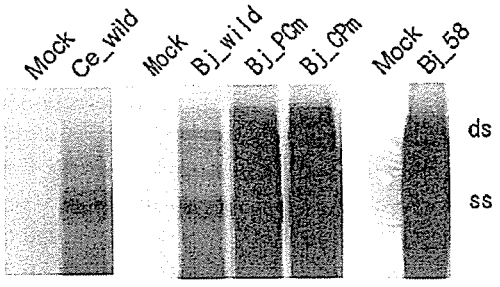

Fig. 3. Southern blot analysis for replicative activity of the wild-type HBV clones (HBV/Ce_wild and Bj_wild), as well as mutants with precore (Bj_PCm) or core-promoter (Bj_CPm) mutation, and Bj_58 with precore stop-codon mutation obtained from a patient with fulminant hepatitis.

densities of migration patterns of the wild-type, precore, and core-promoter mutants in Southern blotting analysis. The wild-type HBV/Bj displayed a band for single-stranded (ss) HBV DNA and an additional band for double-stranded (ds) HBV DNA. Of note, the densities of these bands were far greater for HBV/Bj mutants incorporated with precore or core-promoter mutation, as well as Bj_58 with the precore mutation, thereby indicating much enhanced replicative activity of precore or core-promoter mutant *in vitro*. Although the intracellular HBV DNA level for the wild-type HBV/Bj was comparable with that for the wild-type Ce (Fig. 3), the extracellular HBV DNA level in culture media was approximately threefold higher for Bj than Ce ($P < .01$) (Sugiyama M et al., manuscript in submission).

Discussion

A nationwide survey of genotypes/subgenotypes in patients with acute HBV infection from Japan during the past 2 decades has examined their influence on fulminant and chronic outcomes. The study was feasible in a country where mass vaccination has not been performed because of an extremely high efficacy of immunoprophylaxis on babies born to carrier mothers; it has decreased the persistent HBV carrier rate from 1.4% to 0.3%.[26] Acute HBV infection keeps increasing, however, predominantly through promiscuous sexual contacts in Japan.

Fulminant hepatitis developed rather frequently in 40 of the 301 (13%) patients. This is likely due to selection bias because the study included only patients who were hospitalized for acute hepatitis B. Exclusion of subclinical cases of acute HBV infection would have overestimated the incidence of fulminant hepatitis. Regardless of such a selection bias, influence of HBV genotypes/subgenotypes was evident in comparison with the 40 patients with fulminant and the 261 with acute self-limited hepatitis. Remarkably, none of the 33 patients infected with HBV/Ae developed fulminant hepatitis. In sharp contrast, 12 of the 22 (55%) patients infected with HBV/Bj developed it. Furthermore, both precore (G1896A) and core-promoter (A1762T/G1764A) mutations were detected significantly more frequently in patients with fulminant than acute self-limited hepatitis. In infection with HBV/Bj, in particular, the frequency of core-promoter mutation was much higher in the patients with fulminant (67%) than that reported in those with chronic hepatitis (16%).[27] Precore and core-promoter mutations are very frequent in patients with fulminant hepatitis from Asia[28-30] and the Middle East.[31] The failure in detecting these mutations in Western countries[32-35] could be attributed to frequent HBV/Ae and rare Bj there. In multivariate analysis, HBeAg-negative, HBV/Bj, and the precore stop-codon mutation for G1896A were independent risk factors for the development of fulminant hepatitis (Table 4). Various mutations at nt 1753 for enhanced HBV replication,[36] as well as those adjacent at nt 1754 prevailing in patients with fulminant hepatitis,[37] occurred more frequently in patients with fulminant than acute self-limited hepatitis. Host factors, such as age and total bilirubin, contributed to the development of fulminant hepatitis as well (Table 4).

In vitro replication analysis demonstrated the intracellular HBV DNA level of the wild-type HBV/Bj comparable with that of the wild-type Ce (Fig. 3). The extracellular HBV DNA level of HBV/Bj-clone, however, was much higher than those of the other genotypes, indicating its strong inclination to be secreted from cells (Sugiyama et al., manuscript in submission). Such a high concentration of HBV/Bj in the circulation of patients would rapidly and extensively promote infection of hepatocytes.

Enhanced replication capacities of precore (G1896A) and core-promoter (A1762T/G1764A) mutants for HBeAg-minus and -reduced phenotypes, respectively, were demonstrated in a replication model *in vitro* (Fig. 3). These observations were concordant with those in previous reports[38,39]; however no data are available on the replication of HBV/Bj *in vitro*, either of the wild-type or variants with these mutations. Extremely high intracellular and extracellular expressions of viral DNA were observed for the HBV/Bj clone with precore stop-codon mutation from a patient with fulminant hepatitis. These results might implicate high replication due to mutations of precore region and core-promoter in the induction of fulminant hepatitis. In support of this view, Bocharov et al.[40] have proposed that enhanced HBV replication would efficiently stimulate immune reactions, represented by the cytotoxic T lymphocyte response, suggesting that enhanced replication by HBV/Bj or precore/

core-promoter mutation might lead to fulminant hepatitis.

That HBV DNA levels were lower in patients with fulminant than acute hepatitis, despite a high replication capacity of HBV/Bj incriminated in the development of fulminant hepatic failure, may seem surprising. Because destruction of hepatocytes proceeds swiftly in patients with fulminant hepatitis, hepatic mass for HBV to thrive would have been extremely reduced in them at presentation. As a consequence, some patients with fulminant hepatitis B are without serum HBsAg; they are diagnosed by high-titered IgM anti-HBc.[41] On the contrary, HBV DNA levels were higher in the patients with HBV/Ae than Bj (Table 1); those with Ae tend to delay reducing HBV DNA, some of whom have chronic outcome. Combined, correlating HBV DNA levels with the clinical outcome in acute HBV infection would be difficult.

A wide variation has been seen in the rate of persistence after acute HBV infection in adulthood. No chronic outcomes of acute hepatitis B were seen in female recipients of red blood cells contaminated with HBV (0/28)[42] or patients in an acupuncture-associated outbreak (0/35).[43] In marked contrast, they ranged from 0.2% (14/715) in Greece[44] through 2.7% (1/37) in university students in Taiwan[45] to 10.4% (5/8) in Alaskan Eskimos[46] and 12.1% (7/58) in Germany.[47] HBV genotypes are implicated in a high rate of persistence in European countries where HBV/A is predominant.[48] In Japan, also, adulthood infection tends to persist longer with HBV/A than B or C (23% 3/13 vs. 13% 1/8 or 12% 3/25).[49] In the current series on 256 patients with acute hepatitis B in Japan who were followed rigorously, HBV infection persisted in only three (1%), representing 2 of the 32 (6%) with HBV/Ae and 1 of the 21 (5%) with Ba. Hence, 99% of patients lost their HBsAg by 6 months. Persistence of HBV observed in the patients with HBV/Ae (6%) is less frequent than that in 4 of the 31 (13%) patients with Ae from a hospital in metropolitan Tokyo.[49] The difference would be ascribable, at least in part, to lamivudine given to some patients in this study (18%). All patients treated with lamivudine recovered from acute hepatitis, whereas none of the three patients with chronic outcome had received antiviral treatment during their acute phase of illness, indicating that lamivudine might be able to prevent the chronic outcome. Likewise, some patients from metropolitan Tokyo, in whom HBV persisted,[49,50] had received immunosuppressants in the acute phase of infection before referral to their hospital.

Using cell culture and chimeric mice models for the replication system of different genotype/subgenotype clones, we have observed that the replication of HBV is the highest for HBV/Bj or C and the lowest for Aa/Ae (Sugiyama M et al., manuscript in submission). It is probable that the propensity of HBV/A infection to chronicity would be due to less intensive immune response against its slow viral dynamics. Taken together, the infection with HBV/A appears to persist longer than those with the other genotypes; this needs to be confirmed by further investigation in patients from various countries.

In conclusion, persistence of HBV after acute infection is rare and occurs more often in patients infected with HBV/Ae than others. Fulminant outcome is frequent in hospitalized patients and associated with HBV/Bj accompanied by the lack of serum HBeAg as well as high replication due to precore stop-codon mutation (G1896A), a finding supported by an *in vitro* replication model.

References

1. Lee WM. Hepatitis B virus infection. N Engl J Med 1997;337:1733-1745.
2. Arauz-Ruiz P, Norder H, Robertson BH, Magnius LO. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. J Gen Virol 2002;83:2059-2073.
3. Norder H, Hammas B, Lofdahl S, Courouce AM, Magnius LO. Comparison of the amino acid sequences of nine different serotypes of hepatitis B surface antigen and genomic classification of the corresponding hepatitis B virus strains. J Gen Virol 1992;73:1201-1208.
4. Okamoto H, Tsuda F, Sakugawa H, Sastrosoewignjo RI, Imai M, Miyakawa Y, et al. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. J Gen Virol 1988;69: 2575-2583.
5. Stuyver L, De Gendt S, Van Geyt C, Zoulim F, Fried M, Schinazi RF, et al. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. J Gen Virol 2000;81:67-74.
6. Chu CJ, Lok AS. Clinical significance of hepatitis B virus genotypes. HEPATOLOGY 2002;35:1274-1276.
7. Kao JH. Hepatitis B viral genotypes: clinical relevance and molecular characteristics. J Gastroenterol Hepatol 2002;17:643-650.
8. Miyakawa Y, Mizokami M. Classifying hepatitis B virus genotypes. Intervirology 2003;46:329-338.
9. Sugauchi F, Kumada H, Acharya SA, Shrestha SM, Gamutan MT, Khan M, et al. Epidemiological and sequence differences between two subtypes (Ae and Aa) of hepatitis B virus genotype A. J Gen Virol 2004;85:811-820.
10. Sugauchi F, Orito E, Ichida T, Kato H, Sakugawa H, Kakumu S, et al. Hepatitis B virus of genotype B with or without recombination with genotype C over the precore region plus the core gene. J Virol 2002;76:5985-5992.
11. Huy TT, Ushijima H, Quang VX, Win KM, Luengrojanakul P, Kikuchi K, et al. Genotype C of hepatitis B virus can be classified into at least two subgroups. J Gen Virol 2004;85:283-292.
12. Tanaka Y, Orito E, Yuen MF, Mukaide M, Sugauchi F, Ito K, et al. Two subtypes (subgenotypes) of hepatitis B virus genotype C: A novel subtyping assay based on restriction fragment length polymorphism. Hepatol Res 2005;33:216-224.
13. Kramvis A, Kew MC. Relationship of genotypes of hepatitis B virus to mutations, disease progression and response to antiviral therapy. J Viral Hepatol 2005;12:456-464.
14. Akuta N, Suzuki F, Kobayashi M, Tsubota A, Suzuki Y, Hosaka T, et al. The influence of hepatitis B virus genotype on the development of lamivudine resistance during long-term treatment. J Hepatol 2003;38:315-321.
15. Sugauchi F, Orito E, Ichida T, Kato H, Sakugawa H, Kakumu S, et al. Epidemiologic and virologic characteristics of hepatitis B virus genotype B having the recombination with genotype C. Gastroenterology 2003;124: 925-932.

16. Tanaka Y, Hasegawa I, Kato T, Orito E, Hirashima N, Acharya SK, et al. A case-control study for differences among hepatitis B virus infections of genotypes A (subtypes Aa and Ae) and D. HEPATOLOGY 2004;40:747-755.
17. Lindh M, Andersson AS, Gusdal A. Genotypes, nt 1858 variants, and geographic origin of hepatitis B virus: large-scale analysis using a new genotyping method. J Infect Dis 1997;175:1285-1293.
18. Mitamura K. Epidemiology of HDV infection in Japan. Prog Clin Biol Res 1991;364:81-87.
19. Trey C, Lipworth L, Chalmers TC, Davidson CS, Gottlieb LS, Popper H, et al. Fulminant hepatic failure. presumable contribution of halothane. N Engl J Med 1968;279:798-801.
20. Usuda S, Okamoto H, Tanaka T, Kidd-Ljunggren K, Holland PV, Miyakawa Y, et al. Differentiation of hepatitis B virus genotypes D and E by ELISA using monoclonal antibodies to epitopes on the preS2-region product. J Virol Methods 2000;87:81-89.
21. Kato H, Orito E, Gish RG, Bzowej N, Newsom M, Sugauchi F, et al. Hepatitis B e antigen in sera from individuals infected with hepatitis B virus of genotype G. HEPATOLOGY 2002;35:922-929.
22. Bowyer SM, van Staden L, Kew MC, Sim JG. A unique segment of the hepatitis B virus group A genotype identified in isolates from South Africa. J Gen Virol 1997;78:1719-1729.
23. Hasegawa I, Tanaka Y, Kramvis A, Kato T, Sugauchi F, Acharya SK, et al. Novel hepatitis B virus genotype A subtyping assay that distinguishes subtype Aa from Ae and its application in epidemiological studies. J Virol 2004;78:7575-7581.
24. Sugauchi F, Kumada H, Sakugawa H, Komatsu M, Niitsuma H, Watanabe H, et al. Two subtypes of genotype B (Ba and Bj) of hepatitis B virus in Japan. Clin Infect Dis 2004;38:1222-1228.
25. Abe A, Inoue K, Tanaka T, Kato J, Kajiyama N, Kawaguchi R, et al. Quantitation of hepatitis B virus genomic DNA by real-time detection PCR. J Clin Microbiol 1999;38:2899-2903.
26. Noto H, Terao T, Ryou S, Hirose Y, Yoshida T, Ookubo H, et al. Combined passive and active immunoprophylaxis for preventing perinatal transmission of the hepatitis B virus carrier state in Shizuoka, Japan during 1980-1994. J Gastroenterol Hepatol 2003;18:943-949.
27. Orito E, Mizokami M, Sakugawa H, Michitaka K, Ishikawa K, Ichida T, et al. A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. Japan HBV Genotype Research Group. HEPATOLOGY 2001;33:218-223.
28. Kosaka Y, Takase K, Kojima M, Shimizu M, Inoue K, Yoshiba M, et al. Fulminant hepatitis B: induction by hepatitis B virus mutants defective in the precore region and incapable of encoding e antigen. Gastroenterology 1991;100:1087-1094.
29. Omata M, Ehata T, Yokosuka O, Hosoda K, Ohto M. Mutations in the precore region of hepatitis B virus DNA in patients with fulminant and severe hepatitis. N Engl J Med 1991;324:1699-1704.
30. Sato S, Suzuki K, Akahane Y, Akamatsu K, Akiyama K, Yunomura K, et al. Hepatitis B virus strains with mutations in the core promoter in patients with fulminant hepatitis. Ann Intern Med 1995;122:241-248.
31. Liang TJ, Hasegawa K, Rimon N, Wands JR, Ben-Porath E. A hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. N Engl J Med 1991;324:1705-1709.
32. Karayiannis P, Alexopoulou A, Hadziyannis S, Thursz M, Watts R, Scito S, et al. Fulminant hepatitis associated with hepatitis B virus e antigen-negative infection: importance of host factors. HEPATOLOGY 1995;22: 1628-1634.
33. Laskus T, Persing DH, Nowicki MJ, Mosley JW, Rakela J. Nucleotide sequence analysis of the precore region in patients with fulminant hepatitis B in the United States. Gastroenterology 1993;105:1173-1178.
34. Laskus T, Rakela J, Nowicki MJ, Persing DH. Hepatitis B virus core promoter sequence analysis in fulminant and chronic hepatitis B. Gastroenterology 1995;109:1618-1623.
35. Liang TJ, Hasegawa K, Munoz SJ, Shapiro CN, Yoffe B, McMahon BJ, et al. Hepatitis B virus precore mutation and fulminant hepatitis in the United States. A polymerase chain reaction-based assay for the detection of specific mutation. J Clin Invest 1994;93:550-555.
36. Parekh S, Zoulim F, Ahn SH, Tsai A, Li J, Kawai S, et al. Genome replication, virion secretion, and e antigen expression of naturally occurring hepatitis B virus core promoter mutants. J Virol 2003;77:6601-6612.
37. Imamura T, Yokosuka O, Kurihara T, Kanda T, Fukai K, Imazeki F, et al. Distribution of hepatitis B viral genotypes and mutations in the core promoter and precore regions in acute forms of liver disease in patients from Chiba, Japan. Gut 2003;52:1630-1637.
38. Buckwold VE, Xu Z, Chen M, Yen TS, Ou JH. Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on precore gene expression and viral replication. J Virol 1996;70:5845-5851.
39. Ogata N, Miller RH, Ishak KG, Purcell RH. The complete nucleotide sequence of a pre-core mutant of hepatitis B virus implicated in fulminant hepatitis and its biological characterization in chimpanzees. Virology 1993;194:263-276.
40. Bocharov G, Ludewig B, Bertoletti A, Klenerman P, Junt T, Krebs P, et al. Underwhelming the Immune Response: effect of slow virus growth on CD8+-T-lymphocyte responses. J Virol 2004;78:2247-2254.
41. Shimizu M, Ohyama M, Takahashi Y, Udo K, Kojima M, Kametani M, et al. Immunoglobulin M antibody against hepatitis B core antigen for the diagnosis of fulminant type B hepatitis. Gastroenterology 1983;84:604-610.
42. Rinker J, Galambos JT. Prospective study of hepatitis B in thirty-two inadvertently infected people. Gastroenterology 1981;81:686-691.
43. Kent GP, Brondum J, Keenlyside RA, LaFazia LM, Scott HD. A large outbreak of acupuncture-associated hepatitis B. Am J Epidemiol 1988; 127:591-598.
44. Tassopoulos NC, Papaevangelou GJ, Sjogren MH, Roumeliotou-Karayannis A, Gerin JL, Purcell RH. Natural history of acute hepatitis B surface antigen-positive hepatitis in Greek adults. Gastroenterology 1987;92: 1844-1850.
45. Beasley RP, Hwang LY, Lin CC, Ko YC, Twu SJ. Incidence of hepatitis among students at a university in Taiwan. Am J Epidemiol 1983;117:213-222.
46. McMahon BJ, Alward WL, Hall DB, Heyward WL, Bender TR, Francis DP, et al. Acute hepatitis B virus infection: relation of age to the clinical expression of disease and subsequent development of the carrier state. J Infect Dis 1985;151:599-603.
47. Schomerus H, Wiedmann KH, Dolle W, Peerenboom H, Strohmeyer G, Balzer K, et al. (+)-Cyanidanol-3 in the treatment of acute viral hepatitis: a randomized controlled trial. HEPATOLOGY 1984;4:331-335.
48. Sherlock S. The natural history of hepatitis B. Postgrad Med J 1987;63: S7-S11.
49. Kobayashi M, Arase Y, Ikeda K, Tsubota A, Suzuki Y, Saitoh S, et al. Viral genotypes and response to interferon in patients with acute prolonged hepatitis B virus infection of adulthood in Japan. J Med Virol 2002;68: 522-528.
50. Suzuki Y, Kobayashi M, Ikeda K, Suzuki F, Arfase Y, Akuta N, et al. Persistence of acute infection with hepatitis B virus genotype A and treatment in Japan. J Med Virol 2005;76:33-39.

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Case report

Adult onset type II citrullinemia as a cause of non-alcoholic steatohepatitis

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Adult onset type II citrullinemia (CTLN2) is an autosomal recessive disease accompanied with hyperammonemia and a sudden onset of psychiatric disorders. We demonstrated three male patients with CTLN2 having a liver histology of non-alcoholic steatohepatitis (NASH). Patients with NASH were analyzed for the causative gene of CTLN2, SLC25A13 and discussed.

Keywords: **NASH; CTLN2; Citrullin; Pathology; Mutation**

1. Introduction

Non-alcoholic steatohepatitis (NASH) has been increasingly recognized as one of the major types of progressive liver disease. NASH may be the most common liver disease with a high prevalence in obese individuals and those with type 2 diabetes. In addition to these nutritional or metabolic causes of NASH, drugs and genetic disorders have been reported to induce NASH [1–3]. We herein propose adult onset type II citrullinemia (CTLN2) as a new genetic cause of NASH.

CTLN2 is characterized by episodes of neurological symptoms associated with hyperammonemia involving disorientation, abnormal behavior (aggression, irritability and hyperactivity), seizures, coma, and potentially death from brain edema [4]. The causative gene of CTLN2 has been identified to be SLC25A13 which encodes citrin, a Ca-binding mitochondrial aspartate-glutamate transporter [5], and a citrin deficiency causes not only CTLN2 in adults, but also idiopathic neonatal hepatitis with intrahepatic cholestasis (NICCD) in neonates [4,6]. One of the clinical and pathological features of CTLN2 and NICCD is the presence of a fatty liver [4,7]. We herein show hepatic steatosis associated with fibrosis and inflammatory change compatible with NASH. The pathogenesis of NASH was also investigated based on the mutation of SLC25A13.

2. Patients and methods

2.1. CTLN2 patients

Three patients with genetically and enzymatically diagnosed CTLN2 were analyzed for their clinical and histopathological findings according to the Brunt classification [8]. None of the three male patients had a history of habitual alcohol drinking (Table 1).

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Table 1
Clinical and pathological data of three patients with type II citrullienemia

Patient	1	2	3
Age at onset CTLN2	37	16	42
Sex	M	M	M
Wt. (kg)/Ht (cm), BMI (kg/m^2)	41.5/160,16.2	43/162,16.4	53/172,17.9
Clinical course	Found by a psychiatrist to demonstrate somnolence at night with elevated NH3	NICCD at 2 months of age. Vomiting and consciousness disturbancy at 15 y. o. Fulminant hepatic failure	A liver biopsy revealed steatohepatitis because of liver dysfunction. NH3 was high and a psychiatrist observed dys-orientation with triphasic wave in EEG
NH3 (20–50 nm/dl)	350–600	530	716
Citrulline (28–48 nm/ml)	460	814	119.7
Arginine (70–128 nm/ml)	249.3	183.3	154.3
Mutation of SLC25A13 (see text 2.3)	[IV]: S225X&[V]: IVS13+1G>A, compound hetero	[II], IVS11+1G>A, homo	[V], IVS13+1G>A, homo
ASS in liver (2.59±1.13 U/g)	0.032	0.0038	0.55
PSTI (4.2–12 ng/ml)	Not done	43	150
GOT/GPT (IU/l)	24/33	163/101	52/79
alb (g/dl)	3.6	4.1	3.9
Plt (×10,000/mm^3)	13.5	19.7	20.7
t-chol (mg/dl)/TG (mg/dl)	112/103	193/100	175/nd
Liver histology (#1)			
Grade/steatosis (%)	1/30%	3/70%	2/50%
Stage	1	4	2
Periportal/pericentral	mild/mild	severe/mild	moderate/mild
Mallory body	−	+	+
Liporgranuloma	−	+	+
Glycongen nucleus	+	+	+
Treatment	Sodium glutamate, sodium citrate	Liver transplantation from his father	Argimagte, KM (#2), lactulose
Prognoisis	Good	Fair after transplantation	Fair

#1, modified from Ref. [8]; #2, Kanamycin

2.2. NASH patients

Fourteen patients with NASH, 4 men and 10 women ranging from 28 to 70 years of age (mean±SD=55.3±18.6), who showed various degrees of NASH, were analyzed for 14 loci of the causative gene of CTLN2, SLC25A13, by the method described below.

2.3. Detection of citrin gene SLC25A13 mutations

Fourteen in 21 mutation sites of citrin gene SLC25A13 were analyzed by the conventional method, since 94% of CTLN2 patients have been diagnosed at these 14 loci. The 13 known mutations, [I]–[XI], [XIV] and [XX], were detected by PCR-RFLP and/or multiple GeneScan/SNaPshot methods, as described previously [5,6,9,10], and a novel [XIX] mutation will be reported in the near future elsewhere (Tabata et al. in preparation). Genomic DNA samples from the patients were used after obtaining their written informed consent. This study was performed in accordance with the Declaration of Helsinki and its amendments and was approved by the Ethics Committee of the Faculty of Medicine at Gunma University and Kagoshima University.

3. Results

3.1. Clinical profile of CTLN2 patients (Table 1)

Patient 1 had been healthy until developing night somnolence and thus, visited a psychiatrist for the treatment. Hyperammonemia and deregulation of amino acid including citrullinemia [11] were demonstrated. He was diagnosed to have CTLN2 and had an extremely low activity of argininosuccinate synthetase (ASS) of the liver. After the discovery of SLC25A13 and the identification of mutations [5], compound heterozygous mutations were found. Patient 2 was a 16-year-old boy with fulminant hepatic failure who was diagnosed to have CTLN2 based on a genetic analysis [13]. He underwent a partial liver transplantation from his father and had done well for 5 years after the operation [12]. Thereafter, we found that he suffered from neonatal intrahepatic cholestasis related to NICCD symptoms, at 2 months old of age [13]. Patient 3 was suffered from a sudden onset of consciousness disturbance with hyperammonemia. A serum amino acid analysis revealed hypercitrullinemia and he was finally diagnosed as CTLN2 by a genetic analysis.

3.2. Liver histology of the CTLN2 patients

A liver biopsy under the guide of ultrasonography was performed in patients 1 and 3. The liver histology of patient 2 was obtained from the explanted liver at the transplantation (Table 1 and Fig. 1).

Patient 1 showed grade 1 steatosis (Fig. 1(a)) and stage 1 pericentral and periportal fibrosis (Fig. 1(a)). Focal necrosis

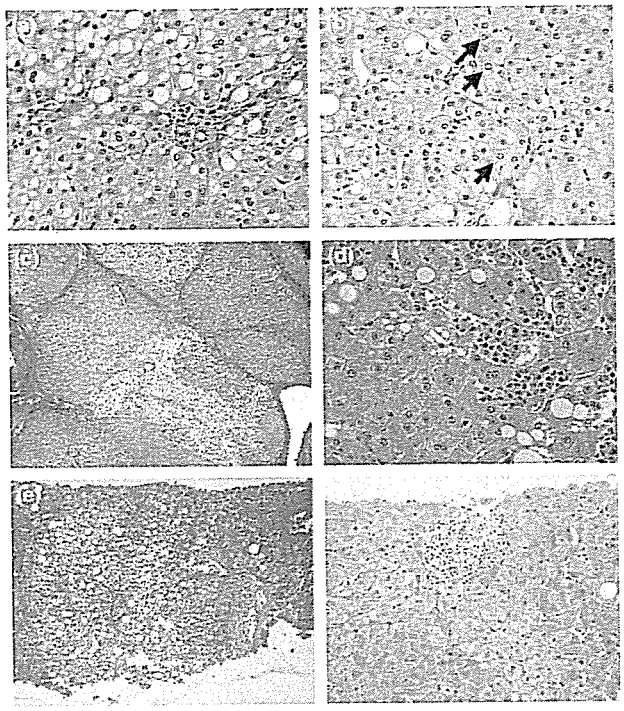

Fig. 1. (a) and (b) Histopathology of patient 1, 37-year-old male. (a) Portal inflammation and periportal fat deposition (HE, 80×, 200×). (b) Glycogen nuclei (arrow), large and small droplets were observed. (c) and (d) Histopathology of patient 2, 16-year-old male. (c) Stage 4 cirrhotic change with pericentral fatty change was observed. Azan Mallory 40×. (d) focal necrosis with neutrophils infiltration. HE 200×. (e) and (f) Histopathology of patient 3, 42-year-old male. (e) Steatosis in zone 2 and 3. Azan Mallory 40×. (f) Granuloma formation was seen. HE, 80×.

with mononuclear cell infiltration (Fig. 1(a)) and lots of glycogen nuclei were found (Fig. 1(b)). Patient 2 had stage 4 fibrosis, namely cirrhosis with pericentral steatosis (Fig. 1(c)) and grade 2 inflammation with neutrophils infiltration (Fig. 1(d)). Mallory body, granuloma formation around central vein and moderate hemosiderosis were noted (not shown). Patient 3 showed pericentral fibrosis accompanied with zone 2–3 steatosis (Fig. 1(e)) and inflammatory change with granuloma formation (Fig. 1(f)).

3.3. Citrin gene analysis in patients with NASH

None of the fourteen NASH patients had a mutation in 14 loci of SLC25A13 gene.

4. Discussion

Due to recent advances in the understanding of NASH, in addition to the acquired causes of NASH such as diabetes mellitus and jejuno-ileal bypass, genetic causes have been reported such as abetalipoproteinemia, galactosemia and so on [1–3]. We herein advocate that CTLN2 is a disease associated with NASH. All three presented cases with CTLN2 demonstrated NASH in different grades of inflammation and stages of fibrosis. Because of the accumulation of NADH in the cytosol due to the dysfunction of citrin, over production of fatty acid and the suppression of the metabolism of fatty acid may simultaneously occur in hepatocytes thereby easily inducing steatosis [4,7]. After the induction of fat deposition, a second hit, which has yet to be clarified, could evoke and inflammatory response, namely NASH.

Previous reports have showed some genetic predisposition of NASH, such as tumor necrosis alpha promoter [14] and a hemochromatosis gene [15] but these changes are only considered to partially contribute to the etiology of the disease. Ordinarily, patients with CTLN2 are lean or not obese regarding their body structure in contrast to most of NASH patients who are tend to be obese. All three patients of CTLN2 patients analyzed in this study had a low body mass index (BMI) under 18 kg/m^2. Five of the 14 patients with NASH were not obese with BMI of under 25, but over 20 kg/m^2. All 14 NASH patients including this five had no detectable known mutations in citrin gene. Although the frequency of NASH by CTLN2 is not supposed to be high, we have heard an episode that one lean patient with a fatty liver who suddenly suffered from a consciousness disturbance and later developed CTLN2 (personal communication).

In conclusion, CTLN2 has thus been histologically proven to be one of the causes of NASH. Although an analysis of the citrin gene in patients with NASH failed to find any mutations, we should therefore include CTLN2 in the differential diagnosis of NASH, especially in lean patients with a fatty liver and mental disturbance.

References

[1] Angulo P. Non Alcoholic fatty liver disease. N Engl J Med 2004;346: 1221–1231.
[2] Sass D, Change P, Chopra KB. Nonalcoholic fatty liver disease: a clinical review. Dig Dis Sci 2005;50:171–180.
[3] Allard JP. Other disease associations with non-alcoholic fatty liver disease (NAFLD). Best Pract Res Clin Gastroenterol 2002;16: 783–795.
[4] Kobayashi K, Saheki T. Aspartate glutamate carrier (Citrin) deficiency. In: Broer S, Wagner CA, editors. Membrane transporter diseases. New York, NY: Kluwer Academic/Plenum Publishers; 2003. p. 147–160.
[5] Kobayashi K, Sinasac DS, Iijima M, Boright AP, Begum L, Lee JR, et al. The gene mutated in adult-onset type II citrullinemia encodes a putative mitochondrial carrier protein. Nat Genet 1999;22159–22163.
[6] Yamaguchi N, Kobayashi K, Yasuda T, Nishi I, Iijima M, Nakagawa M, et al. Screening of SLC25A13 mutations in early and late onset patients with citrin deficiency and in the Japanese population: identification of two novel mutations and establishment of multiple DNA diagnosis methods for nine mutations. Hum Mutat 2002;19:122–130.
[7] Saheki T, Kobayashi K, Iijima M, Horiuchi M, Begum L, Jalil MA, et al. Adult-onset type II citrullinemia and idiopathic neonatal

hepatitis caused by citrin deficiency: involvement of the aspartate glutamate carrier for urea synthesis and maintenance of the urea cycle. Mol Genet Metab 2004;81:S20–S26.

[8] Brunt EM, Janney CG, Di Bisceglie AM. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. Am J Gastroenterol 1999;94:2467–2474.

[9] Lu YB, Kobayashi K, Ushikai M, Tabata A, Iijima M, Li MX, et al. Frequency and distribution in East Asia of 12 mutations identified in the SLC25A13 gene of Japanese patients with citrin deficiency. J Hum Genet 2005;50:338–346.

[10] Takaya J, Kobayashi K, Ohashi A, Ushikai M, Tabata A, Fujimoto S, et al. Variant clinical courses of two neonatal intrahepatic cholestasis patients with a novel mutation of SLC25A13. Metabolism; in press.

[11] Kamiya Y, Horikoshi T, Takagi H, Okada S, Hashimoto K, Kido C, Type II , et al. citrullinemia associated with neutropenia. Int Med 1995;34:679–682.

[12] Tomomasa T, Kobayashi K, Kaneko H, Shimura H, Fukusato T, Tabata M, et al. Possible clinical and histologic manifestations of adult-onset type II citrullinemia in early infancy. J Pediatr 2001;138: 741–743.

[13] Kasahara M, Ohwada S, Takeuchi T, Kaneko H, Tomomasa T, Morikawa A. Living-related liver transplantation for type II citrullinemia using a graft from hetrozygote donor. Transplantation 2001;157:157–159.

[14] Valenti L, Fracanzani AL, Dongiovanni P, Santorelli G, Branchi A, Taioli E, et al. Tumor necrosis factor alpha promoter polymorphisms and insulin resistance in nonalcoholic fatty liver disease. Gastroenterology 2002;122:274–280.

[15] Bonkovsky HL, Jawaid Q, Tortorelli K, LeClair P, Cobb J, Lambrecht RW, Banner BF. Non-alcoholic steatohepatitis and iron: increased prevalence of mutations of the HFE gene in non-alcoholic steatohepatitis. J Hepatol 1999;31:421–429.

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Chapter II

Treatment for the Bone Metastasis of Liver Cancer

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Abstract

Bone metastasis is usually a complication of advanced cancer but sometimes the metastasis preceded before the primary lesion was diagnosed. We report here three cases of bone metastasis of hepatocellular carcinoma and discussed the strategy for the treatment of bone metastasis. They were treated by enderpines for fractured left humerus, surgical resection for Th1 vertebral and right 4th rib metastasis. All the patients were complicated by HCV related liver cirrhosis with hepatocellular carcinoma. Two of the patients were found to have bone metastasis following the treatment of primary lesion in the liver but the third patient was first found by the vertebral metastasis itself. Although hepatocellular carcinoma itself was in an advanced stage, the operation for bone metastasis did not worsen the liver function and achieved a moderate to fair quality of life because the functional reserve of the liver was preserved well enough. A severely disturbed quality of life by the bone metastasis was dramatically improved by surgical treatment although long-term survival could not be gained.

Keywords: Bone metastasis, Hepatocellular carcinoma.

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Introduction

Bone metastasis is usually a complication of advanced cancer but sometimes the metastasis preceded before the primary lesion was diagnosed [1, 2, 3]. Furthermore the choice of the treatment of bone metastasis has not been established because the performance status is usually poor and radical treatment is not tolerated by the patients. The prognostic factors of such patients were summarized in Table 1 and 2. (modified from ref 4). The more the prognostic score, the poorer the prognosis(Table 1). Cumulative survival rate according to the sum of prognostic score of table 1 is shown in Tabe 2 . Namely the sum of the prognostic score is less than 2, long-lasting reconstructive operation should be performed and those with more than 6 should be conservatively treated (4). According to the data, liver is one of the three poorest primary cancers in prognosis (the other two is lung and stomach) to the bone metastasis (4). Especially most of the patients with hepatocellular carcinoma (HCC), which is more than 95% of primary liver cancer [5], were accompanied by chronic liver injury such as liver cirrhosis, the treatment for the metastatic lesions are restricted due to the deteriorated liver functional reserve. We present here the three cases of bone metastasis from HCC. The metastatic bone lesions were surgically treated and the local control for bone fracture, pain and nerve palsy was almost fairy controlled.

Table 1. Multi-variation analysis of the prognosis of bone metastasis. (modified from ref. 4)

	prognostic factor		score
1	primary lesion	lung, liver, stomach	3
		malignant lymphoma, prostate,	0
		breast, multiple myeloma, thyroid	0
		other cancer and sarcoma	2
2	metastasis to the other site including brain		2
3	performance status 3, 4		1
4	previous chemotherapy		1
5	multiple bone meta		1

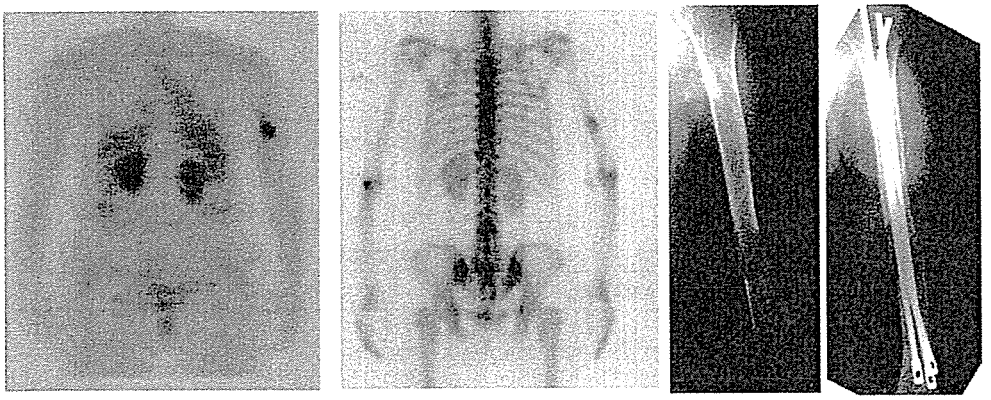
Table 2. Survival rate of patients with bone metastasis according to multivariate analysis. (modified from ref.4)

	Cumulative survival rate		
score	6 months	12 months	24 months
0~2	0.979	0.891	0.753
3~5	0.706	0.488	0.278
6~8	0.313	0.109	0.023

Case Reports

Case 1

Seventy-three-year-old female with HCC accompanied by liver cirrhosis was treated by ethanol injection and transcatheter arterial embolization in September 1993. She has been followed up in the hospital as a case of chronic hepatitis since 1988 and diagnosed later with hepatitis C. In May 1996, she was admitted for ruptured esophageal varices, which were subsequently treated by endoscopic injection sclerotherapy. A solitary metastatic tumor was found in the left humerus in August 1998 on 2-fluorine-18-fluoro-2-deoxy-D glucose positron emission tomography (FDG-PET) (Fig. 1a). A technetium-99m methylene diphosphonate (^{99m}TcMDP) bone scintigram also demonstrated a solitary bone metastatic tumor on the left humerus (Fig. 1b). Radiation therapy applied to the bone metastasis resulted in regression of the tumor. In September 1999, fracture occurred at the site of metastatic tumor in the left humerus (Fig. 1c). Three enderpines were inserted (Fig. 1d). The biopsy specimen taken at operation showed moderately differentiated HCC. The patient complained of severe fracture-related pain preoperatively but after surgical treatment, the pain was completely controlled by pentazosine. She survived 1 year after treatment, but died on September 2, 2000 of liver failure.



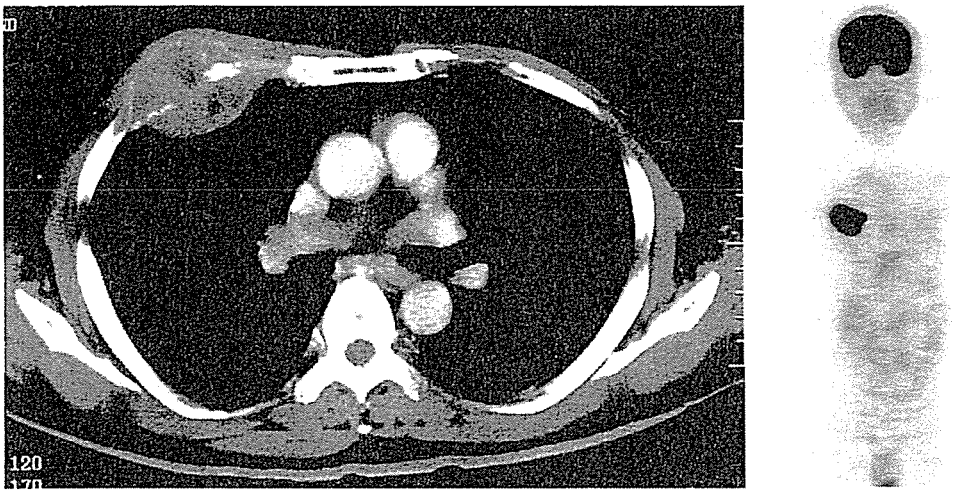
a b c d

Figure 1. 73 year-old female with HCC.
a. FDG-PET showing a solitary metastatic tumor from HCC in the right humerus.
b. ^{99m}TcMDP-bone scintigraphy also detected the solitary bone metastatic tumor from HCC in the middle of the right humerus.
c. Right humerus-showing fracture at the site of metastasis.
d. Three enderpines were inserted into the humerus for the repair of the fracture.

Case 2

Forty-eight-year-old man received IFN therapy for hepatitis C in1995 but failed to eradicate HCV. He was found to have HCC at 54 years old in October 2001 and was treated by transcatheter arterial embolization and radiofrequency ablation. HCC was not completely

eradicated. He came to the hospital with the complaint of right chest wall swelling with pain in February 2003. CT demonstrates 4 x 8 x 6 cm of irregular mass at the right chest wall containing bone density (Fig. 2a). FDG-PET shows dense deposition at the 4th rib without the other accumulation (Fig. 2b). Technetium-99m methylene diphosphonate bone scintigraphy also showed solitary accumulation at the 4th rib. No jaundice or ascites were noted and the functional reserve of the liver was preserved well. The solitary bone metastasis to right 4th rib from HCC was first irradiated and resected with surrounding tissue on March 14, 2003 without any complications. Histopathological examination revealed the metastasis of HCC. The patient survived for 4 months after the resection with no pain concerning the thoracic region in terminal care facility in his vicinity.



a b

Figure 2. 54 year-old male with HCC.
a. CT demonstratess 4 x 8 x 6 cm of irregular mass at right chest wall containing bone density.
b. FDG-PET shows dense deposition at the 4th rib without the other accumulation

Case 3

Sixty four-year-old male admitted to the hospital because of tetraplegia in November 2003. He had been conscious of back pain and paresthesia in his legs for 1 year. MRI showed that a vertebral tumor at Th 1 pressed upon the spinal cord (Fig. 3a). Then the operation to resect the vertebral tumor was performed (Fig. 3b). The pathological diagnosis was metastatic HCC with incomplete resection. Multiple low-density areas in the cirrhotic liver were detected on computed tomography(Fig. 3c) and in laboratory data, HCV-Ab was positive and both of serum AFP (153,433 ng/ml) and PIVKA II (8,008mAU/ml) were highly elevated. He was diagnosed as multiple HCC with liver cirrhosis due to HCV complicated with bone metastasis. Now he is treated with systemic chemotherapy for his multiple HCC and radiation therapy for the residual bone metastasis in Th1 vertebra.

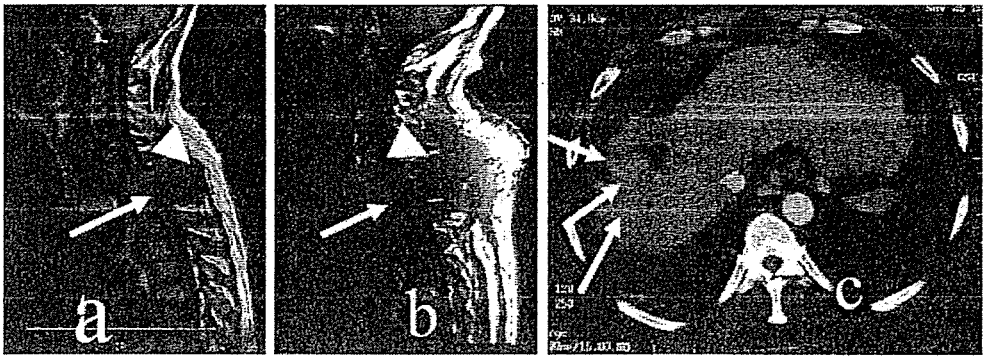


Figure 3. 64 year-old male with HCC appeared with tetraplegia. a. MRI showed a vertebral tumor (arrow head) at Th1 pressed upon the spinal cord (arrow). b. Laminectomy decompressed the spine at Th1 (arrow) but the resected area was swollen by edema after the operation (arrow head). c. Computed tomography revealed multiple liver tumors (arrow) suggesting hepatoma after the vertebral operation.

Discussion

HCC is one of the most intractable cancers in the world. Various treatments including surgical resection, ethanol injection, microwave coagulation, radiofrequency ablation can eradicate the local cancer but the recurrence of HCC in chronic liver disease such as hepatitis B or C worsens the prognosis and causes deterioration of the quality of life [5]. We present here three cases of metastatic bone cancer from HCC. All three patients were surgically treated for their metastatic bone lesions and the successful recovery with functional improvement and pain control was gained after the surgery. Unfortunately, the nerve palsy of the bilateral lower extremities in the third patient was not improved even though radiation and laminectomy was applied in 2 days after the progressive palsy in the lower extremities. According to the scoring by Table 2, the prognostic score of each case is 4, 6 and 5, then the six months survival rate is 0.706, 0,311 and 0.313 respectively. Because the primary lesion was in the liver, the least score surpassed 3. Although the latter two cases were poorer than the first case by this scoring system, the chest pain and nerve palsy accelerated to perform the surgical treatment.

The risk factor of metastasis from HCC is reported as histological poor differentiation, larger tumors and multilobar spread [6]. The frequent sites of HCC metastasis were the lung, bone, adrenal gland, and lymph node [5]. Bone metastasis usually occurs through lung but the bone metastasis without lung metastasis could develop through vertebral venenous plexus because of the lack of venous valve [7, 8]. Poor differentiation, multilobar spread, and size (> or = 5 cm) were the strongest predictors of metastatic disease of HCC [6]. Okazaki et al. reported that male predominance and less frequency of accompanied liver cirrhosis were the predictable factors for HCC bone metastasis [9]

As a diagnostic procedure to detect bone metastasis, FDG-PET could be one of sensitive modality as well as bone scintigraphy [10]. Although our experience is limited to the present case, FDG-PET seems to be useful for the detection of bone metastasis from HCC. Other

studies have previously reported the usefulness of FDG-PET for detection of metastatic bone tumors from primary tumors of the prostate [11] and lung [12] but to the authors' knowledge, this is the first case of metastatic bone tumors from HCC detected by FDG-PET [10]. Bone scintigraphy using technetium 99m is also useful for the detection of such lesions but it is only indicated for bone metastasis. FDG-PET could be used as a first screening modality for metastatic tumors from HCC.

The therapeutic modality for bone metastasis includes irradiation, surgical resection, chemotherapy, arterial infusion or embolization, and ablation using radiofrequency [13, 14]. These non-surgical treatments could control the growth of the metastatic lesion but the residual lesion after any non-surgical treatments could not be clearly prepared and the functional improvement of the involved lesion is usually difficult to fully achieve. No gold standard has been established for the treatment of bone metastasis, however the quality of life should always be considered because most of those cases were in the advanced stage of cancer. Generally the surgical treatment should be indicated for the patients with bone metastasis in the extremities having more than two to three months' survival and more than three to six months prognosis for vertebral metastasis [15, 16]. HCC metastatic lesions do not often cause death [17] but the quality of life worsens precipitously after the development of metastatic bone cancer [18]. The cause of death in such cases is usually HCC-related and includes cancer death, hepatic failure, gastrointestinal bleeding and tumor rupture. Other causes of death that are not related to HCC form only 7.5% of the total HCC according to the newest report from the Japanese Liver Cancer Group [4]. It is often difficult to decide whether metastatic sites should be treated or not when the primary lesion is not controlled as in this case. Tsukeoka et al. [19] reported five preoperative deaths in 59 patients scheduled for palliative operation for metastatic bone lesions. Death in their cases was due to hepatic failure, unexpected cerebral tumor embolism, intraoperative cardiac arrest secondary to pulmonary embolism, disseminated intravascular coagulation with acute renal failure and superior vena cava syndrome with life-threatening airway obstruction during general anesthesia. Although the rate of unexpected events in such patients is less than 10%, surgical procedures in patients with poor general condition always need strict evaluation. When the primary lesion is not controlled, bone fractures at the site of metastatic lesion are often not treated because of poor condition and unsuitability for the surgical treatment. Good prognosis would be gained in case as follows; 1) intrahepatic lesions was cleared or well controlled, 2) the treatment for extrahepatic metastases was effective and 3) extrahepatic metastasis was recognized in Child A by Imamura [20]. If the general condition allows general anesthesia and operative surgery, the patient should be orthopedically or surgically treated to enhance the quality of life. This rule should be applicable even in elderly patients.

References

[1] Edmondson HA, Peters RL: Neoplasms of the liver. In: *Diseases of the liver,* Edited by Schiff L and Schiff ER. 5th Edition. JB Lippincott Company, Philadelphia, 1982, p 1081-1157.

[2] Orr FW, Lee J, Duivenvoorden WC, Singh G. Pathophysiologic interactions in skeletal metastasis. *Cancer* 2000; 88: Suppl:2912-8.

[3] Okazaki N, Yoshino M, Yoshida T, Hirohashi S, Kishi K, Shimosato Y. Bone metastasis in hepatocellular carcinoma. *Cancer* 1985; 55: 1991-4.

[4] Katagiri H, Takahashi M, Takagi T. Prognostic factor and diagnostic strategy for skeletal metastasis. *J Joint Surg* 2003; 22: 974-982 (in Japanese).

[5] Liver Cancer study Group of Japan. Survey and follow-up study of primary liver cancer in Japan. *Acta Hepatol Jap. 41*: 799-811, 2000(in Japanese and abstract in English).

[6] Si MS, Amersi F, Golish SR, Ortiz JA, Zaky J, Finklestein D, Busuttil RW, Imagawa DK. Prevalence of metastases in hepatocellular carcinoma: risk factors and impact on survival. *Am Surg.* 2003; 69: 879-85.

[7] Batson OV. The function of the vertebral veins and their role in the spread of metastases. *Ann Surg 112*: 138-149, 1940.

[8] Yamada T, Soga K, Ohta H, Nomoto M, Kamimura T, Ichida F. Clinicopathological study of hepatocellular carcinoma with bone metastasis. *Acta Hepatol Japn.* 271550-1558, 1985.

[9] Okazaki N, Yoshino I, Yoshida T, Hirohashi S, Kishi K, Shimosato Y. Bone metastasis in hepatocellular carcinoma. *Cancer* 1985:55: 1991-4.

[10] Takagi H, Nagai H, Minashi K, Monden T, Kanda D, Nakajima H, Otsuka T, Mori M. Orthopedic treatment of metastatic bone fracture from hepatocellular carcinoma. *HepatoGastroenterology* 2002; 49: 1526-27.

[11] Oyama N, Akino H, Suzuki Y, Kanamaru H, Sadato N, Yonekura Y, Okada K. Increased accumulation of [^{18}F]fluorodeoxyglucose in untreated prostate cancer. *Jpn J Clin Oncol 29*:623-9, 1999.

[12] Bury T, Barreto A, Daenen F, Barthelemy N, Ghaye B, Rigo P. Fluorine-18 deoxyglucose positron emission tomography for the detection of bone metastases in patients with non-small cell lung cancer. *Eur J Nucl Med 25*:1244-7, 1998.

[13] Maruyama M, Asano T, Kenmochi T, Kainuma O, Iwasaki K, Miyauchi H, Saigo K, Miura F, Kobayashi S, Ochiai T. Radiofrequency ablation therapy for bone metastasis from hepatocellular carcinoma: case report. *Anticancer Res.* 2003 May-Jun; 23(3C): 2987-9.

[14] Nakane H, Iwata K, Nishizawa S, Yoshikane M, Anan A, Irie M, Suzuki N, Sohda T, Watanabe H, Sakisaka S.Nippon Shokakibyo Gakkai Zasshi. 2003 Jul; 100(7):873-7. Percutaneous radiofrequency ablation of bone metastasis to the rib from hepatocellular carcinoma: a case report.

[15] Harrington KD. New trends in the management of lower extremity metastasis. *Clin Orthop.* 1982: 169: 53-61.

[16] Tomita K, Kawahara N, Kobayashi T, et al. Surgical Strategy for spinal metastasis. *Spine 26*: 298-306, 2001.

[17] Sohara N, Yamada T, Ichikawa T, Abe T, Ito H, Takagi H. Three cases of hepatocellular carcinoma died of extrahepatic metastatic lesions. *Liver Cancer. 3*: 164-169, 1997 (in Japanese and Abstract in English).

[18] Iwamoto Y, Sugioka Y, Chuman H, Shiba K, Yuge I. Surgical treatment of metastatic tumors of long bones and the spine. *Adv Exp Med Biol 324*:295-303, 1992.

[19] Tsukeoka T, Kochi T, Koide K, Tahara M, Koyama T, Kitou M, Umeda T. The risk of palliative operation for bone metastasis. *Masui 46*:1634-8, 1997.

[20] Imamura I. Prognostic efficacy of treatment for extrahepatic metastasis after surgical treatment of hepatocellular carcinoma. *Kurume Med J.* 2003; 50:41-48.

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Basic Studies

Lack of macrophage migration inhibitory factor protects mice against concanavalin A-induced liver injury

Nakajima H, Takagi H, Horiguchi N, Toyoda M, Kanda D, Otsuka T, Emoto Y, Emoto M, Mori M. Lack of macrophage migration inhibitory factor protects mice against concanavalin A-induced liver injury. Liver International 2006: 26:346–351. © Blackwell Munksgaard 2006

Abstract: *Background:* Macrophage migration inhibitory factor (MIF) is involved in inflammatory and immune-mediated diseases but the role of MIF in liver injury has not yet been elucidated. *Methods:* We investigated biochemically, histologically and immunologically the character of MIF in concanavalin A (Con A)-induced T-cell-mediated liver injury using MIF knockout (KO) mice and wild-type (WT) mice. *Results:* MIF KO mice showed significantly decreased serum alanine aminotransferase values and suppressed histological change with massive necrosis of the hepatic parenchymal cells and infiltration of inflammatory cells compared with their WT counterparts. This protection was not mediated by either tumor necrosis factor-α or interferon-γ, which are critical mediators of Con A-induced liver injury, as their serum concentrations were shown to be similar in MIF KO and WT mice. On the other hand, a flow cytometric analysis demonstrated that the number of activated hepatic leukocytes decreased more in the MIF KO mice than in the WT mice. *Conclusions:* A lack of MIF protected the mice from Con A-induced liver injury. Controlling the MIF activity may be a useful therapeutic strategy for treating such T-cell activation-associated liver diseases as autoimmune hepatitis and viral hepatitis.

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Key words: concanavalin A – interferon-γ – liver – macrophage migration inhibitory factor – mouse – tumor necrosis factor–α

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Concanavalin A (Con A), a plant lectin from the jack bean (*Canavalia ensiformis*), is known to mitogenically stimulate T lymphocytes and cause T-cell-mediated acute hepatic injury in mice (1–3). $CD4^+$ T lymphocytes as well as macrophages were identified as effector cells, and injection of Con A led to liver-specific single organ injury in a dose-dependent manner without any further sensitization (1–3). Con A-induced liver injury could provide a model for investigating the pathophysiology of T-cell activation-associated hepatitis in humans such as autoimmune hepatitis (1). The induction of the liver injury is associated with the production of various cytokines (interleukin (IL)-2, tumor necrosis factors (TNFs), IL-1, IL-6, IL-10, IL-12, granulocyte macrophage-colony stimulating factor (GM-CSF) and interferon (IFN)-γ) (2–6). Two proinflammatory cytokines, TNF-α and IFN-γ, might act synergistically and play a critical role in the development of massive hepatocellular apoptosis and necrosis (2, 4, 7, 8). Administration of either anti-TNF-α or anti-IFN-γ antibody inhibits development of Con A-induced hepatitis (2–4, 9). In addition, in chronic active hepatitis B or C, the activity of the disease has been shown to correlate with T helper 1-like cytokine response of intrahepatic $CD4^+$ T-cells (10, 11).

Macrophage migration inhibitory factor (MIF) was first discovered about 40 years ago as a T-cell-derived lymphokine that inhibited the random migration of macrophages, and was involved in the mechanism of delayed-type hypersensitivity (12, 13). However, recent studies have revealed that MIF is a pleiotropic cytokine expressed in a variety of cells and tissues other than T-cells during inflammatory responses, and that monocytes and macrophages are important sources of

Abbreviations: Con A, concanavalin A; IFN, interferon; LPS, lipopolysaccharide; MIF, macrophage migration inhibitory factor; TNF, tumor necrosis factor; KO, knockout; WT, wild-type.

MIF production (14, 15). MIF is characterized as an inflammatory cytokine, a neuroendocrine hormone and an enzyme (16, 17). MIF is also a unique counter-regulator of the immunosuppressive and anti-inflammatory activities of glucocorticoids (18).

Endotoxemia after challenge with lipopolysaccharide (LPS) induces MIF release from macrophages and pituitary cells. MIF is a critical mediator of host defenses, with a role in septic shock (14) and acute inflammatory diseases (19–21) as a proinflammatory cytokine. On the other hand, Honma et al. (22) showed that MIF was not crucial for LPS-induced immune responses leading to shock by studies in MIF-deficient mice.

To our knowledge, there are no reports of the role of MIF in Con A-induced hepatic injury. In the present study, we examined the role of MIF in Con A-induced hepatic injury using MIF Knockout (KO) mice.

Materials and methods

Reagents

Con A was purchased from Sigma (St. Louis, MO). Diethyl ether and the Isogen RNA extraction kit were purchased from Wako (Osaka, Japan). Mouse MIF cDNA probe was a generous gift from Dr. J. Nishihira (Hokkaido University School of Medicine, Japan).

Animals

MIF KO mice were provided by Dr. T. Nakayama and M. Taniguchi (Graduate School of Medicine, Chiba University, Japan). Wild-type (WT) counterpart BALB/c female mice (7–9 weeks) were purchased from Japan SLC (Hamamatsu, Japan). The animals were kept in an air-conditioned room, and given standard chow and water *ad libitum*. All animal studies were performed according to the guidelines for animal care and use established by Gunma University Graduate School of Medicine.

Assessment of liver injury

Con A was dissolved to a concentration of 1 mg/ml in pyrogen-free saline and was administered to mice via the tail vein at a dose rate of 10 mg/kg. After mice were anesthetized by inhalation of diethyl ether, their blood was collected from axillary vessels and livers were excised. One section of the liver was fixed in buffered formalin, and the other section was frozen for Northern blot analysis. Liver damage was evaluated by measuring the serum activity of alanine aminotransferase (ALT) assayed by using a standard clinical autoanalyzer (model 7450, Hitachi, Tokyo, Japan).

Assay of serum levels of TNF-α and IFN-γ

Serum levels of TNF-α and IFN-γ were assayed by an enzyme-linked immunosorbent assay (ELISA) kit (American Research Products, Belmont, MA) according to the instructions provided by the manufacturer.

Histological examination

Liver tissue from individual mice was fixed in 10% formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin for histological examination.

Analysis of RNA transcripts

The MIF transcript was detected by Northern blot hybridization as described previously (23). The mouse MIF cDNA probe was synthesized by PCR as a template and the following set of primers: 5′-CACCATGCCTATGTTCATCGTGAACA-3′ and 5′-AGCGAAGGTGGAACCGTTCCA-3′. Total RNA was extracted from liver tissues using an Isogen RNA extraction kit according to the instructions provided by the manufacturer, and 20 μg was loaded per lane onto 1% agarose/formaldehyde gels and transferred onto nylon membranes after electrophoresis.

Cell preparation and flow cytometry

Hepatic leukocytes (HL) were prepared as described previously (24). In brief, the liver was passed through a stainless-steel mesh after perfusion. The cell suspensions were centrifuged at 50g for 30 s and supernatants were harvested. The cells were then suspended in 40% Percoll (1.124 g/ml; Biochrome, Berlin, Germany) and layered onto 70% Percoll. The tubes were centrifuged at 600g at 20 °C for 25 min, and HL (interface between 40% and 70% layer of Percoll) were isolated. After blocking with anti-FcγR mAb, cells were stained with conjugated mAbs. Fluoresein isothiocyanate-conjugated anti-CD69mAb and biotinylated anti-CD3mAb were purchased from BD PharMingen (Hamburg, Germany). Streptavidin-conjugated CyChrome was obtained from BD PharMingen. After staining, cells were washed with PBS containing 0.1% bovine serum albumin (Serva, Heidelberg, Germany) and 0.1% sodium azide (Merck, Darmstadt, Germany), and then fixed with 1% paraformaldehyde (Merck). Stained cells were acquired by FACSCalibur® (BD Biosciences, Mountain View, CA) and analyzed with CellQuest software.

Statistical analysis

Data are presented as mean ± SD. The significance of differences between groups was determined by two-way analysis of variance (ANOVA). All analyses were performed using the Statview software, and differences were considered significant when $P<0.05$.

Results

Confirmation of targeted disruption of the MIF gene in MIF KO mice

As shown in Fig. 1, brain tissue from a WT mouse was used as a control. As expected, no MIF mRNA was detected in MIF KO livers, whereas MIF expression was detected in livers and a brain from WT mice.

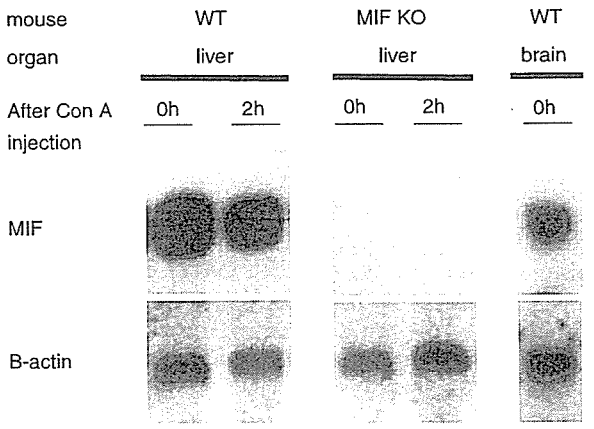

Fig. 1. Northern blot hybridization of liver RNA obtained from wild-type (WT) and macrophage migration inhibitory factor knockout (MIF KO) mouse at 0 and 2 h after 10 mg/kg concanavalin A injection via the tail vein was performed using probes for MIF and β-actin.

Suppressed Con A-induced liver injury in KO mice

Biochemical liver damage was confirmed in WT mice by elevation of serum ALT at 12 and 24 h after intravenous administration of Con A. Notably, MIF KO mice were found to be significantly less susceptible to liver injury compared with their WT counterparts ($P = 0.0021$, Fig. 2).

Basal liver histology of control (Fig. 3a) and MIF KO mice (Fig. 3b) before treatment was almost identical, with no specific changes. Twenty-four hours after Con A injection, massive necrosis of hepatic parenchymal cells and infiltration of inflammatory cells were seen in WT mice (Fig. 3c). In contrast, these changes were very mild in MIF KO mice (Fig. 3d).

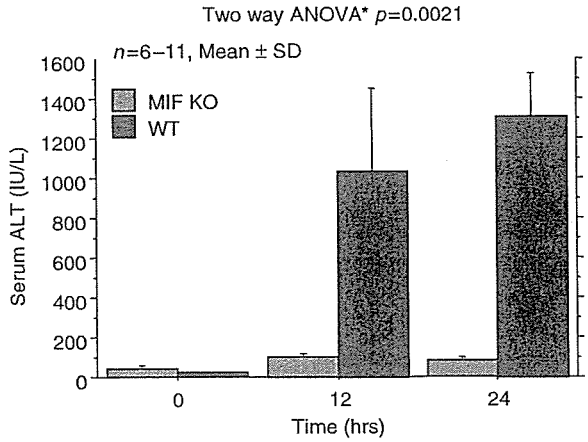

Fig. 2. Time course of serum alanine aminotransferase (ALT) levels in wild-type (WT) and macrophage migration inhibitory factor knockout (MIF KO) mice before and after Concanavalin A (Con A) injection. Mice were injected via the tail vein with 10 mg/kg Con A. Results are mean ± SD of six to 11 mice per group. ANOVA, analysis of variance.

Inflammatory mediator expression in MIF KO and WT mice

As shown in Fig. 4a, serum TNF-α content increased at 2 h after Con A injection and then quickly decreased at 12 h. This change was almost the same in both WT and MIF KO mice ($P = 0.7272$). On the other hand, serum IFN-γ content increased 2 h after Con A injection, reached a peak level at 8 h and then decreased at 24 h (Fig. 4b). This change was almost identical in WT and MIF KO mice ($P = 0.2194$).

CD69 expression on $CD3^+$and $CD3^-$ cells before and after Con A injection in livers of WT and MIF KO mice (Fig. 5)

Because activated leukocytes play a decisive role in liver injury, we examined the influence of MIF deficiency on activated T-cells. Increased proportion of $CD69^+$cells was detected among hepatic leukocytes from WT mice at 24 h after Con A administration regardless of CD3 expression, which were markedly diminished in MIF KO mice. These results suggest that MIF plays a central role in activation of T-cells.

Discussion

MIF was recently reported to be involved in inflammatory, autoimmune, and neoplastic process in human diseases (16). The contribution of MIF has also been reported in liver diseases such as drug-induced liver injury (25), hepatocellular carcinoma (26), and chronic hepatitis B (27). Despite findings suggesting the involvement of MIF in various models of liver injury, its precise physiological role is still unknown as no MIF

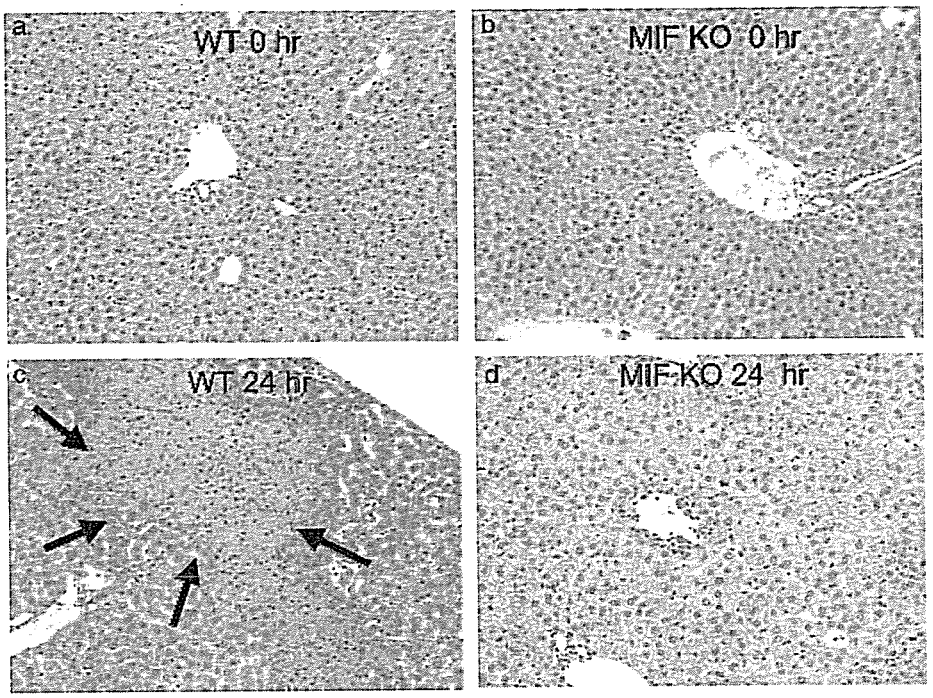

Fig. 3. Microscopic appearance of the liver from wild-type (WT) and macrophage migration inhibitory factor knockout (MIF KO) mice before and after Concanavalin A (Con A) injection (hematoxylin and eosin (HE), × 200). The livers were removed 0 and 24 h after 10 mg/kg Con A injection via the tail. Light micrographs of livers stained with HE are shown for each representative experiment. (a) Liver tissue of untreated WT mouse. (b) Liver tissue of untreated MIF KO mouse. (c) Liver tissue of WT mouse 24 h after Con A injection. Arrows show a necrotic lesion. (d) Liver tissue of MIF KO mouse 24 h after Con A injection.

receptor has thus far been identified (17), although CD74 was reported to be a candidate of MIF receptor (28). In this study, we have demonstrated that lack of MIF protects mice from Con A-induced liver injury, namely, that MIF is necessary for Con A-induced hepatic injury.

MIF KO mice were overtly normal, fertile, and healthy in appearance. The numbers and proportions of splenic T and B lymphocytes were comparable with those in WT mice, indicating that MIF is not involved in the process of lymphocyte development (22).

Bozza et al. (20) report that MIF KO mice were protected from LPS-induced liver injury and had lower plasma levels of TNF-α than did WT mice when stimulated with LPS, and that MIF was required for the optimal production of TNF-α during endotoxemia. MIF may be secreted from infiltrated Kupffer cells and macrophages in response to LPS, which in turn induces TNF-α production and T-cell infiltration (15, 17, 21). Anti-MIF antibody suppressed TNF-α production (17). Within the cytokine network, TNF-α and IFN-γ up-regulate MIF production in macrophages, and conversely, MIF induces TNF-α and IFN-γ production (15, 17, 21). Trautwein et al. (29) showed that the rise of aminotransaminase level was significantly reduced by anti-TNF-α blocking in Con A-induced liver injury in mice. Two reports previously demonstrated that IFN-γ played a central role in Con A-induced hepatitis (4, 8). TNF-α and IFN-γ could induce apoptosis in Con A-induced acute liver failure because the liver injury was prevented by anti-TNF and anti-IFN in this model (3, 7). Thus, the cascade of inflammatory mediators triggered by Con A is important in the pathogenesis of Con A-induced liver injury, we measured cytokine levels in the serum of MIF KO mice compared with WT mice by Con A stimulation. We speculated that the mechanism of resistance of MIF KO mice to Con A-induced liver injury was by blocking TNF-α or IFN-γ. However, in this study, TNF-α and IFN-γ production was not inhibited in the MIF KO mice. Bourdi et al. (25) reported that the decreased drug-induced liver injury in the MIF KO mice correlated with a reduction in the mRNA levels of IFN-γ. These unique differences could be related to a different signaling pathway because of differences in the animal model of hepatitis (drug-induced liver injury and Con A hepatitis) or in animal strains (C57Bl/6 × 129F1 and BALB/c). In order to further clarify the mechanism of the attenuation of Con A-induced liver injury in MIF KO mice,

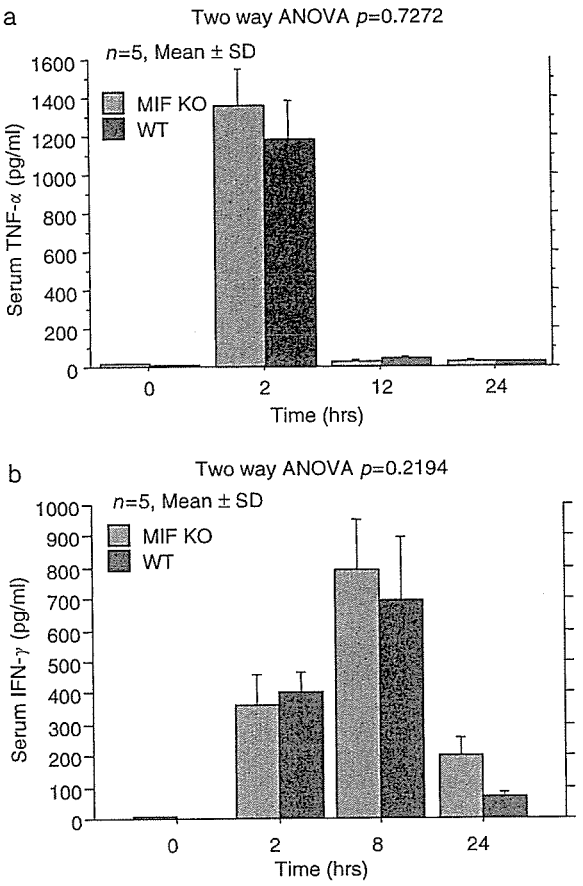

Fig. 4. Time course of serum tumor necrosis factor-α (TNF-α) (a) and interferon-γ (IFN-γ) (b) levels in wild-type (WT) and macrophage migration inhibitory factor knockout (MIF KO) mice challenged by Concanavalin A (Con A) injection. Mice were injected via the tail vein with 10 mg/kg Con A. Levels were assayed by specific enzyme-linked immunosorbent assay (ELISA) kits in duplicate, exactly according to the manufacturer's instructions. Results are mean ± SD of five mice per group. ANOVA, analysis of variance.

the hepatic lymphocyte subpopulation was analyzed by flowcytometry. As a result, the number of activated T-cells decreased in the MIF KO mice in the liver both before and after Con A-induced liver injury. MIF is reported to activate T-cells via the T-cell receptor but not via the IL-2 receptor (30). Our results support the findings of this previous report regarding the necessity of MIF in T-cell activation.

In summary, we have demonstrated that the lack of MIF protected mice from Con A-induced liver injury. In explanation of this, we showed that TNF-α and IFN-γ did not play an important part in the pathogenesis of this process but that the activation of hepatic lymphocytes was low in MIF KO than WT mice after Con A injection. Further studies are needed to elucidate the mechanisms involved. In any case, control of MIF

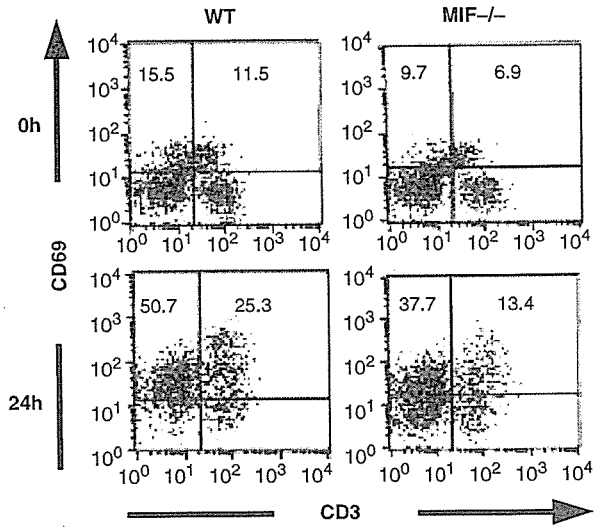

Fig. 5. CD69 expression on $CD3^+$ and $CD3^-$ cells before and after Concanavalin A (Con A) injection in livers of wild-type (WT) and macrophage migration inhibitory factor knockout (MIF KO) mice. Data are displayed as dot plots after gating on liver cells of WT or MIF KO mice. Numbers in dot plots represent percentages of $CD3^+$ and $CD3^-$ CD69 cells.

activity may be a useful therapeutic strategy in T-cell activation-associated liver diseases such as autoimmune hepatitis and viral hepatitis.

Acknowledgments

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References

1. Tiegs G, Hentschel J, Wendel A. A T cell-dependent experimental liver injury in mice inducible by concanavalin A. J Clin Invest 1992; 90: 196–203.
2. Gantner F, Leist M, Lohse AW, Germann P G, Tiegs G. Concanavalin A-induced T-cell-mediated hepatic injury in mice: the role of tumor necrosis factor. Hepatology 1995; 21: 190–8.
3. Mizuhara H, O'Neill E, Seki N, et al. T cell activation-associated hepatic injury: mediation by tumor necrosis factors and protection by interleukin 6. J Exp Med 1994; 179: 1529–37.
4. Kusters S, Gantner F, Kunstle G., Tiegs G. Interferon gamma plays a critical role in T cell-dependent liver injury in mice initiated by concanavalin A. Gastroenterology 1996; 111: 462–71.