detection reagent were obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). All other reagents were purchased from Sigma Chemical or Wako Pure Chemical.

### Animals and Experimental Protocol

Pathogen-free male Wistar rats (7–8 weeks of age) were obtained from SLC (Shizuoka, Japan). Animals were housed at a constant temperature and supplied with laboratory chow and water *ad libitum*. The experimental protocol was approved by the Animal Research Committee of Osaka City University (Guide for Animal Experiments, Osaka City University).

The rats were fed a CD (group C,  $n=5$ ) or MCDD (group M,  $n=5$ ) for 10 weeks. The contents of MCDD and CD are listed in Supplementary Table 1. A recovery model (group R,  $n=5$ ) was produced by administering MCDD for 8 weeks and thereafter CD for 2 weeks. During the experimental period, individual body weights were recorded twice per week (see Supplementary Table 2).

### Sample Harvesting

At the end of the tenth week, rats were killed under ether anesthesia, and the portal vein was cannulated using an 18-G Teflon catheter. Blood samples were collected from the inferior vena cava, centrifuged at 3000 r.p.m. for 30 min at 4°C, and the obtained sera were kept at –70°C until further use for serum chemistry tests. The liver of each animal was perfused with 100 ml of phosphate-buffered saline (pH 7.0) to remove blood and then washed with ice-cold saline, dried using filter paper, and weighed in a wet state. A portion of the liver was fixed with 4% paraformaldehyde, embedded in paraffin, and frozen. Another portion of the liver was snap-frozen in liquid nitrogen and stored at –70°C until use for reverse transcription-PCR (RT-PCR) and immunoblots.

### Serum Chemistries

Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglycerides (TGs), and FFAs were measured at Special Reference Laboratories (Osaka, Japan).

### Histochemical and Immunohistochemical Analyses of the Rat Liver

Paraformaldehyde-fixed specimens were cut into 5- $\mu$ m-thick sections and stained for 1 h with 0.1% (w/v) Sirius Red (Direct Red 80; Aldrich, Milwaukee, WI, USA), Oil red O, or hematoxylin and eosin (H&E). Immunohistochemistry was performed according to the methods described elsewhere.<sup>26</sup> Briefly, sections were deparaffinized, washed, and pre-incubated in blocking solution, followed by incubation with anti-4-HNE (15  $\mu$ g/ml), HO-1 (1:50), CD68 (1:100),  $\alpha$ -SMA (1:100), cytoglobin (1:400), or caspase-12 (1:100) antibodies. Sections were then incubated with HRP-conjugated secondary antibodies (1:1000), washed, covered with DAB, and counterstained with hematoxylin. Some of the immunostainings were performed at the Biopathology Institute (Oita, Japan). The red area on Oil red O staining was image analyzed by LuminaVision (Mitani Corporation, Tokyo, Japan).

### TdT-Mediated dUTP Nick-End Labeling Assays

For the detection of apoptotic cells, paraffin-embedded sections were stained with the TdT-Mediated dUTP Nick-End Labeling (TUNEL) technique using an *In Situ* Apoptosis Detection kit (Takara Shuzo, Ohtsu, Japan) according to the manufacturer's instructions. For semi-quantitative analysis, the number of TUNEL-positive cells was counted in five randomly selected fields by viewing each slide at a magnification of  $\times 400$ , and the average number in each group was calculated, as described previously.<sup>27</sup>

### BrdU Assay for Hepatocyte Proliferation

For the detection of hepatocyte proliferation, rats were i.p. injected with BrdU (100  $\mu\text{g}/\text{kg}$ ) 2 h before being killed. *In situ* detection of the incorporation of BrdU into the nuclei was conducted immunohistochemically.<sup>28</sup> For semi-quantitative analysis, as for TUNEL, the number of BrdU-positive cells was counted in five randomly selected fields by viewing each slide at a magnification of  $\times 200$ , and the average number in each group was calculated.

### Quantitative RT-PCR

mRNA expressions of tumor necrosis factor-alpha (TNF- $\alpha$ ), transforming growth factor-beta 1 (TGF- $\beta$ 1),  $\alpha$ -SMA, collagen 1A2 (COL1A2), matrix metalloproteinases (MMPs)-2, -9, and -13, tissue inhibitor of MMP-1 (TIMP-1), caspases-3, -7, -9, and -12, GRP78, interleukin-6 (IL-6), BAX, BAK, Bcl-xl, Bcl-2, and ERp57 were assessed by quantitative RT-PCR. Total RNA was extracted from liver tissues using Isogen (Nippon Gene, Tokyo, Japan).<sup>29</sup> The expression of mRNA was measured using TaqMan One-Step RT-PCR Master Mix Reagents (Applied Biosystems, Foster City, CA, USA) or using the One-Step SYBR RT-PCR Kit (Perfect Real Time; Takara Bio, Ohtsu, Japan), and Applied Biosystems Prism 7700 (Applied Biosystems) according to a previously reported procedure.<sup>27</sup> Primers and oligonucleotide probes were designed using Primer Express (Sigma Chemical), and are listed in Table 1. Each PCR amplification was performed on five rats in both experimental and control groups. Individual gene expression was normalized by GAPDH. The conditions for the TaqMan One-Step RT-PCR were as follows: 30 min at 48°C (stage 1, RT), 10 min at 95°C (stage 2, RT inactivation and Ampli Taq Gold activation), and then 40 cycles of amplification for 15 s at 95°C and 1 min at 60°C (stage 3, PCR). The conditions for the One-Step SYBR RT-PCR (Perfect Real Time) were as follows: an initial step of 15 min at 42°C, 2 min at 95°C, and then 40 amplification cycles of denaturation at 95°C for 15 s, and annealing and extension at 60°C for 1 min.

### Immunoblot Analysis

Some protein levels were assessed by immunoblot analysis, as described previously.<sup>29</sup> The liver tissue was lysed by RIPA buffer containing 50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM EDTA, and 1 mM PMSF and then homogenized in ice-cold water. After centrifugation for

10 min at 4°C and 12 000 r.p.m., the protein concentration of the obtained supernatant was determined using the Bio-Rad Dc protein Assay Reagent (Bio-Rad, Hercules, CA, USA). Protein was electrophoretically resolved in 10 or 12% SDS polyacrylamide gel, and successively transferred to Hybond-ECL nitrocellulose membranes. The membranes were blocked by 5% non-fat dietary milk solution in Tris-buffered saline (20 mM Tris and 150 mM NaCl, pH 7.4) with 0.1% Tween-20. They were then incubated overnight with primary antibodies at 4°C and successively with secondary antibodies at room temperature for 1 h. The following dilutions of primary antibodies were used: mouse monoclonal antibody to  $\alpha$ -SMA, 1:1000; rabbit polyclonal antibodies to cytoglobin, 1:200; rat monoclonal antibody to caspase-12, 1:200; goat polyclonal antibodies to GRP78, 1:200; rabbit polyclonal antibodies to caspase-7, 1:1000; rabbit polyclonal antibodies to cleaved caspase-7 (Asp198), 1:1000; rabbit polyclonal antibodies against PDI, 1:200; mouse monoclonal antibody to GAPDH, 1:30 000. Immune complexes were visualized using a SuperSignal West Pico Chemiluminescent Substrate (ECL, Pierce, Rockford, IL, USA). Finally, band intensity was determined by scanning video densitometry.

### Statistical Analysis

All results are expressed as mean  $\pm$  s.d. Statistical analysis was performed using Student's *t*-test ( $P < 0.05$  was considered significant).

## RESULTS

### Accumulation of Fat in the Liver and Recovery after Dietary Change

As shown in Figure 1a and b, Oil red O staining clearly indicated that the MCDD diet (group M) for 10 weeks induced fat accumulation in the liver, especially in hepatocytes. It was also recognized that fat accumulation immediately decreased in group R ( $P < 0.01$ ), whose diet was changed from MCDD to CD during the last 2 weeks. The serum level of FFA was reduced in group M ( $183 \pm 31.6 \mu\text{equiv./l}$ ) compared with control group C ( $274 \pm 35.7 \mu\text{equiv./l}$ ) and recovery group R ( $267 \pm 36.5 \mu\text{equiv./l}$ ) ( $P < 0.01$ ). Similarly, the serum level of TG was reduced in group M ( $4.33 \pm 1.21 \text{ mg/dl}$ ) compared with control group C ( $23.5 \pm 4.95 \text{ mg/dl}$ ) and group R ( $28.2 \pm 4.97 \text{ mg/dl}$ ) ( $P < 0.01$ ) (Figure 1c and d,  $P < 0.01$ ). Changes in the body weights of rats in each group are presented in Supplementary Table 2.

### Liver Histology and Kupffer Cell Activation

H&E staining showed that the livers in group C showed an intact tissue structure, whereas those in group M showed the apparent vacuolization of hepatocytes, focal necrosis, and inflammatory cell accumulation in the parenchyma. These pathological changes were clearly improved in group R (Figure 2a). In accordance with this histological recovery, serum levels of AST and ALT were reduced from  $97.7 \pm 13.5$



**Table 1 Primer pairs and probes used for real-time PCR**

Primer name	Sequence	Note
<i>TNF-α</i>		TaqMan
Forward	5'-GCT CCC TCT CAT CAG TTC CAT G-3'	
Reverse	5'-TAC GGG CTT GTC ACT CGA GTT TTG-3'	
Probe	5'-CCC AGA CCC TCA CAC TCA GAT CAT CTT C-3'	
<i>Heme oxygenase-1</i>		SYBR Green
Forward	5'-CGT GGC AGT GGG AAT TTA TG-3'	
Reverse	5'-AGG CTA CAT GAG ACA GAG TTC ACA-3'	
<i>TGF-β1</i>		TaqMan
Forward	5'-TGC TTC CGC ATC ACC GT-3'	
Reverse	5'-TAG TAG ACG ATG GGC AGT GGC-3'	
Probe	5'-CTG CGT GCC GCA GGC TTT GG-3'	
<i>Collagen IA2</i>		TaqMan
Forward	5'-AAG GGT CCT TCT GGA GAA CC-3'	
Reverse	5'-TCG AGA GCC AGG GAG ACC CA-3'	
Probe	5'-CAG GGT CTT CTT GGT GCT CCC GGT AT-3'	
<i>α-SMA</i>		TaqMan
Forward	5'-GAG GAG CAT CCG ACC TTGC-3'	
Reverse	5'-TTT CTC CCG GTT GGC CTTA-3'	
Probe	5'-AAC GGA GGC GCC GCT GAA CC-3'	
<i>MMP-2</i>		TaqMan
Forward	5'-CCG AGG ACT ATG ACC GGG ATA A-3'	
Reverse	5'-CTT GTT GCC CAG GAA AGT GAA G-3'	
Probe	5'-TCT GCC CCG AGA CCG CTA TGT CCA-3'	
<i>MMP-9</i>		SYBR Green
Forward	5'-GAC AAT CCT TGC AAT GTG GAT G-3'	
Reverse	5'-CCG ACC GTC CTT GAA GAA ATG-3'	
<i>MMP-13</i>		SYBR Green
Forward	5'-TGA CCT GGG ATT TCC AAA AGA G-3'	
Reverse	5'-TCT TCC CCG TGT CCT CAA A-3'	
<i>TIMP-1</i>		SYBR Green
Forward	5'-TCA GCC ATC CCT TGC AAA-3'	
Reverse	5'-GAG CCC ATG AGG ATC TGA TCT-3'	

**Table 1 Continued**

Primer name	Sequence	Note
<i>Caspase-12</i>		SYBR Green
Forward	5'-GGC AGA CAT ACT GGT ACT ATT TGG G-3'	
Reverse	5'-GCT CAA CAC ACA TTC CTC ATC TGT-3'	
<i>Caspase-9</i>		SYBR Green
Forward	5'-TGG ACA TTG GTT CTG GCA GAG-3'	
Reverse	5'-GTG TAT GCC ATA TCT GCA TGT CTC-3'	
<i>Caspase-7</i>		SYBR Green
Forward	5'-TAC AAG ATC CCG GTG GAA GCT-3'	
Reverse	5'-CTG GGT TCC TCC ACG AAT AAT AG-3'	
<i>Caspase-3</i>		SYBR Green
Forward	5'-AGA AAT TCA AGG GAC GGG TC-3'	
Reverse	5'-TGC GCG TAC AGT TTC AGC A-3'	
<i>GRP78</i>		SYBR Green
Forward	5'-CCA TCA CCA ATG ACC AAA ACC-3'	
Reverse	5'-GCG CTC TTT GAG CTT TTT GTC T-3'	
<i>ERp57</i>		SYBR Green
Forward	5'-TGAATGCTGAAGACAAGGACGTG-3'	
Reverse	5'-CATTGGCTGTGGCATCCATC-3'	
<i>BAX</i>		SYBR Green
Forward	5'-TAAAGTGCCCGAGCTGATCAGAACC-3'	
Reverse	5'-CCTGGTCTTGATCCAGACAAGCA-3'	
<i>BAK</i>		SYBR Green
Forward	5'-GAGTTTGCGTAGAGACCCATCCT-3'	
Reverse	5'-CCACAAATTGGCCCAACAGAACCA-3'	
<i>Bcl-xl</i>		SYBR Green
Forward	5'-TGCCTGGAAGCGTAGACAAGGA-3'	
Reverse	5'-AAGGCTCTAGGTGGTCTTCCAGGT-3'	

Table 1 Continued

Primer name	Sequence	Note
<i>Bcl-2</i>		SYBR Green
Forward	5'-TCGCGACTTTGCAGAGATGTCC-3'	
Reverse	5'-ACCCCATCCCTGAAGAGTTCCT-3'	
<i>IL-6</i>		TaqMan
Forward	5'-TGT CTC GAG CCC ACC AGG-3'	
Reverse	5'-TGC GGA GAG AAA CTT CAT AGC TG-3'	
Probe	5'-CGA AAG TCA ACT CCA TCT GCC CTT CAG G-3'	
<i>STAT3</i>		SYBR Green
Forward	5'-CAA TAC CAT TGA CCT GCC GAT-3'	
Reverse	5'-CCC CGT TAT TTC CAA ACT GC-3'	
<i>GAPDH</i>		TaqMan
Forward	5'-AAG ATG GTG AAG GTC GGT GTG-3'	
Reverse	5'-GAA GGC AGC CCT GGT AAC-3'	
Probe	5'-CGG ATT TGG CCG TAT CGG ACGC-3'	
<i>GAPDH</i>		SYBR Green
Forward	5'-AAT GCA TCC TGC ACC ACC AAC TGC-3'	
Reverse	5'-GGA GGC CAT GTA GGC CATG AGG TC-3'	

and  $98.5 \pm 14.9$  IU/ml in group M and from  $51.2 \pm 13.8$  and  $35.4 \pm 3.3$  IU/ml in group R, respectively ( $P < 0.01$ ). Cells positive for CD68, a marker of activated Kupffer cells, increased in number (Figure 2b) in group M. The expression of TNF- $\alpha$  mRNA, which is known to be derived from activated Kupffer cells in the liver, increased to 2.5-fold in group M (Figure 2e,  $P < 0.01$ ) compared with group C, and returned to the normal range in group R ( $P < 0.01$ ). These results indicate that the change in diet from MCDD to CD reduced inflammatory reactions, Kupffer cell activation, and AST/ALT release from hepatocytes.

#### Oxidative Stress and its Recovery in Steatohepatitis

We used the immunostaining of 4-HNE to detect the production of oxidative aldehyde by lipid peroxidation in the liver. As shown in Figure 3a, the control liver of group C exhibited negligible staining by anti-4-HNE antibodies. In contrast, the liver in group M showed brown-colored hepatocytes and stained cells also accumulated at necrotic foci (magnified area). The number of 4-HNE-positive cells and

the amount of granulomatous accumulation in cells, presumably macrophages, were clearly reduced in group R.

These results were reproduced on immunostaining of HO-1. As shown in Figure 3b, HO-1 expression is restricted in hepatic sinusoids, presumably in Kupffer cells (magnified area). However, in group M, HO-1-positive cells additionally led to an aggregation of cells next to vacuolized hepatocytes (magnified area). These reactions immediately disappeared in group R. In accordance with these results, HO-1 mRNA expression determined by RT-PCR was significantly augmented in group M compared with group C ( $5.19 \pm 1.08$  vs  $3.13 \pm 0.26$ , respectively,  $P < 0.01$ ) and returned to the normal level in group R.

Taken together, it can be stated that the accumulation of Oil red O-stained lipids, 4-HNE, and CD68- and HO-1-positive macrophages by administering an MCDD diet generates oxidative stress and hepatocyte damage (increase in AST and ALT levels), resulting in inflammatory gene expression, such as TNF- $\alpha$ . However, switching the diet from MCDD to CD immediately triggers the inhibition of inflammatory reactions.

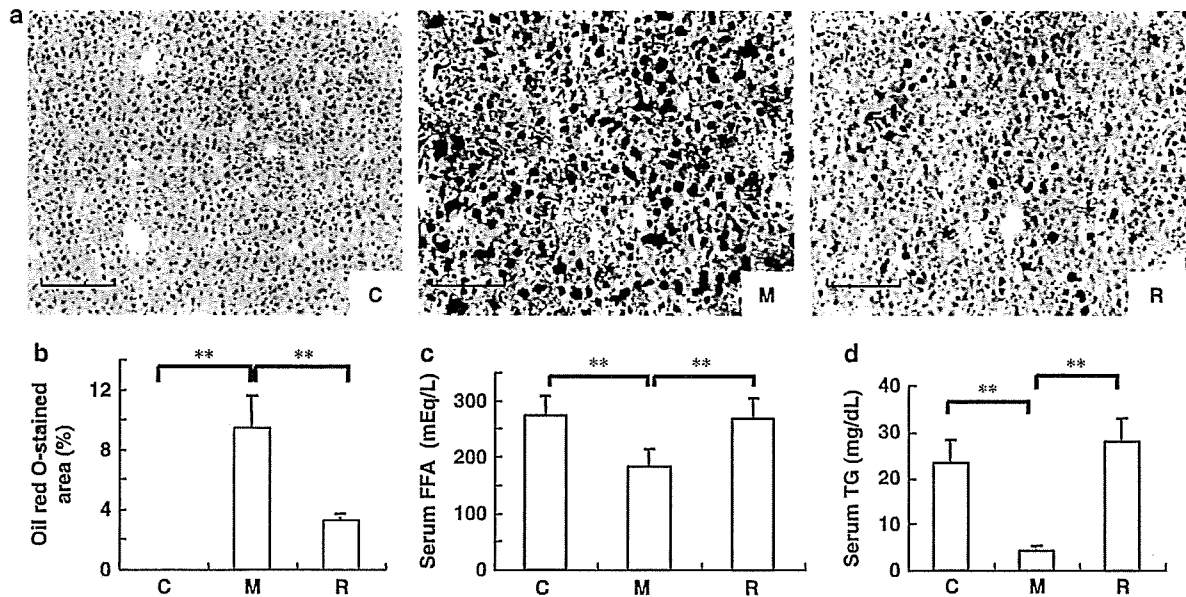
#### Regression of Fibrosis and Hepatic Stellate Cell Activation in Steatohepatitis

Sirius Red staining (Figure 4a) clearly revealed collagen deposition with C-C and P-C bridges, near to cirrhosis, in the fibrotic septum of group M, although collagen deposition was observed only around veins in the intact liver (group C). An immediate and marked recovery of this advanced fibrosis was observed in group R, indicating that the change in diet from MCDD to CD triggers the regression of fibrosis in the liver within 2 weeks.

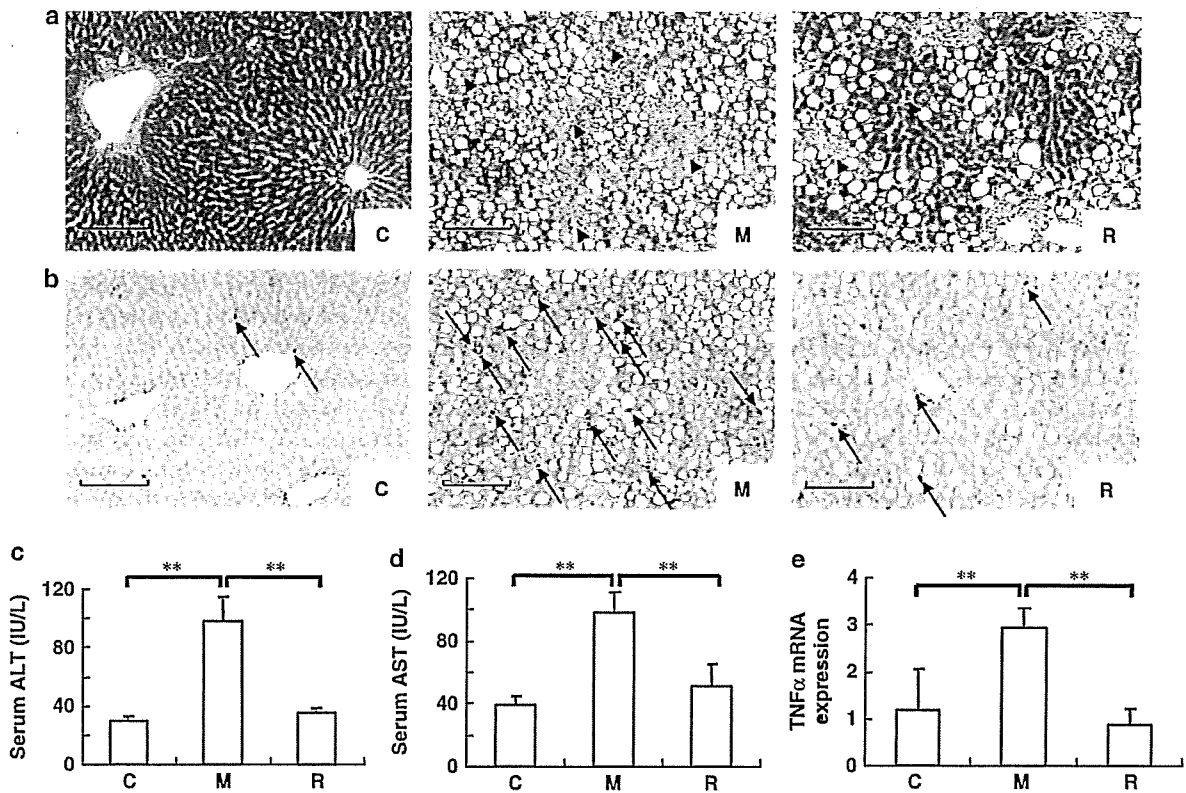
Immunohistochemistry of  $\alpha$ -SMA and cytoglobin, markers of activated stellate cells and liver myofibroblasts, showed that hepatic fibrotic cell activation occurred in group M, whereas the process ceased immediately in group R (Figure 4b and c). Immunoblot analyses confirmed these immunohistochemical observations (Figure 4d). Furthermore, as shown in Figure 4e, RT-PCR analyses indicated that mRNA expressions of all  $\alpha$ -SMA, TGF- $\beta$ 1, Col1A2, TIMP-1, MMP-2, and MMP-9 increased significantly in group M, and thereafter returned to the original level in group R. Conversely, the expression of MMP-13 decreased significantly in group M ( $0.98 \pm 0.30$  vs  $3.39 \pm 1.24$ ,  $P < 0.01$ ) and recovered significantly in group R ( $2.30 \pm 1.11$ ). Thus, the recovery of fibrosis is evident at levels of histology, fibrotic protein, and fibrotic gene expression after switching the diet from MCDD to CD.

#### The Change of Hepatocyte Apoptosis and Proliferation

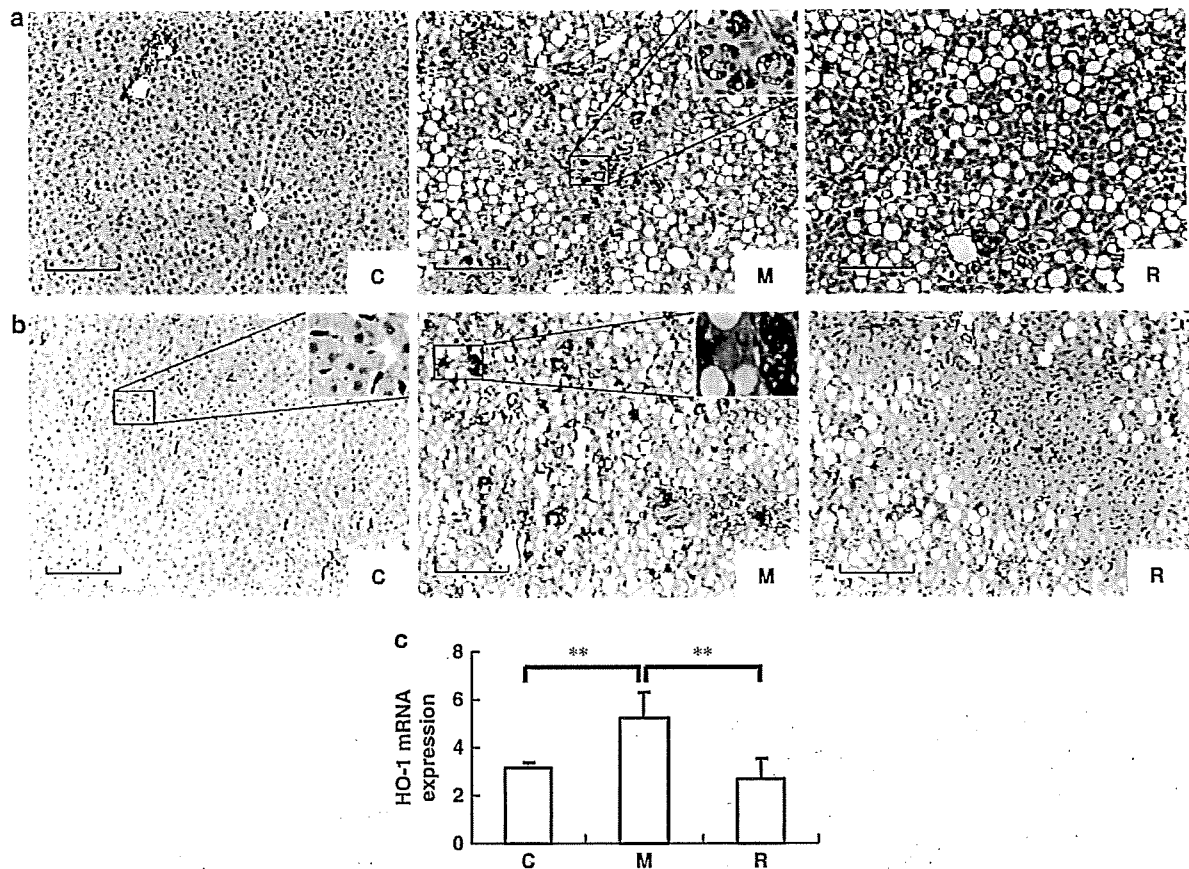
TUNEL staining showed that the number of apoptotic hepatocytes slightly increased in group M compared with group C ( $68.2 \pm 15.9$  vs  $40.3 \pm 8.33$  cells per field, respectively,  $P < 0.05$ ), whereas it decreased significantly in group R ( $48.0 \pm 6.48$  cells per field) (Figure 5a and c).



**Figure 1** Oil red O staining in the liver and the serum level of FFA and TG. (a) Oil red O staining ( $\times 200$ ). Five sections per group were measured. Bar,  $25 \mu\text{m}$ . (b) Percentage of Oil red O-stained area was determined using an image analyzer (LuminaVision). Group C, 0%; group M, 9.45% ( $P < 0.01$  compared with group C); group R, 3.27% ( $P < 0.01$  compared with group M). (c) Serum level of FFA ( $\mu\text{equiv./l}$ ).  $**P < 0.01$ . (d) Serum level of TG (mg/dl).  $**P < 0.01$ .



**Figure 2** Kupffer cell activation and inflammation. (a) H&E staining ( $\times 200$ ). In group M, hepatocytes with fatty degeneration and inflammatory cell foci (arrowheads) were distributed in the parenchyma, whereas their number decreased in group R. Bar,  $25 \mu\text{m}$ . (b) CD68 immunostaining (arrows) ( $\times 200$ ). CD68, a macrophage marker and low-density lipoprotein binding site, was rare in group C, whereas CD68-positive cells increased in number in group M, and decreased in group R. Bar,  $25 \mu\text{m}$ . (c and d) Serum levels of ALT and AST. (e) The relative TNF- $\alpha$  mRNA level measured by RT-PCR. The TNF- $\alpha$  mRNA level was normalized by the GAPDH mRNA level.



**Figure 3** Immunohistochemistry of 4-HNE and HO-1, and the expression of HO-1 mRNA. (a) Immunohistochemistry of 4-HNE ( $\times 200$ ). Bar,  $25 \mu\text{m}$ . There is a magnified view of the squared area ( $\times 1000$ ). Foamed macrophages are evident. (b) Immunohistochemistry of HO-1 ( $\times 200$ ). The box shows a higher magnification of the squared area. Bar,  $25 \mu\text{m}$ . (c) HO-1 mRNA level.  $**P < 0.01$ . The HO-1 mRNA level was normalized by the GAPDH mRNA level.

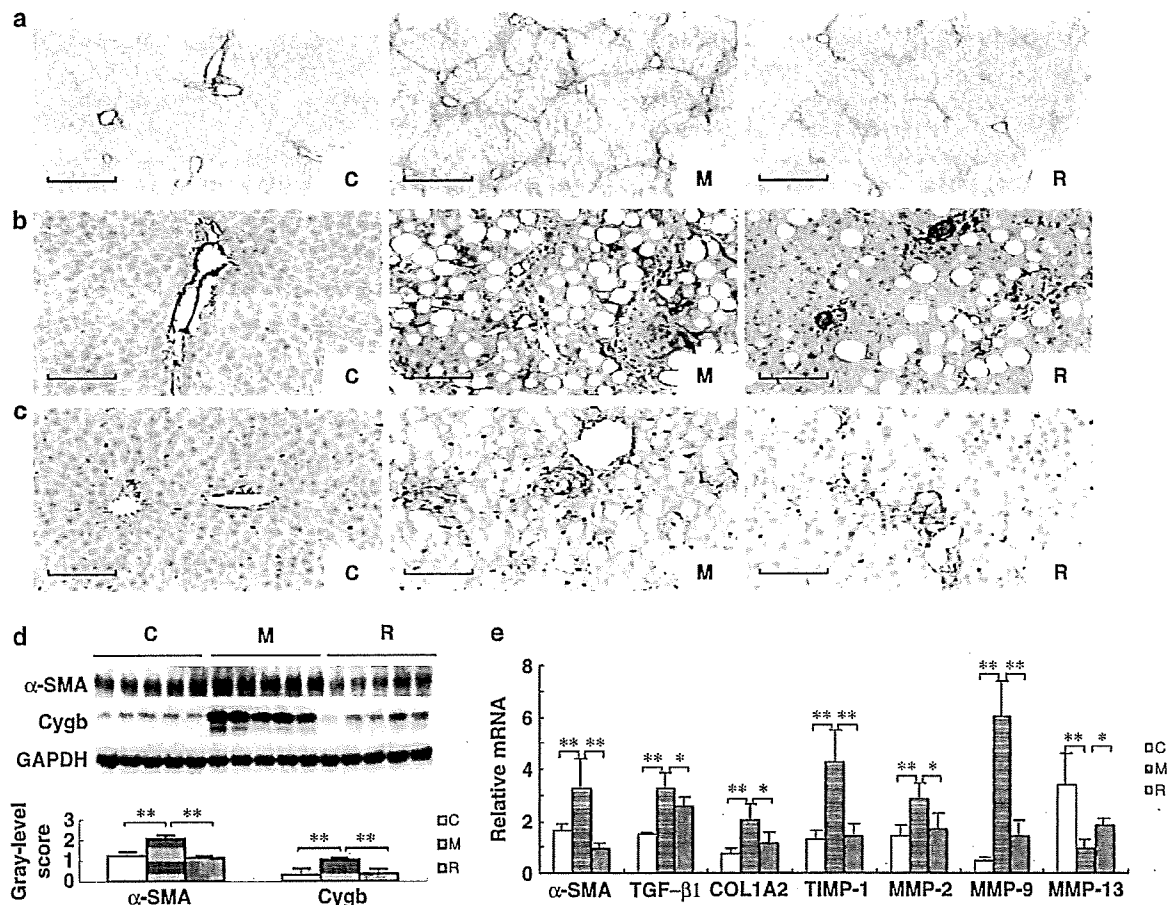
Conversely, the *in situ* BrdU incorporation assay showed that the number of proliferated hepatocytes underwent no marked changes in group M compared with group C ( $7.5 \pm 3$  vs  $7.6 \pm 1.0$  cells per field, respectively,  $P > 0.05$ ), whereas it increased significantly in group R ( $17.2 \pm 4.35$  cells per field) (Figure 5b and d). Furthermore, the ratio of hepatocyte proliferation (BrdU-positive cells)/apoptosis (TUNEL-positive cells) decreased significantly in group M compared with control group C ( $0.10 \pm 0.03$  vs  $0.19 \pm 0.03$ , respectively,  $P < 0.01$ ), whereas it increased significantly in group R compared with group M ( $0.41 \pm 0.09$ ,  $P < 0.01$ , Figure 5e). In addition, as shown in Figure 5f, mRNA expression of IL-6, which is known to initiate hepatocyte growth through the STAT3 transcription factor, markedly decreased in group M and thereafter increased in group R, whereas STAT-3 expression remained unchanged (data not shown). Furthermore, mRNA expression of BAX and BAK, proapoptotic genes, markedly increased in group M and returned to the normal range in group R, whereas Bcl-xl and Bcl-2 mRNA expression remained unchanged (data not shown). These results suggest that hepatocyte proliferation/apoptosis undergoes a dynamic transition on changing the diet from MCDD to CD.

### Involvement of ER Stress in Fibrosis of Steatohepatitis and its Recovery

Caspase-12 is known as a member of the IL-1 $\beta$ -converting enzyme subfamily of caspases. In rodents, the homolog of this gene mediates apoptosis in response to ER stress. Immunohistochemistry using anti-caspase-12 antibodies revealed that caspase-12 showed negligible expression in group C, whereas it was abundant in hepatocytes with fatty degeneration in group M, indicating the occurrence of ER stress in fatty hepatocytes (Figure 6a). The dietary change clearly reduced the content of caspase-12 in hepatocytes (Figure 6a).

Immunoblot analyses of ER stress-marker proteins such as caspase-12, caspase-7 and cleaved caspase-7, and GRP78 showed that all of these protein expressions were significantly increased in group M and successively reduced in group R (Figure 6b and c). In addition, PDI, an ER-residing protein that catalyzes protein folding and thiol-disulfide interchange reactions,<sup>30,31</sup> was markedly induced in group M compared with groups C and R (Figure 6d).

RT-PCR analyses supported these observations by showing that mRNAs of GRP78, caspase-12, caspase-7, c-Jun, and



**Figure 4** Hepatic stellate cell activation and liver fibrosis. (a) Sirius Red staining ( $\times 100$ ). Bar,  $50 \mu\text{m}$ . (b)  $\alpha$ -SMA immunostaining ( $\times 400$ ).  $\alpha$ -SMA-positivity was seen only around large vessels in group C. In group M,  $\alpha$ -SMA-positive cells colocalized in and around granulomas close to fatty hepatocytes and perisinusoidal spaces, indicating they were activated stellate cells. The number of  $\alpha$ -SMA-positive cells was clearly reduced in group R. Bar,  $12.5 \mu\text{m}$ . (c) Cygb immunostaining ( $\times 400$ ). Cygb-positivity was seen only along sinusoids in group C. In group M, Cygb-positive cells colocalized in and around granulomas close to fatty hepatocytes and perisinusoidal spaces, indicating that they were activated stellate cells. The number of Cygb-positive cells was clearly reduced in group R. Bar,  $12.5 \mu\text{m}$ . (d) Immunoblotting for  $\alpha$ -SMA and Cygb. The gray-level score indicates the histogram of immunoblotting for  $\alpha$ -SMA and Cygb.  $^{**}P < 0.01$ . (e) Fibrotic gene expressions in the liver determined by qRT-PCR. Relative mRNA levels of  $\alpha$ -SMA, TGF $\beta$ 1, Col1A2, TIMP-1, MMP-2, MMP-9, and MMP-13. mRNA levels were normalized by GAPDH.  $^{*}P < 0.05$ ,  $^{**}P < 0.01$ .

ERp57 increased significantly in group C and decreased after 2 weeks of CD diet administration (Figure 6e).

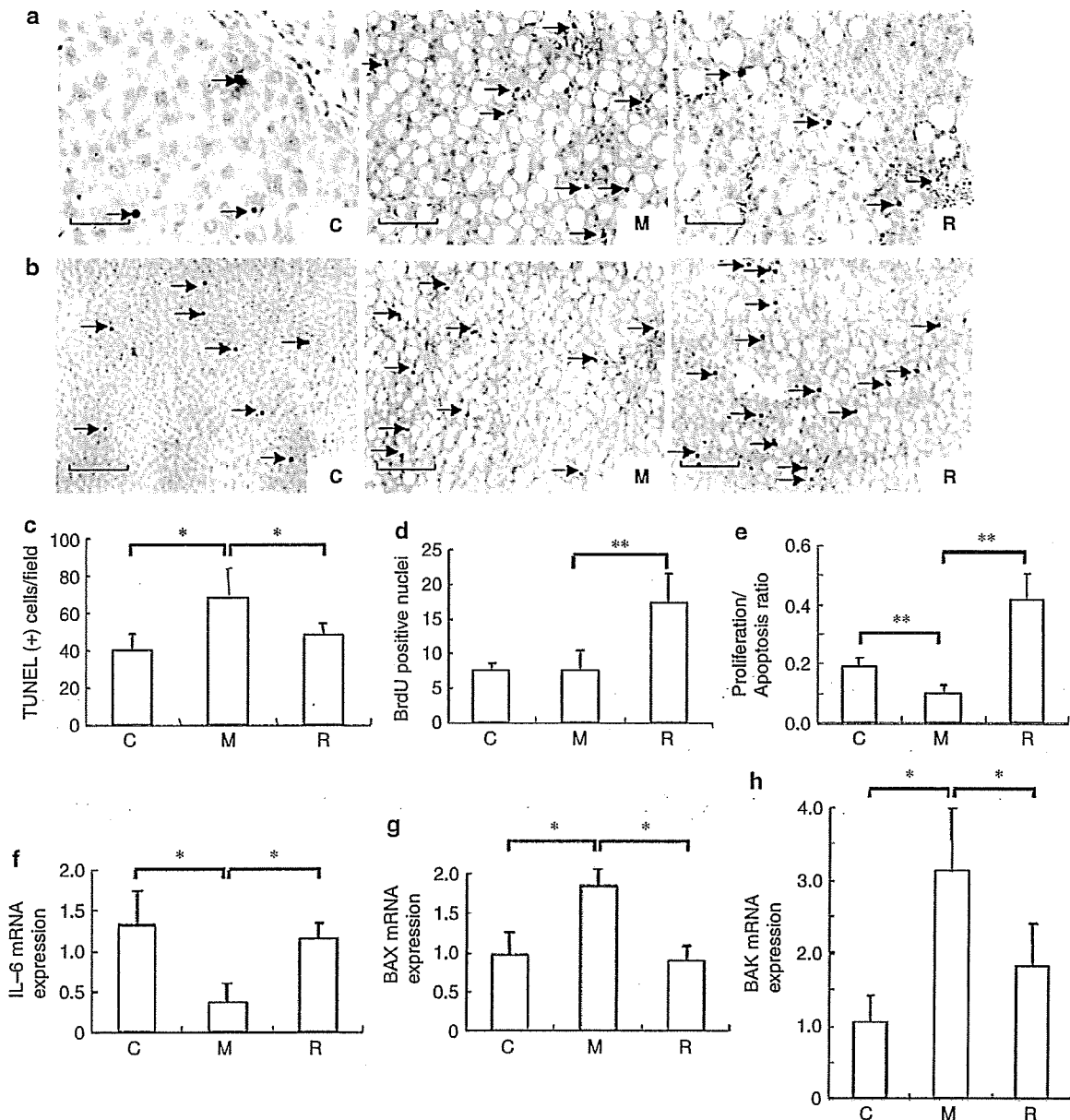
## DISCUSSION

### Hepatic Steatosis: the 'Primer' for Fibrosis of NASH Induced by MCDD

As both methionine and choline are essential precursors of hepatic phosphatidylcholine synthesis, their deficiency provokes hepatic steatosis by limiting the availability of its substrates, which, in turn, inhibits VLDL assembly and blocks TG secretion from hepatocytes.<sup>32,33</sup> Our study showed that serum levels of TG and FFA in MCDD-induced fatty liver fibrosis (group M) decreased to one-fifth and two-thirds, respectively, of that in the CD group (group C), indicating the impaired secretion of TG and FFA from hepatocytes. Hepatic steatosis facilitates the mitochondrial

uptake of FFA, the overflow of which triggers  $\beta$ -oxidation, resulting in the generation of reactive oxygen radicals to trigger lipid peroxidation.<sup>34,35</sup> This leads to the production of toxic substances that damage the mitochondria and stimulate further production of reactive oxygen species.<sup>34</sup> Such a positive feedback loop results in cellular damage, activation of liver macrophages (Kupffer cells), and generation of proinflammatory cytokines that initiate hepatic inflammation.<sup>36,37</sup>

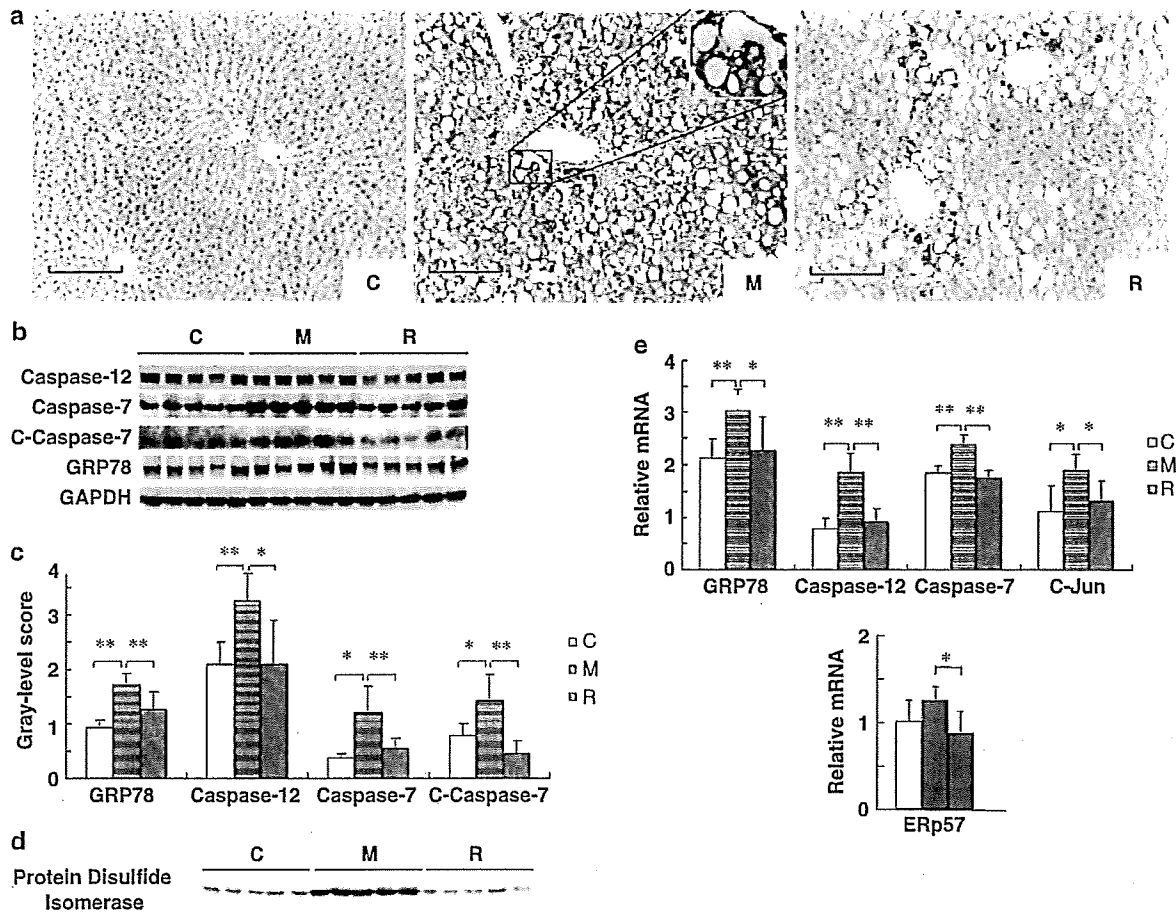
Kupffer cells could be the main source of TNF- $\alpha$  in MCDD-induced liver injury. In addition, MMP-9 (gelatinase B), a member of MMPs, is derived mainly from Kupffer cells in the liver,<sup>38</sup> and its expression is significantly increased together with the activation of cells, indicating that MMP-9 could be a marker of Kupffer cell activation instead of its activity as a collagenase. A number of studies have shown increased serum and plasma levels of MMP-9 in various types



**Figure 5** Changes in hepatocyte apoptosis and proliferation. (a) A typical photograph of apoptotic hepatocytes (TUNEL staining, arrows) ( $\times 400$ ). TUNEL-positive cells were counted under a microscope in  $> 100$  unselected  $20 \times$  microscopic fields per liver. Bar,  $12.5 \mu\text{m}$ . (b) A typical photograph of proliferating hepatocytes (BrdU staining, arrows) ( $\times 200$ ). Bar,  $25 \mu\text{m}$ . (c) Bar graphs indicate the number of TUNEL-positive cells per field.  $*P < 0.05$ . (d) Bar graphs indicate the number of BrdU-positive cells per field.  $**P < 0.01$ . (e) The ratio of proliferation/apoptosis of hepatocytes.  $*P < 0.05$ .  $**P < 0.01$ . (f-h) Levels of IL-6, BAX, and BAK mRNA. The IL-6, BAX, and BAK mRNA levels determined by RT-PCR. The mRNA level was normalized by the GAPDH mRNA level.  $**P < 0.01$ .

of liver injury, including ischemic reperfusion injury<sup>39</sup> and chronic viral hepatitis.<sup>40,41</sup> Furthermore, the mutation of MMP-9 was reported to inhibit hepatic fibrogenesis in mice.<sup>42</sup> This study showed that MMP-9 and TNF- $\alpha$  mRNA expression increased significantly with the activation of Kupffer cells that were positive for CD68 in group M, whereas switching the diet from MCDD to CD triggered the immediate recovery of MMP-9, suggesting that MMP-9 is associated with the activation status of Kupffer cells.

In contrast, Kupffer cells have been reported to have an important role in the regression of fibrosis through the expression of MMP-13 in other experimental models.<sup>43,44</sup> The current study showed that the number of CD68- and HO-1-positive cells, which are identical to activated Kupffer cells, significantly increased in group M, whereas MMP-13 mRNA expression markedly decreased. Switching the diet from MCDD to CD triggered the immediate recovery of the liver histology, accompanied by a marked increase in the expression



**Figure 6** Endoplasmic reticulum stress. (a) Caspase-12 immunostaining. Caspase-12-positive cells were rare in group C, whereas hepatocytes with fatty degeneration exhibited caspase-12 positivity in group M. In group R, caspase-12 was retained in hepatocytes with fat, whereas it disappeared in intact hepatocytes. Bar, 25  $\mu$ m. The box shows an enlarged view of caspase-12-positive hepatocytes with fatty degeneration. (b) Immunoblotting of caspase-12, caspase-7, cleaved caspase-7, and GRP78. (c) The gray-level score determined by densitometry of the bands on immunoblotting for GRP78, caspase-12, caspase-7, and cleaved caspase-7. (d) Immunoblotting of protein disulfide isomerase. (e) Relative mRNA levels of GRP78, caspase-12, caspase-7, c-Jun, and ERp57 in groups C, M, and R. mRNA levels were normalized by GAPDH. \* $P < 0.05$ , \*\* $P < 0.01$ .

of MMP-13 mRNA, indicating the active role of MMP-13 derived from Kupffer cells in the regression of liver fibrosis.<sup>45</sup>

**Stellate Cell Activation: a Crucial Role in Fibrosis Development in NASH**

Hepatic stellate cells are present in the normal liver in a quiescent state, and their major function seems to be the storage of vitamin A. After injury, stellate cells activate or trans-differentiate into proliferating myofibroblast-like cells that produce abundant levels of fibrillar collagen and TIMP-1.<sup>46</sup> The secretion of abundant levels of TIMP-1 by activated stellate cells inhibits hepatic collagenase activity, and thereby promotes a net increase in extracellular matrix materials. TIMP-1 also promotes hepatic fibrogenesis by inhibiting the apoptosis of activated stellate cells.<sup>47</sup> MMP-2 (gelatinase A), derived mainly from activated stellate cells, is

involved in degradation of the basement membrane in the early stage of liver fibrosis.<sup>48</sup> This study showed that both hepatic collagen deposition and stellate cell activation markers, such as  $\alpha$ -SMA and cytoglobin, increased significantly with a markedly elevated expression of Col1A2, TIMP-1, MMP-2, and TGF $\beta$ -1 mRNAs. After switching to CD feeding, hepatic collagen deposition decreased significantly, accompanied by the marked reduction of these mRNAs, suggesting that MCDD-induced fatty liver fibrosis was closely related to stellate cell activation.

**ER Stress: an Important Participant in Advancement of Liver Fibrosis in NASH**

Obesity is associated with the induction of ER stress predominantly in the liver and adipose tissues.<sup>49</sup> Previous research has shown that fatty liver induced by a high sucrose

diet and high saturated fat diet shows clear ER stress events in a rodent model.<sup>50</sup> In addition, CHOP (a key component in ER stress-mediated apoptosis) deficiency attenuates cholestasis-induced liver fibrosis by reducing hepatocyte injury in bile duct ligation mice.<sup>51</sup> However, the roles of ER stress in fatty liver induced by MCDD have not been reported. Initial mediators of ER stress responses are ER resident type I transmembrane serine/threonine protein kinases, PKR-like ER kinase, and inositol-requiring enzyme-1 (IRE-1); the accumulation of unfolded proteins in ER induces the oligomerization-dependent autophosphorylation of these kinases,<sup>52,53</sup> and thereby initiates cytoplasmic signal transduction. It was recently shown that activated IRE-1 on the ER membrane recruits TNF receptor-associated factor 2, and thus activates c-Jun amino-terminal kinase.<sup>54</sup> In addition, the survival response activates genes that encode ER-residing chaperones such as GRP78/Bip, which uses energy derived from ATP hydrolysis to prevent the aggregation of ER proteins and is considered the classical marker of UPR activation.<sup>55</sup> The current study showed that the expression of ER stress markers GRP78 (protein and mRNA) and c-Jun mRNA increased significantly in the MCDD group, whereas the expression of all of them decreased markedly after changing the diet. Another ER stress-related molecule, PDI,<sup>30,31</sup> showed similar behavior (Figure 6d and e). Thus, ER stress may be a key factor in MCDD-induced fatty liver fibrosis.

Caspases also participate in ER stress-induced apoptosis. In mice, procaspase-12 is localized on the cytoplasmic side of the ER and is cleaved and activated specifically by ER stress, but not by death receptor or mitochondria-mediated apoptotic signals.<sup>56</sup> Caspase-7, which translocates from the cytosol to the cytoplasmic side of the ER membrane in response to ER stress, has been reported to interact with and cleave procaspase-12, leading to its activation.<sup>57</sup> Activated caspase-12 then cleaves and activates procaspase-9, which in turn activates the downstream caspase cascade, including caspase-3, DNA fragmentation, and cell death.<sup>58</sup> Our results also showed that protein and gene expressions of caspase-12, caspase-7, and cleaved caspase-7 were significantly elevated during steatohepatitis together with the increased number of apoptotic hepatocytes, whereas they were reduced markedly after changing the diet. Furthermore, although the proliferation of hepatocytes did not show a clear change in the MCDD group, the ratio of hepatocyte proliferation/apoptosis decreased significantly, whereas it increased markedly after changing the diet to CD, indicating that the apoptotic caspase-12 pathway may also inhibit hepatocyte proliferation in MCDD-induced steatohepatitis. The marked elevation of BAX and BAK may also participate in stimulating the apoptosis of hepatocytes by MCDD.

In conclusion, this study shows that, although the underlying mechanisms involved in MCDD-induced fatty liver fibrosis are complicated, they may include not only well-known factors, such as steatosis, oxidative stress, and the activation of Kupffer and stellate cells, but also ER stresses

and the balance between hepatocyte proliferation and apoptosis. The reversibility of liver fibrosis is also shown in steatohepatitis, at least in the present MCDD model.

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

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#### DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

- Angulo P. Nonalcoholic fatty liver disease. *N Engl J Med* 2002; 346:1221–1231.
- Clark JM, Diehl AM. Hepatic steatosis and type 2 diabetes mellitus. *Curr Diab Rep* 2002;2:210–215.
- Festi D, Colecchia A, Sacco T, *et al*. Hepatic steatosis in obese patients: clinical aspects and prognostic significance. *Obes Rev* 2004;5:27–42.
- Bedogni G, Miglioli L, Masutti F, *et al*. Prevalence of and risk factors for nonalcoholic fatty liver disease: the Dionysos nutrition and liver study. *Hepatology* 2005;42:44–52.
- Boccatto S, Pistis R, Noventa F, *et al*. Fibrosis progression in initially mild chronic hepatitis C. *J Viral Hepat* 2006;13:297–302.
- Fartoux L, Chazouillères O, Wendum D, *et al*. Impact of steatosis on progression of fibrosis in patients with mild hepatitis C. *Hepatology* 2005;41:82–87.
- Leandro G, Mangia A, Hui J, *et al*. Relationship between steatosis, inflammation and fibrosis in chronic hepatitis C: a meta-analysis of individual patient data. *Gastroenterology* 2006;130:1636–1642.
- Sass DA, Chang P, Chopra KB. Nonalcoholic fatty liver disease: a clinical review. *Dig Dis Sci* 2005;50:171–180.
- Bugianesi E, Leone N, Vanni E, *et al*. Expanding the natural history of nonalcoholic steatohepatitis: from cryptogenic cirrhosis to hepatocellular carcinoma. *Gastroenterology* 2002;123:134–140.
- Shimada M, Hashimoto E, Taniai M, *et al*. Hepatocellular carcinoma in patients with non-alcoholic steatohepatitis. *J Hepatol* 2002;37: 154–160.
- Bugianesi E. Review article: steatosis, the metabolic syndrome and cancer. *Aliment Pharmacol Ther* 2005;22(Suppl 2):40–43.
- Kelley DE, McKolanis TM, Hegazi RA, *et al*. Fatty liver in type 2 diabetes mellitus: relation to regional adiposity, fatty acids, and insulin resistance. *Am J Physiol Endocrinol Metab* 2003;285:E906–E916.
- Shiratori Y, Imazeki F, Moriyama M, *et al*. Histologic improvement of fibrosis in patients with hepatitis C who have sustained response to interferon therapy. *Ann Intern Med* 2000;132:517–524.
- Poynard T, McHutchison J, Davis GL, *et al*. Impact of interferon alfa-2b and ribavirin on progression of liver fibrosis in patients with chronic hepatitis C. *Hepatology* 2000;32:1131–1137.
- Yuan M, Konstantopoulos N, Lee J, *et al*. Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta. *Science* 2001;293:1673–1677.
- Uysal KT, Wiesbrock SM, Marino MW, *et al*. Protection from obesity-induced insulin resistance in mice lacking TNF- $\alpha$  function. *Nature* 1997;389:610–614.
- Hirosami J, Tuncman G, Chang L, *et al*. A central role for JNK in obesity and insulin resistance. *Nature* 2002;420:333–336.
- Hampton RY. ER stress response: getting the UPR hand on misfolded proteins. *Curr Biol* 2000;10:R518–R521.
- Mori K. Tripartite management of unfolded proteins in the endoplasmic reticulum. *Cell* 2000;101:451–454.
- Harding HP, Calton M, Urano F, *et al*. Transcriptional and translational control in the mammalian unfolded protein response. *Annu Rev Cell Dev Biol* 2002;18:575–599.



21. Ji C, Kaplowitz N. Betaine decreases hyperhomocysteinemia, endoplasmic reticulum stress, and liver injury in alcohol-fed mice. *Gastroenterology* 2003;124:1488–1499.
22. Rodrigues CM, Ma X, Linehan-Stieers C, *et al*. Ursodeoxycholic acid prevents cytochrome c release in apoptosis by inhibiting mitochondrial membrane depolarization and channel formation. *Cell Death Differ* 1999;6:842–854.
23. Pavo N, Romano PR, Graczyk TM, *et al*. Protein synthesis and endoplasmic reticulum stress can be modulated by the hepatitis C virus envelope protein E2 through the eukaryotic initiation factor 2 alpha kinase PERK. *J Virol* 2003;77:3578–3585.
24. Tardif KD, Mori K, Siddiqui A. Hepatitis C virus subgenomic replicons induce endoplasmic reticulum stress activating an intracellular signaling pathway. *J Virol* 2002;76:7453–7459.
25. Kawada N, Kristensen DB, Asahina K, *et al*. Characterization of a stellate cell activation-associated protein (STAP) with peroxidase activity found in rat hepatic stellate cells. *J Biol Chem* 2001;276:25318–25323.
26. Nakatani K, Seki S, Kawada N, *et al*. Expression of SPARC by activated hepatic stellate cells and its correlation with the stages of fibrogenesis in human chronic hepatitis. *Virchows Arch* 2002;441:466–474.
27. Otagawa K, Kinoshita K, Fujii H, *et al*. Erythrophagocytosis by liver macrophages (Kupffer cells) promotes oxidative stress, inflammation, and fibrosis in a rabbit model of steatohepatitis: implications for the pathogenesis of human nonalcoholic steatohepatitis. *Am J Pathol* 2007;170:967–980.
28. Wang YQ, Ikeda K, Ikebe T, *et al*. Inhibition of hepatic stellate cell proliferation and activation by the semisynthetic analogue of fumagillin TNP-470 in rats. *Hepatology* 2000;32:980–989.
29. Uyama N, Shimahara Y, Okuyama H, *et al*. Carbenoxolone inhibits DNA synthesis and collagen gene expression in rat hepatic stellate cells in culture. *J Hepatol* 2003;39:749–755.
30. Riemer J, Bulleid N, Herrmann JM. Disulfide formation in the ER and mitochondria: two solutions to a common process. *Science* 2009;324:1284–1287.
31. Nakamura T, Lipton SA. Cell death: protein misfolding and neurodegenerative diseases. *Apoptosis* 2009;14:455–468.
32. Vance JE, Vance DE. The role of phosphatidylcholine biosynthesis in the secretion of lipoproteins from hepatocytes. *Can J Biochem Cell Biol* 1985;63:870–881.
33. Yao ZM, Vance DE. The active synthesis of phosphatidylcholine is required for very low density lipoprotein secretion from rat hepatocytes. *J Biol Chem* 1988;263:2998–3004.
34. Fromenty B, Robin MA, Igoudjil A, *et al*. The ins and outs of mitochondrial dysfunction in NASH. *Diabetes Metab* 2004;30:121–138.
35. Pessayre D, Fromenty B. NASH: a mitochondrial disease. *J Hepatol* 2005;42:928–940.
36. Albano E, Mottaran E, Occhino G, *et al*. Review article: role of oxidative stress in the progression of non-alcoholic steatosis. *Aliment Pharmacol Ther* 2005;22:71–73.
37. Day CP. From fat to inflammation. *Gastroenterology* 2006;130:207–210.
38. Winwood PJ, Schuppan D, Iredale JP, *et al*. Kupffer cell-derived 95 kDa type IV collagenase/gelatinase B: characterisation and expression in cultured cells. *Hepatology* 1995;22:304–315.
39. Kuyvenhoven JP, Verspaget HW, Gao Q, *et al*. Assessment of serum matrix metalloproteinases MMP-2 and MMP-9 after human liver transplantation: increased serum MMP-9 level in acute rejection. *Transplantation* 2004;77:1646–1652.
40. Leroy V, Monier F, Bottari S, *et al*. Circulating matrix metalloproteinases 1, 2, 9 and their inhibitors TIMP-1 and TIMP-2 as serum markers of liver fibrosis in patients with chronic hepatitis C: comparison with PIIINP and hyaluronic acid. *Am J Gastroenterol* 2004;799:271–279.
41. Chung TW, Kim JR, Suh JJ, *et al*. Correlation between plasma levels of matrix metalloproteinase (MMP)-9/MMP-2 ratio and alpha-fetoproteins in chronic hepatitis carrying hepatitis B virus. *J Gastroenterol Hepatol* 2004;19:565–571.
42. Roderfeld M, Weiskirchen R, Wagner S, *et al*. Inhibition of hepatic fibrogenesis by matrix metalloproteinase-9 mutants in mice. *FASEB J* 2006;20:444–454.
43. Hironaka K, Sakaida I, Matsumura Y, *et al*. Enhanced interstitial collagenase (matrix metalloproteinase-13) production of Kupffer cell by gadolinium chloride prevents pig serum-induced rat liver fibrosis. *Biochem Biophys Res Commun* 2000;267:290–295.
44. Sakaida I, Hironaka K, Terai S, *et al*. Gadolinium chloride reverses dimethylnitrosamine (DMN)-induced rat liver fibrosis with increased matrix metalloproteinases (MMPs) of Kupffer cells. *Life Sci* 2003;72:943–959.
45. Friedman S. Mac the knife? Macrophages —the double-edged sword of hepatic fibrosis. *J Clin Invest* 2005;115:29–32.
46. Iredale JP, Benyon RC, Arthur MJ, *et al*. Tissue inhibitor of metalloproteinase-1 messenger RNA expression is enhanced relative to interstitial collagenase messenger RNA in experimental liver injury and fibrosis. *Hepatology* 1996;24:176–184.
47. Iredale JP, Murphy G, Hembry RM, *et al*. Human hepatic lipocytes synthesize tissue inhibitor of metalloproteinases-1. Implications for regulation of matrix degradation in liver. *J Clin Invest* 1992;90:282–287.
48. Winwood PJ, Schuppan D, Iredale JP, *et al*. Kupffer cell-derived 95-kd type IV collagenase/gelatinase B: characterization and expression in cultured cells. *Hepatology* 1995;22:304–315.
49. Ozcan U, Cao Q, Yilmaz E, *et al*. Hotamisligil. Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science* 2004;306:457–461.
50. Wang D, Wei Y, Pagliassotti MJ. Saturated fatty acids promote endoplasmic reticulum stress and liver injury in rats with hepatic steatosis. *Endocrinology* 2006;147:943–951.
51. Tamaki N, Hatano E, Taura K, *et al*. CHOP deficiency attenuates cholestasis-induced liver fibrosis by reduction of hepatocyte injury. *Am J Physiol Gastrointest Liver Physiol* 2008;294:G498–G505.
52. Bertolotti A, Zhang Y, Hendershot LM, *et al*. Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nat Cell Biol* 2000;2:326–332.
53. Liu CY, Schröder M, Kaufman RJ. Ligand-independent dimerization activates the stress response kinases IRE1 and PERK in the lumen of the endoplasmic reticulum. *J Biol Chem* 2000;275:24881–24885.
54. Urano F, Wang X, Bertolotti A, *et al*. Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science* 2000;287:664–666.
55. Iwawaki T, Hosoda A, Okuda T, *et al*. Translational control by the ER transmembrane kinase/ribonuclease IRE1 under ER stress. *Nat Cell Biol* 2001;3:158–164.
56. Nakagawa T, Yuan J. Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. *J Cell Biol* 2000;150:887–894.
57. Rao RV, Hermel E, Castro-Obregon S, *et al*. Coupling endoplasmic reticulum stress to the cell death program: mechanism of caspase activation. *J Biol Chem* 2001;276:33869–33874.
58. Tan Y, Dourdin N, Wu C, *et al*. Ubiquitous calpains promote caspase-12 and JNK activation during endoplasmic reticulum stress-induced apoptosis. *J Biol Chem* 2006;281:16016–16024.

## Special Report

# Management of hepatitis C; Report of the Consensus Meeting at the 45th Annual Meeting of the Japan Society of Hepatology (2009)

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The consensus meeting for the diagnosis, management and treatment for hepatitis C was held in 45<sup>th</sup> annual meeting for the Japan Society of Hepatology (JSH) in June 2009 where the recommendations and informative statements were discussed including organizers and presenters. The several important informative statements and recommendations have been shown. This was the fourth JSH consensus meeting of hepatitis C, however, the recommendations have not been published in English previously. Thus, this is the first report of JSH consensus of hepatitis C. The rate of development of hepatocellular carcinoma (HCC) in HCV-infected patients in Japan is higher than in the USA, because the average age

of the HCV-infected patients is greater and there are more patients with severe fibrosis of the liver than in the USA. In Japan, more than 60% of HCV-infected patients are genotype 1b infection, and they show lower response to perinterferon and ribavirin combination treatment. To improve the response rate is also an important issue in our country. To establish the original recommendations and informative statements to prevent the development of HCC is a very important issue in Japan.

**Key words:** chronic hepatitis C, peginterferon, ribavirin, fibrosis of the liver, hepatocellular carcinoma, HCV mutation

## INTRODUCTION

**H**EPATITIS C VIRUS (HCV) infection is a major public health problem and a leading cause of death from liver disease in Japan. Two million people are infected, and more than 30 000 patients die from hepatocellular carcinoma (HCC) and/or liver cirrhosis every

year. HCC is the fourth leading cause of death from malignant neoplastic disease, and prevention of the development of HCC is an urgent issue in Japan. The purpose of this consensus is to provide clinicians with consensus-based approaches to diagnosis and treatment of HCV infection.

The consensus meeting for the diagnosis, management and treatment for hepatitis C was held during the 45th annual meeting of the Japan Society of Hepatology (JSH) in June 2009 (Congress President: M. Kudo), where the recommendations and informative statements were discussed and compared with AASLD practice guidelines which has been published in *Hepatology*.<sup>1</sup> This was the fourth JSH consensus meeting of hepatitis C, however, the recommendations have not been published in English previously. This is the first report of the JSH consensus of hepatitis C. Established information regarding the pathogenesis and contributing factors for disease

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*Disclaimer Statement:* The view expressed in these consensus do not necessarily represent the view of the National Health Insurance of Japan, or the Japanese Government. This article was previously published in *Kanzo* 50:11, pp 665–677 (November 2009). Received 24 September 2009; revision 1 December 2009; accepted 1 December 2009.

Table 1 Grading system for recommendations

	Description
Classification	
Class I	Conditions for which there is evidence and/or general agreement that a given diagnostic evaluation procedure or treatment is beneficial, useful and effective
Class II	Conditions for which there is conflicting evidence and/or a divergence of opinion about the usefulness/efficacy of a diagnostic evaluation, procedure or treatment
Class IIa	Weight of evidence/opinion is in favor of usefulness/efficacy
Class IIb	Usefulness/efficacy is less well established by evidence/opinion
Class III	Conditions for which there is no evidence and/or general agreement that a diagnostic evaluation, procedure/treatment is not useful/effective and in some cases may be harmful
Level of evidence	
Level A	Data derived from multiple randomized clinical trials or meta-analysis
Level B	Data derived from a single randomized trial, or non-randomized studies
Level C	Only consensus opinion of experts, case studies or standard of care

progression which were agreed by the organizers and presenters are shown as informative statements, and clinically useful consensus are shown as "Recommendations". The rate of development of HCC in HCV-infected patients in Japan is higher than that in the USA, because the average age of the patient is greater and there are more patients with severe fibrosis of the liver than in the USA. To establish original recommendations and informative statements to prevent the development of HCC is a very important issue in our country. The quality of recommendations or informative statements is required to show a "class" (reflecting benefit vs risk) and "level" (assessing strength or certainty) of evidence according to AASLD practice guidelines (Table 1).<sup>1,2</sup>

## PATHOGENESIS OF HEPATITIS C

**H**EPATITIS C VIRUS infection causes acute and chronic hepatitis (CH), cirrhosis and HCC. The severity and rate of progression of the disease are highly variable and may reflect both host and viral factors, but

the mechanisms of pathogenesis are incompletely understood. Thus, understanding the mechanisms of HCV pathogenesis is an important goal of HCV research.

### Entry pathway of HCV

For the virus, the first step in propagation is enter into hepatocytes. A decade ago, the HCV envelop protein E2 was shown to bind human CD81, a tetraspanin expressed on various cell types including hepatocytes and B lymphocytes.<sup>3</sup> Next, two other essential proteins, scavenger receptor class B type I (SR-B1)<sup>4</sup> and claudin-1 (CLDN1),<sup>5</sup> and potentially additional accessory factors such as glycosaminoglycans and low-density protein receptors<sup>6</sup> were identified as receptors involved in HCV entry. Finally, the crucial factor was identified as the tight junction protein occludin (OCLN).<sup>7</sup> Interestingly, both CLDN1 and OCLN are components of tight junctions which are structures forming firm seals between adjacent cells. The initial adhesion of HCV to hepatocytes may be mediated by accessory factors and/or direct interaction with SR-B1 and CD81 proteins. On transfer to a tight junction complex, HCV may interact directly with CLDN1 and/or OCLN, allowing viral uptake into the cell.

Hepatitis C virus infects only humans and chimpanzees. Once these HCV entry factors were identified, the next concern was to determine which factors dictate species-specific tropism. CD81 proteins from other mammals, such as the mouse, are used inefficiently by HCV.<sup>8</sup> Although HCV does not discriminate between human and mouse SR-B1 and CLDN1, mouse OCLN like CD81 cannot substitute for the related human protein in aiding viral entry. These findings indicate that CD81 and OCLN represent minimal human-specific entry factors.

*Informative statement: CLDN1 and OCLN in addition to CD81 and SR-B1 are required for entering of HCV into hepatocytes, and especially CD81 and OCLN represent minimal human-specific entry factors. (Grade A.)*

### Evasion of intracellular host defense by HCV

One of the mechanisms by which HCV infection is likely to lead to be persistent is evasion of intracellular host defense through a complex combination of processes that include interference of interferon (IFN) signaling, modulation of its effectors and continual viral genetic variation. The HCV genome contains pathogen-associated molecular pattern (PAMP) signatures which

are recognized by the retinoic-inducible gene I (*RIG-I*) and specific Toll-like receptors when introduced into naïve cells.<sup>9–11</sup> Viral signaling through *RIG-I* and its adaptor protein, IFN promoter-stimulator 1 (IPS-1), activates IFN regulatory factor-3 (IRF-3) and the host IFN- $\alpha/\beta$  response that limits virus infection.<sup>12,13</sup> HCV NS3/4A protease cleaves IPS-1, releasing IPS-1 from the mitochondrial membrane.<sup>14</sup> Cleavage results in subcellular redistribution of IPS-1 and loss of interaction with *RIG-I*, thereby preventing downstream activation of IRF-3 and induction of IFN $\beta$ .<sup>15</sup>

Secreted IFN $\beta$  engages the local tissue through the autocrine and paracrine processes of binding the IFN- $\alpha/\beta$  receptors. This results in activation of the Jak-signal transducer and activator of transcription (STAT) pathway, in which the receptor-associated Jak and Tyk1 protein kinases catalyze the phosphorylation of STAT proteins. The resulting IFN-stimulated gene factor-3 (ISGF3) transcription factor complex localizes in the cell nucleus, where it binds to the IFN-stimulated response element (ISRE) within the promoter/enhancer region of IFN-stimulated genes (ISG). Jak-STAT signaling leads to a second wave of transcriptional activity stimulating ISG expression in the infected cells. Expression of the HCV core protein has been associated with increased expression levels of suppressor of cytokine signaling (SOCS)-3.<sup>16</sup> The SOCS proteins are known for their role as negative regulators and inhibitors of Jak-STAT signaling, where they mediate a classic negative feedback loop on IFN- $\alpha/\beta$  receptor signaling events.<sup>17</sup> The HCV NS5A protein has been shown to induce interleukin (IL)-8 production leading to partial inhibition of the IFN-induced antiviral response, probably through the alteration of ISG expression.<sup>18</sup> The HCV NS5A and E2 proteins also bind double-strand RNA-activated protein kinase (PKR) and inhibit its catalytic activity,<sup>19,20</sup> which allows HCV to evade in part the translational-suppressive actions of IFN. Thus, HCV evasion of the host response includes various strategies directed by viral proteins to control IFN signaling, ISG expression or function.

*Informative statement: HCV evades intracellular host defenses through a complex combination of processes that include IFN signaling, modulation of its effectors and continual viral genetic variation. These mechanisms include cleavage of IPS-1 by the NS3/4A protease, inhibition of Jak-STAT signaling by HCV-induced SOCS3, inhibition of the IFN-induced antiviral response by NS5A-induced IL-8, and/or inhibition of catalytic activity of PKR by the NS5A and E2 proteins. (Grade A.)*

### Oxidative stress induced by HCV

Oxidative stress is well known to be present in CH-C to a greater degree than in other inflammatory liver diseases. Although the mechanisms underlying oxidative stress induced by HCV have not been elucidated fully, there are several lines of evidence suggesting that HCV directly generates reactive oxygen species (ROS) *in vitro* and *in vivo*. Hepatic ROS production is significantly higher in HCV core transgenic mice than in normal control mice in the absence of hepatic inflammation.<sup>21</sup> HCV core protein also produces ROS in human hepatoma Huh-7 cells and HeLa cells.<sup>22</sup> Analysis of the interaction of HCV core protein with mitochondria in transgenic mice and direct interaction of recombinant core protein and isolated mitochondria indicated oxidation of the mitochondrial glutathione pool and an increase in ROS production by the mitochondrial electron transport complex I, suggesting that direct interaction of core protein with mitochondria is an important cause of the oxidative stress seen in CH-C.<sup>23</sup>

*Informative statement: Mitochondrial dysfunction induced by HCV leads to ROS generation that causes the oxidative stress seen in CH-C. (Grade A.)*

### Metabolic disorders caused by HCV

Epidemiological studies have suggested a link between type 2 diabetes and chronic HCV infection, which implies HCV-induced insulin resistance. A high level of tumor necrosis factor (TNF)- $\alpha$  and disturbance of tyrosine phosphorylation of the insulin receptor substrate (IRS)1 by TNF- $\alpha$  has been demonstrated in HCV core transgenic mice.<sup>24</sup> Another possible mechanism is that HCV core-induced SOCS3 promotes proteasomal degradation of IRS1 and IRS2 through ubiquitination.<sup>25</sup> Hepatic steatosis is one of the histopathological features in CH-C. HCV core protein inhibits microsomal triglyceride transfer protein activity and secretion of very low density lipoprotein.<sup>26</sup> HCV core protein also upregulates the sterol regulatory element binding protein (SREBP)1c promoter activity through the enhanced binding of the LXR $\alpha$ /RXR $\alpha$  to LXR-response element,<sup>27</sup> which leads to an increase in transcription of genes involved in hepatic fatty acid synthesis. Hepatic iron accumulation is also a histopathological feature of CH-C, even though its levels are not extremely high. HCV-induced ROS downregulates the transcriptional activity of hepcidin, a negative regulator in iron homeostasis, in transgenic mice expressing the HCV polyprotein<sup>28</sup> and in HCV replicon cells<sup>29</sup> (Fig. 1).