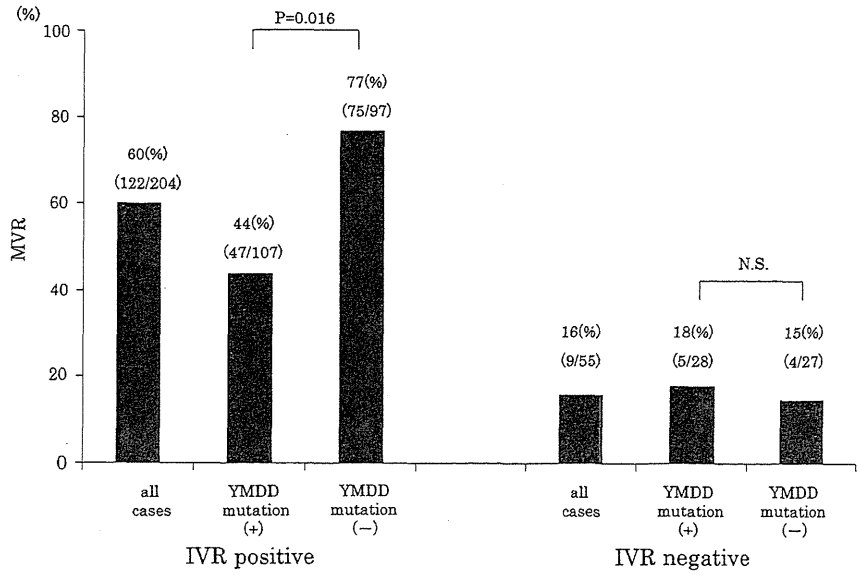

Fig. 3 Relationship between IVR and MVR. *IVR* Initial viral response, *MVR* maintained viral response, *N.S.* not significant

Many investigators have reported that serum HBV DNA levels higher than 4.0–4.5 log copies/ml before HBV treatment serve as a strong risk predictor of HCC [23–25]. Di Marco et al. [26] have reported that the incidence of HCC was higher in patients with serum HBV levels of more than 5.0 log copies/ml, at least once, during LAM therapy than in those in whom serum HBV levels were maintained at 5.0 log copies/ml or less. However, the add-on ADV therapy had not been adopted when the study of Di Marco et al. was reported. When the use of ADV is possible, an evaluation method is needed to measure the antiviral effects of nucleoside/nucleotide analogues against HBV-related liver disease. In the present study, we set the cut-off value for HBV-DNA at 4.0 log copies/ml. The basis of this cut-off value is that a serum HBV DNA level higher than 4.0 log copies/ml before HBV treatment was reported to serve as a strong risk predictor of HCC [23]. MVR, defined as a median HBV-DNA level of less than 4.0 log copies/ml measured every 6 months during therapy, was adopted as an indicator of viral replication, and non-MVR (median HBV-DNA >4.0 log copies/ml) during LAM therapy was shown to be significantly associated with the development of HCC in HBV-infected patients. We also found that a median HBV-DNA level of >4.0 log copies/ml during LAM therapy was a risk factor for HCC development. On the other hand, IVR, defined as HBV-DNA of <4.0 log copies/ml in the first 6 months of the follow-up period after the initiation of therapy, was not associated with the development of HCC in HBV patients in this study. As shown in Fig. 3, 84% of the IVR-negative patients could not achieve an MVR, suggesting that it is crucial to achieve an IVR in order to achieve an MVR. The reason why IVR was not a significant factor for MVR seemed to be the appearance of the YMDD mutation, which reduced the antiviral effect of LAM for HBV in IVR-positive patients. The LAM-resistant YMDD mutant virus was found in 52% of the IVR-positive patients. Although ADV was added to LAM treatment for 73 patients, only 33% of these patients could achieve an MVR. We speculate that the antiviral effect of ADV is not very strong [27] and it takes time to reduce the YMDD mutant virus, which may explain the low MVR rate (33%) in patients with the add-on ADV therapy. The immediate administration of ADV when the LAM-resistant YMDD mutant virus appears can be important [28]. A switch to ETV, which induces resistant virus less frequently, could also raise MVR rates among IVR-positive patients without the YMDD mutant virus.

As the duration of the add-on ADV therapy was included in this study, we compared the cumulative incidence of HCC in patients receiving LAM monotherapy with that in patients who also received the add-on ADV therapy. Sixteen (10%) of the 164 patients who received the LAM monotherapy developed HCC and the cumulative carcinogenesis rate was 6% at 3 years, 10% at 5 years, and 15% at 7 years. On the other hand, 16 (12%) of the 129 patients who received LAM plus ADV developed HCC and the cumulative carcinogenesis rate was 6% at 3 years, 12% at 5 years, and 14% at 7 years. No significant difference was found between these two groups ($p = 0.986$). In addition, we examined the cumulative incidence of HCC development according to the effectiveness of treatment (MVR vs. non-MVR) in patients for whom the observation period was terminated when ADV was added, and the same results were obtained (data not shown).

Older age (≥50 years), cirrhosis, and low platelet count ($<14 \times 10^4/mm^3$) were shown to be significantly associated with the development of HCC in patients with HBV infection. These results were consistent with those of

previous reports [29–31], suggesting that patients of older age with advanced fibrosis should be followed up carefully for longer periods in order to detect early stages of HCC even if LAM therapy does effectively suppress HBV. Of note, in the present study we estimated the cumulative HCC incidence according to the initial diagnosis of chronic hepatitis or cirrhosis. In the chronic hepatitis patients, older age (≥50 years), HBe Ag negativity, and low platelet count ($<14 \times 10^4/mm^3$) were significant risk factors for the development of HCC, but this was not the case in the cirrhosis patients. Because liver biopsies had not been performed, the liver fibrosis stage could not be evaluated with respect to the risk factors for HCC in this study. Instead, the factors of age, HBe Ag status, and platelet count may reflect the degree of liver fibrosis in chronic hepatitis patients. In fact, cirrhotic patients, in comparison with chronic hepatitis patients, were of older age (chronic hepatitis vs. cirrhosis: 46.3 ± 10.7 vs. 51.9 ± 9.8 years, $p < 0.001$), had higher rates of HBe Ag negativity (chronic hepatitis vs. cirrhosis: 39 vs. 51%, $p = 0.065$), and had lower platelet counts (chronic hepatitis vs. cirrhosis: 15.6 ± 4.9 vs. $9.3 \pm 3.8 \times 10^4/mm^3$, $p < 0.001$). This seems to explain why none of these factors were significant risk factors for HCC in cirrhotic patients. On the other hand, in the chronic hepatitis patients, MVR was not a significant factor for HCC development, while MVR was a significant factor for HCC development in the cirrhotic patients. We speculate that HBV suppression induced by LAM therapy could reduce the incidence of HCC in patients infected with HBV, especially those with cirrhosis, who displayed higher malignant potential. Investigation over a longer period is needed to clarify the effect of HBV suppression on the development of HCC in chronic hepatitis patients.

In conclusion, the present study shows that the attainment of an MVR induced by LAM therapy has a significant beneficial effect on the clinical course of HBV-infected patients by decreasing the incidence of HCC. The newer nucleotide analogues, such as ETV and tenofovir, should be able to further reduce the incidence of HCC, given their greater potency.

Acknowledgments This work was supported by Grants-in-Aid for Research on Hepatitis and BSE from the Ministry of Health Labour and Welfare of Japan and for Scientific Research from the Ministry of Education, Science, and Culture of Japan.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Lok AS, McMahon BJ. Chronic hepatitis B. Hepatology. 2007;45:507–39.
2. Lavanchy D. Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. J Viral Hepat. 2004;11:97–107.
3. Lee WM. Hepatitis B virus infection. N Engl J Med. 1997;337:1733–45.
4. Beasley RP. Hepatitis B virus: the major etiology of hepatocellular carcinoma. Cancer. 1988;61:1942–56.
5. Lok SF. Chronic hepatitis B. N Engl J Med. 2002;346:1682–3.
6. Tang B, Kruger WD, Chen G, Shen F, Lin WY, Mboup S, et al. Hepatitis B viremia is associated with increased risk of hepatocellular carcinoma in chronic careers. J Med Virol. 2004;72:35–40.
7. Kim SR, Kudo M, Hino O, Han KH, Chung YH, Lee HS. Epidemiology of hepatocellular carcinoma in Japan and Korea. A review. Oncology. 2008;75:13–6.
8. Marcellin P, Chang TT, Lim SG, Tong MJ, Sievert W, Shiffman ML, et al. Adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B. N Engl J Med. 2003;348:808–16.
9. Taura K, Ikai I, Hatano E, Fujii H, Uyama N, Shimahara Y. Implication of frequent local ablation therapy for intrahepatic recurrence in prolonged survival of patients with hepatocellular carcinoma undergoing hepatic resection: an analysis of 610 patients over 16 years old. Ann Surg. 2006;244:265–73.
10. Shimada K, Sano T, Sakamoto Y, Kosuge T. A long-term follow-up and management study of hepatocellular carcinoma patients surviving for 10 years or longer after curative hepatectomy. Cancer. 2005;104:1939–47.
11. Takayasu K, Arii S, Ikai I, Omata M, Okita K, Ichida T, et al. Prospective cohort study of transarterial chemoembolization for unresectable hepatocellular carcinoma in 8510 patients. Gastroenterology. 2006;131:461–9.
12. Dienstag JL, Perrillo RP, Schiff ER, Bartholomew M, Vicary C, Rubin M. A preliminary trial of lamivudine for chronic hepatitis B infection. N Engl J Med. 1995;333:1657–61.
13. Nevens F, Main J, Honkoop P, Tyrrell DL, Barber J, Sullivan MT, et al. Lamivudine therapy for chronic hepatitis B: a six-month randomized dose-ranging study. Gastroenterology. 1997;113:1258–63.
14. Rapti I, Dimou E, Mitsouka P, Hadziyannis SJ. Adding-on versus switching-to adefovir therapy in lamivudine-resistant HBVAg-negative chronic hepatitis B. Hepatology. 2007;45:307–13.
15. Chang TT, Lai CL, Kew Yoon S, Lee SS, Coelho HS, Carriho FJ, et al. Entecavir treatment for 5 years in patients with hepatitis Be antigen-positive chronic hepatitis B. Hepatology. 2010;51:422–30.
16. Kiyosawa K, Tanaka E, Sodeyama T. Hepatitis C virus and hepatocellular carcinoma. Curr Stud Hematol Blood Transfus. 1998;62:161–80.
17. Ohkoshi S, Norio O, Ichida T. The long-term clinical outcome of 1-year treatment of chronic hepatitis B with lamivudine-5 years observation. Hepatol Res. 2003;27:13–7.
18. Chu CM, Liaw YF. Hepatitis B virus-related cirrhosis: natural history and treatment. Semin Liver Dis. 2006;26:142–52.
19. Matsumoto A, Tanaka E, Rokuhara A, Kiyosawa K, Kumada H, Omata M, et al. Efficacy of lamivudine for preventing hepatocellular carcinoma in chronic hepatitis B: a multicenter retrospective study of 2795 patients. Hepatol Res. 2005;32:173–84.
20. Shamliyan TA, MacDonald R, Shaukat A, Taylor BC, Yuan JM, Johnson JR, et al. Antiviral therapy for adults with chronic hepatitis B: a systematic review for a National Institutes of Health Consensus Development Conference. Ann Intern Med. 2009;150(2):111–24.
21. Liaw YF, Sung JJ, Chow WC, Farrell G, Lee CZ, Yuen H, et al. Lamivudine for patients with chronic hepatitis B and advanced liver disease. N Engl J Med. 2004;351(15):1521–31.

22. Perrillo R, Hann HW, Mutimer D, Willems B, Leung N, Lee WM, et al. Adefovir dipivoxil added to ongoing lamivudine in chronic hepatitis B with YMDD mutant hepatitis B virus. Gastroenterology. 2004;126:81–90.
23. Chen CJ, Yang HI, Su J, Jen CL, You SL, Lu SN, et al. Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. JAMA. 2006;295:65–73.
24. Chan HL, Hui AY, Wong ML, Tse AM, Hung LC, Wong VW, et al. Genotype C hepatitis B virus infection is associated with increased risk of hepatocellular carcinoma. Gut. 2004;53:1494–8.
25. Chan HL, Tse CH, Koh FM, Wong VW, Wong GL, Chan SL, et al. High viral load and hepatitis B virus subgenotype Ce are associated with increased risk of hepatocellular carcinoma. J Clin Oncol. 2008;26:177–82.
26. Di Marco V, Marzano A, Lampertico P, Andreone P, Santantonio T, Almasio PL, et al. Clinical outcome of HBeAg-negative chronic hepatitis B in relation to virological response to lamivudine. Hepatology. 2004;40:883–91.
27. Marcellin P, Chang TT, Lim SG, Tong MJ, Sievert W, Shiffman ML, et al. Adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B. N Engl J Med. 2003;348:808–16.
28. Kurashige N, Hiramatsu N, Ohkawa K, Yakushijin T, Kiso S, Kanto T, et al. Factors contributing to antiviral effect of adefovir dipivoxil therapy added to ongoing lamivudine treatment in patients with lamivudine-resistant chronic hepatitis B. J Gastroenterol. 2009;44(6):601–7.
29. Chen CJ, Yu MW, Liaw YF. Epidemiological characteristics and risk factors of hepatocellular carcinoma. J Gastroenterol Hepatol. 1997;12:5294–308.
30. Yun LF. Natural history of chronic hepatitis B virus infection and long-term outcome under treatment. Liver Int. 2009;29:100–7.
31. Sung JJ, Tsoi KK, Wong VW, Li KC, Chan LL. Meta-analysis: treatment of hepatitis B infection reduces risk of hepatocellular carcinoma. Aliment Pharmacol Ther. 2008;28:1067–77.

Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Bak deficiency inhibits liver carcinogenesis: A causal link between apoptosis and carcinogenesis

Hayato Hikita[1], Takahiro Kodama[1], Satoshi Shimizu[1], Wei Li[1], Minoru Shigekawa[1], Satoshi Tanaka[1], Atsushi Hosui[1], Takuya Miyagi[1], Tomohide Tatsumi[1], Tatsuya Kanto[1], Naoki Hiramatsu[1], Eiichi Morii[2], Norio Hayashi[3], Tetsuo Takehara[1,*]

[1]*Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, Suita, Osaka 565-0871, Japan;*
[2]*Department of Pathology, Osaka University Graduate School of Medicine, Suita, Osaka 565-0871, Japan;*
[3]*Kansai-Rosai Hospital, Amagasaki, Hyogo 660-8511, Japan*

Background & Aims: Hepatocyte apoptosis is a key feature of chronic liver disease including viral hepatitis and steatohepatitis. A previous study demonstrated that absence of the Bcl-2 family protein Mcl-1 led to increased hepatocyte apoptosis and development of liver tumors in mice. Since Mcl-1 not only inhibits the mitochondrial pathway of apoptosis but can also inhibit cell cycle progression and promote DNA repair, it remains to be proven whether the tumor suppressive effects of Mcl-1 are mediated by prevention of apoptosis.

Methods: We examined liver tumor development, fibrogenesis, and oxidative stress in livers of hepatocyte-specific knockout (KO) of *Mcl-1* or *Bcl-xL*, another key antagonist of apoptosis in hepatocytes. We also examined the impact of additional KO of *Bak*, a downstream molecule of Mcl-1 towards apoptosis but not the cell cycle or DNA damage pathway, on tumor development, hepatocyte apoptosis, and inflammation.

Results: *Bcl-xL* KO led to a high incidence of liver tumors in 1.5-year-old mice, similar to *Mcl-1* KO. Bcl-xL- or Mcl-1-deficient livers showed higher levels of TNF-α production and oxidative stress than wild-type livers at as early as 6 weeks of age and oxidative DNA damage at 1.5 years. Deletion of Bak significantly inhibited hepatocyte apoptosis in *Mcl-1* KO mice and reduced the incidence of liver cancer, coinciding with reduction of TNF-α production, oxidative stress, and oxidative DNA damage in non-cancerous livers.

Conclusions: Our findings strongly suggest that chronically increased apoptosis in hepatocytes is carcinogenic and offer genetic evidence that inhibition of apoptosis may suppress liver carcinogenesis in chronic liver disease.

© 2012 European Association for the Study of the Liver. Published by Elsevier B.V. All rights reserved.

Keywords: Bcl-xL; Mcl-1; 8-OHdG.
Received 26 September 2011; received in revised form 19 January 2012; accepted 21 January 2012; available online 10 March 2012

* Corresponding author. Address: Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan. Tel.: +81 6 6879 3621; fax: +81 6 6879 3629.
E-mail address: takehara@gh.med.osaka-u.ac.jp (T. Takehara).

Abbreviations: HCC, hepatocellular carcinoma; ALT, alanine aminotransferase; RT-PCR, reverse-transcription PCR; HO-1, heme oxygenase-1; NQO1, NAD(P)H:quinone oxidoreductase 1; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling.

Introduction

Apoptosis of epithelial cells, as well as infiltration of inflammatory cells or deposits of fibers, is frequently observed in the chronic diseased liver, which is a high-risk condition for hepatocellular carcinoma (HCC) [1]. For example, Fas-mediated hepatocyte apoptosis is a mechanism of cell death in chronic hepatitis C virus infection and hepatitis B virus infection [2,3]. Hepatocyte apoptosis shows correlation with inflammation and fibrosis in non-alcoholic steatohepatitis [4]. Cytokeratin 18 neoepitope, a well-established marker of caspase activity in serum, is elevated and associated with liver injury in chronic viral hepatitis and non-alcoholic steatohepatitis [5–7]. Although viral factors and overt organ inflammation linked to liver cancer development have been extensively studied [8,9], less information is available on the involvement of hepatocyte apoptosis in liver cancer development.

Bcl-xL and Mcl-1 are among the anti-apoptotic members of the Bcl-2 family, which antagonizes the pro-apoptotic function of Bak and/or Bax at the mitochondrial outer membrane. We previously reported that hepatocyte-specific *Bcl-xL* or *Mcl-1* knockout (KO) mice showed persistent apoptosis of hepatocytes in the adult liver and mild fibrotic responses [10,11]. A very recent study by Weber *et al.* [12] demonstrated that hepatocyte-specific *Mcl-1* KO mice developed liver tumors in old age. This observation raised the important possibility that apoptosis in hepatocytes could lead to the development of liver cancer. However, as Mcl-1 has been reported to possess functions other than anti-apoptosis, such as cell cycle inhibition [13,14] and DNA damage repair [15,16], it is difficult to conclude that the phenotypes observed in *Mcl-1* KO are simply ascribable to apoptosis. Indeed, *Mcl-1* KO mice showed not only increased apoptosis but also increased regeneration in the liver [12]. In the present study, we demonstrated that hepatocyte-specific *Bcl-xL* KO mice also develop liver cancer in old age and that deficiency of Bak, a downstream effector molecule of Mcl-1 towards the

ELSEVIER

Journal of Hepatology **2012** vol. 57 | 92–100

Table 1. Incidence of liver tumors in KO mice.

Age (yr)	Genotype	Tumor incidence
1.5		
	Bcl-xL$^{+/+}$	0% (0/10)
	Bcl-xL$^{-/-}$	88% (7/8)*
1		
	Bcl-xL$^{+/+}$	0% (0/4)
	Bcl-xL$^{-/-}$	27% (3/11)
1.5		
	Mcl-1$^{+/+}$	0% (0/22)
	Mcl-1$^{-/-}$	100% (16/16)*
1		
	Mcl-1$^{-/-}$ *Bak*$^{+/+}$	64% (14/22)
	Mcl-1$^{-/-}$ *Bak*$^{-/-}$	0% (0/7)*

*p <0.05 *vs.* control.

mitochondrial pathway of apoptosis, clearly suppresses hepatocyte apoptosis and liver carcinogenesis in *Mcl-1* KO mice. We also considered possible mechanisms involving oxidative stress that underlie elevated malignant transformation in the apoptosis-prone liver. The present study offers strong support for the hypothesis that chronically increased apoptosis in hepatocytes is carcinogenic. It also provides genetic evidence that inhibition of apoptosis may suppress liver carcinogenesis in chronic liver disease.

Materials and methods

Mice

Conditional *Bcl-xL* KO mice (*bcl-x*$^{flox/flox}$ *Alb-Cre*) and *Mcl-1* KO mice (*mcl-1*$^{flox/flox}$ *Alb-Cre*) were previously described [11]. We purchased *Bak* KO mice (*bak*$^{-/-}$) from the Jackson Laboratory (Bar Harbor, ME). We generated hepatocyte-specific Bak/Mcl-1 double KO mice (*bak*$^{-/-}$ *mcl-1*$^{flox/flox}$ *Alb-Cre*) by mating the strains. They were maintained in a specific pathogen-free facility and treated with humane care with approval from the Animal Care and Use Committee of Osaka University Medical School. Measurement of serum alanine aminotransferase (ALT) level, caspase-3/7 activity and histological analyses have been previously described [11].

Western blot analysis

For immunodetection, the following antibodies were used: anti-Bcl-xL antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Mcl-1 antibody (Rockland, Gilbertsville, PA), anti-Bak antibody (Millipore, Billerica, MA), anti-Bax antibody, anti-ERK antibody, anti-phospho-ERK antibody, anti-p38 antibody, anti-phospho-p38 antibody, anti-JNK antibody, anti-phospho-JNK antibody, anti-PCNA antibody (Cell Signaling Technology, Danvers, MA), and anti-beta-actin antibody (Sigma-Aldrich, Saint Louis, MO).

Real-time reverse-transcription PCR (RT-PCR)

The following TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) were used: mouse-AFP (Mm00431715_m1), mouse-glypican-3 (Mm00516722_m1), mouse-IL-6 (Mm00446190_m1), mouse-TNF-α (Mm00443258_m1), mouse-MCP-1 (Mm00441242_m1), mouse-CD68 (Mm03047343_m1), mouse-CD4 (Mm00442754_m1), mouse-CD8 (Mm01182108_m1), mouse-heme oxygenase-1 (HO-1) (Mm00516005_m1), mouse-NAD(P)H:quinone oxidoreductase 1 (NQO1) (Mm00500821_m1), and mouse-Beta actin (Mm00607939_s1). All expression levels were corrected with the quantified expression level of beta actin.

Immunohistochemistry

8-Hydroxy-2′-deoxyguanosine (8-OHdG), cleaved caspase-9, PCNA, and ki-67 were labeled in paraffin-embedded liver sections using anti-8-OHdG antibody (Nikken Seil, Tokyo, Japan), anti-cleaved caspase-9 antibody, anti-PCNA antibody (Cell Signaling Technology), and anti-ki-67 antibody (Dako, Tokyo, Japan), respectively. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) was performed according to a previously reported procedure [17].

Statistical analysis

Data are presented as mean ± SD. Differences between two groups were determined using the Student's *t*-test for unpaired observations. Carcinogenesis rates were analyzed using the Chi-square test. Multiple comparisons of *Bak/Mcl-1* double KO mice were performed by ANOVA followed by Scheffe *post hoc* correction. Fisher *post hoc* correction was used for the other multiple comparisons. A p <0.05 was considered statistically significant.

Results

Bcl-xL KO mice develop liver tumors in old age

We previously reported that hepatocyte-specific *Bcl-xL* KO mice developed spontaneous hepatocyte apoptosis by the mitochondrial pathway (Supplementary Fig. 1A) at as early as 1 month of age with a gradual increase in the liver fibrotic response from 3 to 7 months [10]. To examine the phenotypes at later time points, we sacrificed *Bcl-xL* KO mice and their control littermates at 1 and 1.5 years of age. Macroscopic tumors had developed in the liver of 27% and 88% of the KO mice, respectively, but not in the control littermates (Fig. 1A and Table 1). Most of the *Bcl-xL* KO mice had multiple tumors and the liver body-weight ratio for *Bcl-xL* KO mice was significantly higher than that of the control mice (Fig. 1B and C). Tumors were histologically defined as well-differentiated HCCs (Fig. 1D). To find out whether the *bcl-x* gene is really targeted in the tumors, we performed Western blot analysis for the expression of the Bcl-2 family proteins (Fig. 1E and Supplementary Fig. 2A). The tumors were confirmed to be deficient for Bcl-xL, excluding the possibility that transformed cells arising from hepatocytes in which the *bcl-x* gene was not deleted had expanded to form tumors. Interestingly, most of these tumors showed apparently higher levels of Mcl-1 expression than the wild-type liver or the non-cancerous surrounding tissues. Reciprocal overexpression of Mcl-1 may explain the possible survival advantage of these tumors. Tumors in *Bcl-xL* KO mice expressed higher levels of α-fetoprotein (Fig. 1F) and frequently showed activation of ERK and JNK (Fig. 1G), which are observed in human HCC [18,19].

Liver tumors in Mcl-1 KO mice show similar characteristics to human HCC

We have previously reported phenotypes of hepatocyte-specific *Mcl-1* KO mice, which display spontaneous hepatocyte apoptosis by the mitochondrial pathway (Supplementary Fig. 1B) and liver fibrotic responses at an early age [11]. Since our *Mcl-1* floxed mice differed from those of Weber *et al.* [12] in origin, we next examined the development of liver tumors in our hepatocyte-specific *Mcl-1* KO mice. All the *Mcl-1* KO mice, but none of the control littermates, developed liver tumors at 1.5 years of age, with a significant increase of liver body-weight ratio (Fig. 2A–C

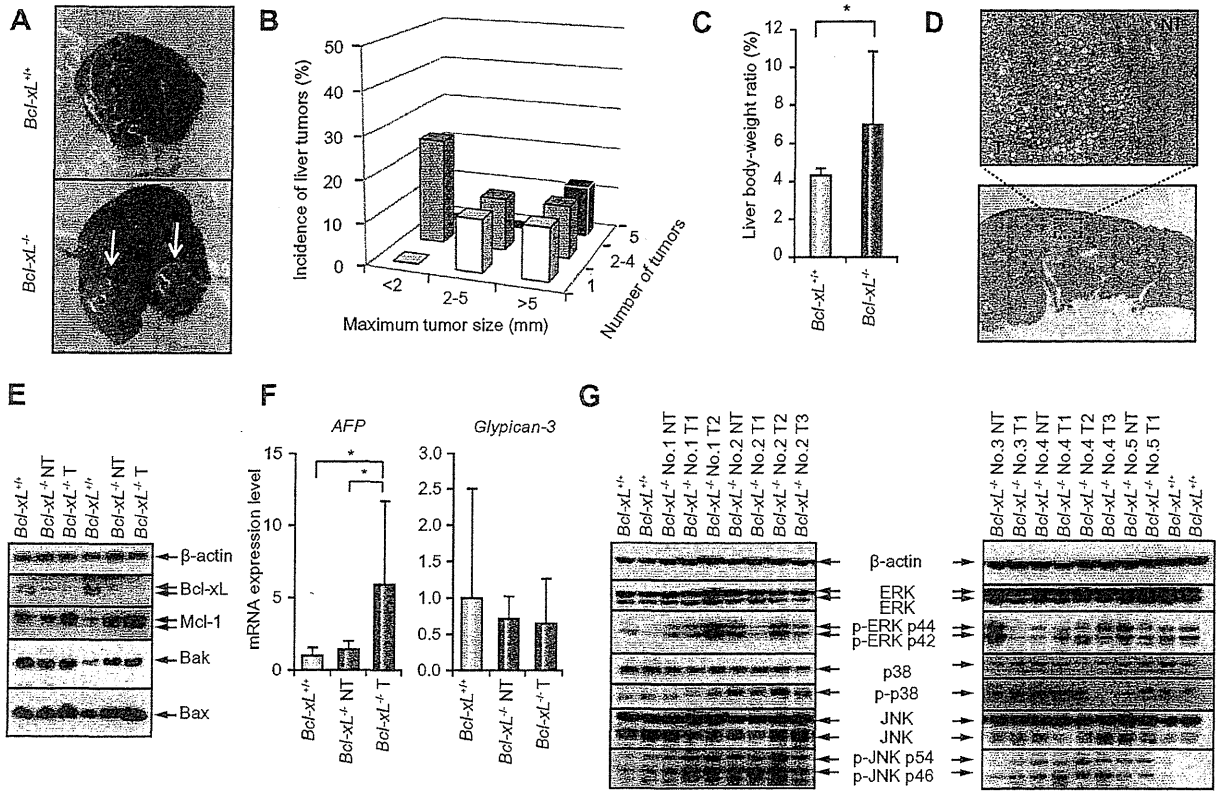

Fig. 1. Liver tumors in *Bcl-xL* KO mice. (A–E) Hepatocyte-specific *Bcl-xL*-deficient mice (*Bcl-xL*$^{-/-}$) (N = 8) and their control littermates (*Bcl-xL*$^{+/+}$) (N = 10) were sacrificed at 1.5 years of age. (A) Representative macroscopic view of the livers with arrows indicating tumors. (B) Incidence of liver tumors separated by maximum tumor size and number of tumors. (C) Liver body-weight ratio. (D) Representative histology of liver tumors in *Bcl-xL* KO mice. (E) Western blot of the Bcl-2 family proteins in tumors (T) and surrounding non-cancerous livers (NT) of *Bcl-xL* KO mice and livers of control mice. (F and G) Characteristics of liver tumors in *Bcl-xL* KO mice. (F) Real-time RT-PCR analysis of the expression levels of α-fetoprotein (*AFP*) and *glypican-3* mRNA (N = 9 or 10 per group). (G) Expression and activation of mitogen-activated protein kinases. *p <0.05.

and Table 1). As in the case of tumors of *Bcl-xL* KO mice, liver tumors that developed in *Mcl-1* KO mice were deficient for Mcl-1 expression and, in most cases, reciprocally overexpressed Bcl-xL (Fig. 2E and Supplementary Fig. 2B). These tumors expressed higher levels of α-fetoprotein and glypican-3 (Fig. 2F) and frequently showed activation of ERK and JNK (Fig. 2G).

Inflammatory response and oxidative stress occur in Bcl-xL- or Mcl-1-KO livers

To examine the molecular mechanism of tumor development, we examined gene expression in the livers of 6-week-old *Bcl-xL* or *Mcl-1* KO mice. Real-time RT-PCR analysis revealed increases of inflammatory cytokine TNF-α, but not IL-6, and chemokine MCP-1 in *Bcl-xL* and *Mcl-1* KO livers (Fig. 3A and B), despite overt histological inflammation (data not shown). Together with an increase of MCP-1, CD68 expression was significantly higher in KO livers than in control livers (Fig. 3C and D). In contrast, there was no difference in the expression of CD4 and CD8 between the groups. These findings suggest that activation or infiltration of myeloid-derived cells and production of TNF-α are characteristic of the *Bcl-xL* or *Mcl-1* KO liver. Together with the previous study reporting that TNF-α promotes cellular transformation [20], these results suggest that the increase in TNF-α may be one of the mechanisms of tumor development.

Since oxidative stress is also reported to cause carcinogenesis [21], we examined the expression of HO-1 and NQO1, inducible anti-oxidant enzymes, and 8-OHdG in the liver tissues. Real-time RT-PCR analysis revealed that HO-1 and NQO-1 expressions were significantly increased in *Mcl-1* KO livers at 6 weeks (Fig. 3E). 8-OHdG staining revealed that there were few 8-OHdG positive nuclei in both *Mcl-1* KO and the control liver at 6 weeks of age. However, scattered positive nuclei were observed in KO livers at 1.5 years of age, but not in the tumors, and the number of positive nuclei was significantly higher in KO livers than in control livers (Fig. 3F and Supplementary Fig. 3). Similarly, the number of 8-OHdG positive nuclei was significantly higher in *Bcl-xL* KO livers at 1.5 years of age than in control livers (Fig. 3G). These results suggest that oxidative stress may occur at as early as 6 weeks of age in KO livers and that oxidative injury arises at a later time point.

Bak deficiency significantly ameliorates hepatocyte apoptosis and reduces tumor development in Mcl-1 KO mice

Bak is a proapoptotic Bcl-2 family protein, which is able to oligmerize to form pores at the outer membrane of mitochondria. To understand whether inhibition of apoptosis could reduce the carcinogenic potential, we crossed *Mcl-1* KO mice and *Bak* KO mice and generated *Bak Mcl-1* double KO mice. As expected, *Bak* KO significantly suppressed hepatocyte apoptosis in *Mcl-1*

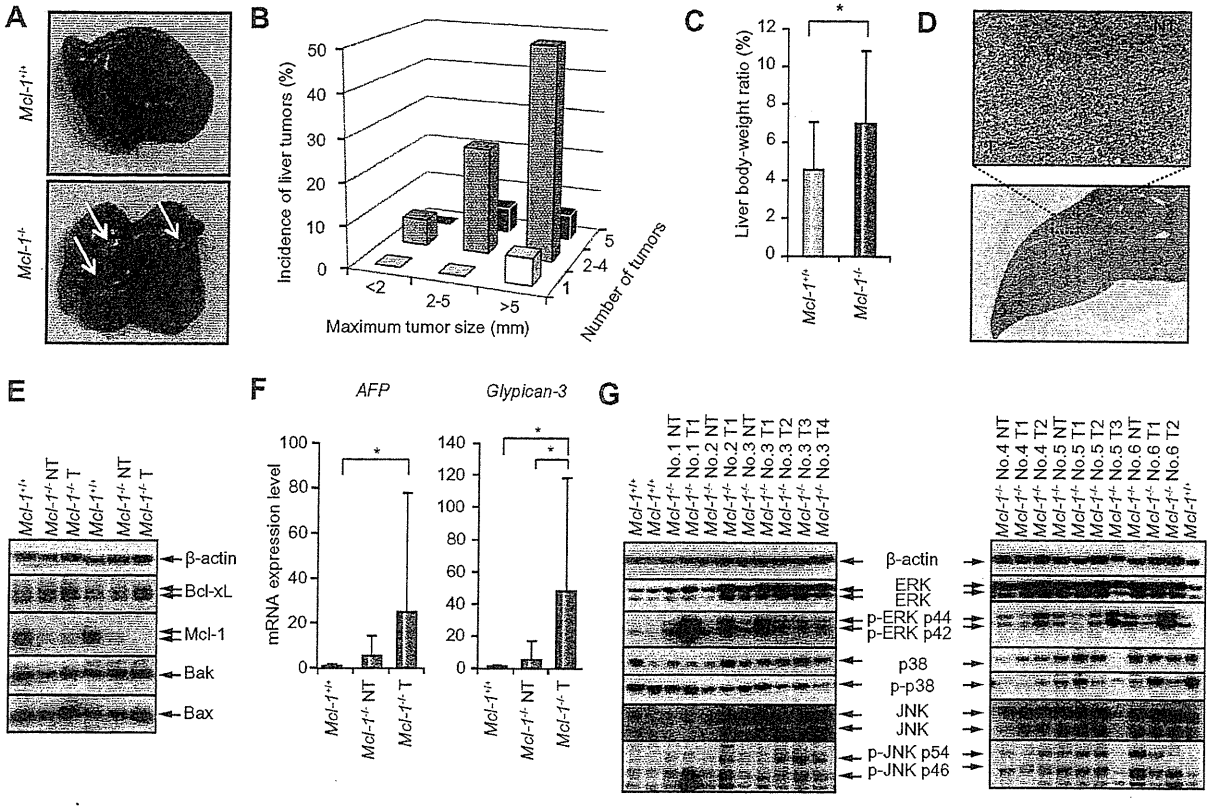

Fig. 2. Liver tumors in *Mcl-1* KO mice. (A–E) Hepatocyte-specific *Mcl-1*-deficient mice (*Mcl-1*$^{-/-}$) (N = 16) and their control littermates (*Mcl-1*$^{+/+}$) (N = 22) were sacrificed at 1.5 years of age. (A) Representative macroscopic view of the livers with arrows indicating tumors. (B) Incidence of liver tumors separated by maximum tumor size and number of tumors. (C) Liver body-weight ratio. (D) Representative histology of liver tumors in *Mcl-1* KO mice. (E) Western blot of the Bcl-2 family proteins in tumors (T) and surrounding non-cancerous livers (NT) of *Mcl-1* KO mice and livers of control mice. (F and G) Characteristics of liver tumors in *Mcl-1* KO mice. (F) Real-time RT-PCR analysis of the expression levels of α-fetoprotein (*AFP*) and *glypican-3* mRNA (N = 16 per group). (G) Expression and activation of mitogen-activated protein kinases. *p <0.05.

KO mice as evidenced by TUNEL staining of liver sections, serum ALT levels and caspase-3/7 activity at 6 weeks of age (Fig. 4A–C). Weber *et al.* [12] previously described hepatocyte regeneration in the *Mcl-1* KO liver. In agreement with this, *Mcl-1* KO livers showed higher expression of cell cycle markers PCNA and ki-67, than those from control littermates (Fig. 4A, B, and D and Supplementary Fig. 4). Importantly, the levels of PCNA and ki-67 expression decreased with a *Bak* KO background in *Mcl-1* KO mice. While *Mcl-1* KO livers show a mild fibrotic change [11], the levels of col1a1 expression at 6 weeks of age and Sirius red staining at 1 year of age decreased with a *Bak* KO background in *Mcl-1* KO livers (Fig. 4E and Supplementary Fig. 5). Bak deficiency also reduced expression levels of TNF-α, MCP-1, and CD68 at 6 weeks of age (Fig. 4F). Next, we examined the impact of apoptosis inhibition by Bak deficiency on oxidative stress markers, which were increased in *Mcl-1* KO livers. Real-time RT-PCR revealed that Bak deficiency reduced the levels of HO-1 and NQO1 expression at 6 weeks of age (Fig. 4G). Consistent with these observations, *Bak* KO significantly lowered the number of 8-OHdG-positive nuclei in *Mcl-1* KO livers at 1 year of age (Fig. 4H). These results suggested that inhibition of hepatocyte apoptosis reduced oxidative stress in the liver. Finally, to examine the impact of apoptosis inhibition on liver tumor development, we compared the carcinogenetic rates in *Mcl-1* KO mice with or without *Bak* KO background at 1 year of age and found that *Bak* KO significantly suppressed liver tumor development (Fig. 5A and B and Table 1).

Discussion

Mcl-1 was first identified as a gene induced during myeloid cell differentiation. Compared with other anti-apoptotic members such as Bcl-2, Bcl-xL, Bcl-w, and Bfl-1, Mcl-1 possesses a unique N-terminus containing two PEST domains, which are found in proteins displaying rapid turnover, and its expression is tightly regulated by growth factors and a variety of other stimuli. Mice systemically deficient for Bcl-xL suffered embryonic death due to massive apoptosis in hematopoietic organs and developing neurons [22]. On the other hand, systemic *Mcl-1* KO resulted in peri-implantation lethality, but *Mcl-1* KO embryos showed no alterations in the extent of apoptosis [23], suggesting that Mcl-1 may play a role early in development that is distinct from its anti-apoptotic functions. Indeed, *in vitro* studies have shown that Mcl-1 interacts with PCNA and Cdk1 in the nucleus and inhibits proliferation [13,14]. Recently, the early responding gene *IEX-1*

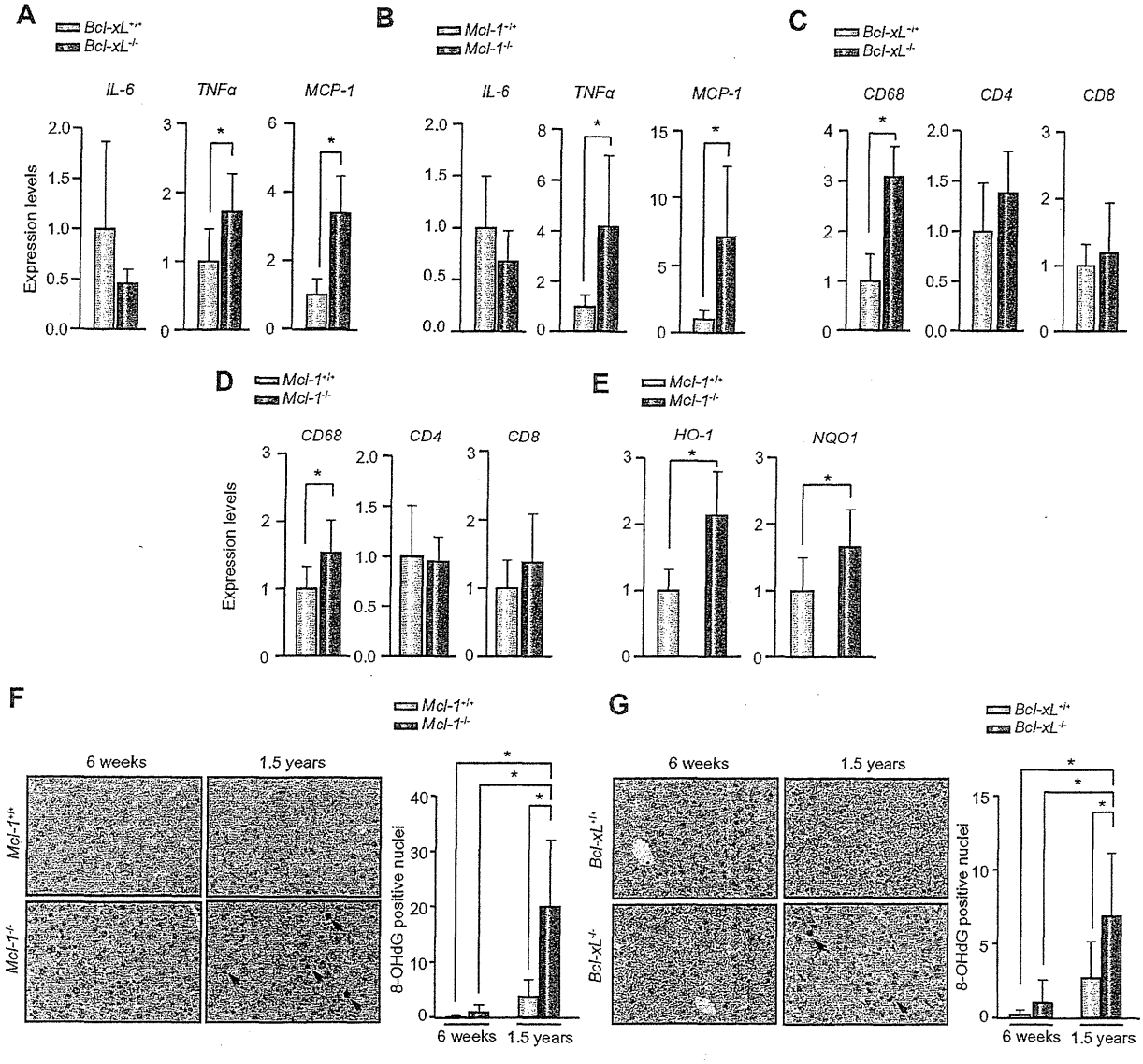

Fig. 3. Inflammatory response and oxidative stress in *Bcl-xL* or *Mcl-1* KO liver. (A–D) Inflammatory responses in KO livers. (A and C) Hepatocyte-specific *Bcl-xL* KO mice (*Bcl-xL*$^{-/-}$) and their control littermates (*Bcl-xL*$^{+/+}$) (N = 6 per group) as well as (B and D) hepatocyte-specific *Mcl-1* KO (*Mcl-1*$^{-/-}$) mice and their control littermates (*Mcl-1*$^{+/+}$) (N = 9 per group) were sacrificed at 6 weeks of age. Expression levels of (A and B) inflammatory molecules and (C and D) cell surface markers of immune cells were analyzed by real-time RT-PCR. (E–G) Oxidative injury in KO livers. (E) Real-time RT-PCR analysis of the expression levels of *HO-1* and *NQO1* of *Mcl-1* KO and control livers at 6 weeks of age (N = 9 per group). (F) Liver sections of *Mcl-1* KO or (G) *Bcl-xL* KO and the control liver at the indicated ages stained with anti-8-OHdG and statistics of the number of positive nuclei (N = 6 and more per group) (G). *p <0.05.

was found to be induced upon DNA damage and to be bound to and to transport Mcl-1 from the cytosol to the nucleus [15]. Mcl-1 was also reported to be induced upon DNA damage and to regulate the DNA damage response through activation of Chk1 [16]. These findings suggest that Mcl-1 possesses additional functions in cell cycle progression and the DNA damage response pathway. This raised concern as to whether the hepatocarcinogenesis observed in *Mcl-1* KO mice was actually related to increased apoptosis in the liver.

In the present study, we demonstrated that hepatocyte-specific destruction of Bcl-xL led to the development of liver cancer similarly to that in hepatocyte-specific *Mcl-1* KO mice. Although we could not completely exclude the possibility that Bcl-xL may have additional effects other than apoptosis, this finding clearly shows that hepatocarcinogenesis observed in the apoptosis-prone liver is not a specific finding of loss of Mcl-1 but is also observed with the knockout of other genes that are critically involved in hepatocyte integrity. Tumors observed in these murine livers frequently showed activation of ERK and JNK, similar to the activation observed in human HCC [18,19]. While 64% of *Mcl-1* KO mice (14/22) developed liver tumors within 1 year, only 27% of *Bcl-xL* KO mice (3/11) did so within 1 year (Table 1). These finding indicate that the incidence rate of carcinogenesis in *Bcl-xL* KO mice is lower than that of *Mcl-1* KO mice. This may be

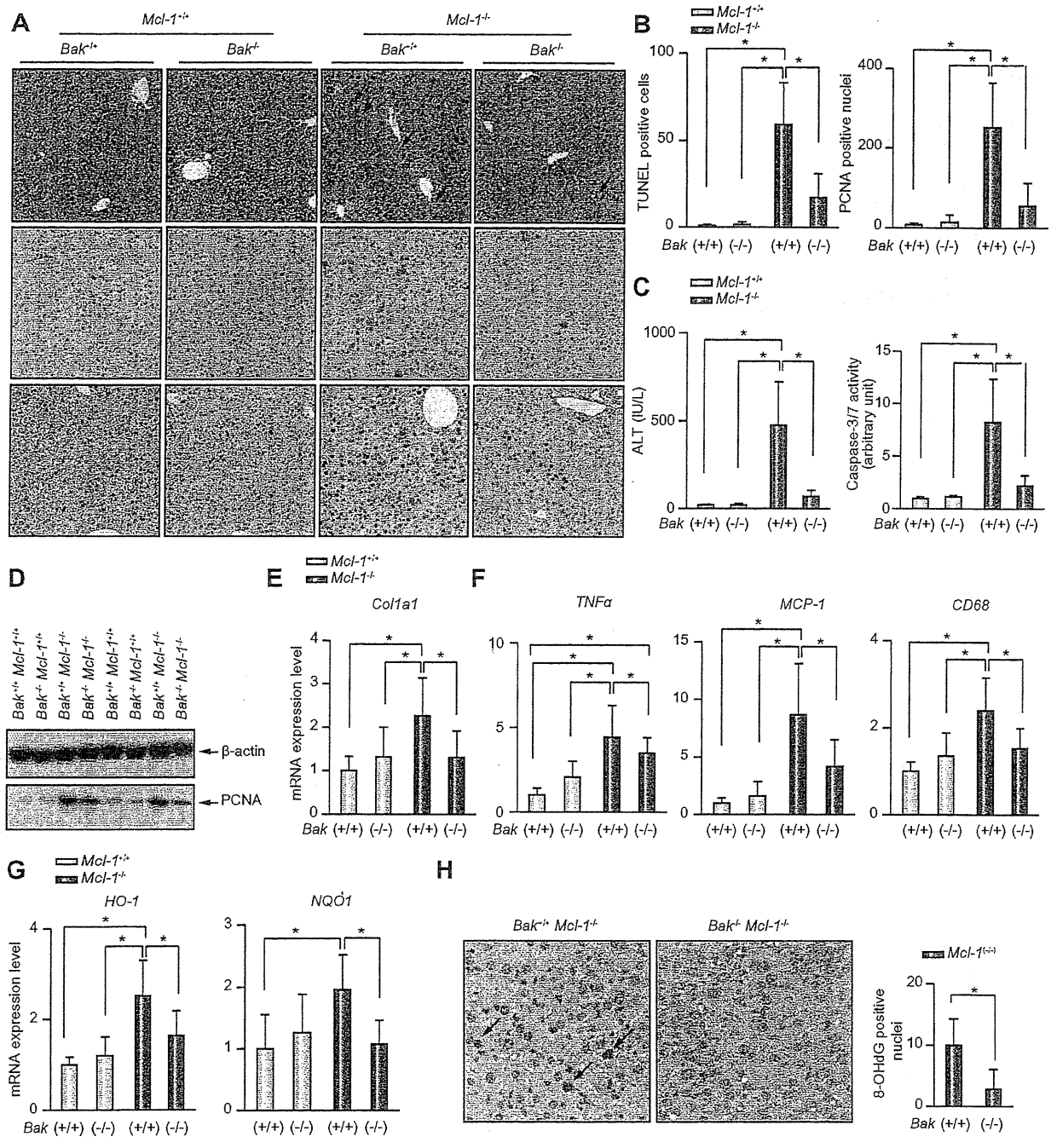

Fig. 4. Impact of Bak deficiency in *Mcl-1* KO mice. (A–G) *Bak*-deficient hepatocyte-specific *Mcl-1* KO mice ($Bak^{-/-}$ $Mcl\text{-}1^{-/-}$) were sacrificed at 6 weeks of age. (A) Representative pictures of hematoxylin–eosin with arrows indicating typical apoptotic cells (upper), TUNEL (middle) and PCNA staining (lower) and (B) statistics of TUNEL and PCNA staining of liver sections (N = 6 or 8 per group). (C) Serum levels of ALT and caspase-3/7 activity (N = 12 per group). (D) Western blot for PCNA expression. Real-time RT-PCR analysis for expression levels of (E) *Col1a1*, (F) *TNF-α*, *MCP-1*, *CD68*, (G) *HO-1* and *NQO1* in the livers at 6 weeks of age (N = 12 per group). (H) Liver sections of the *Bak*-deficient *Mcl-1* KO and control *Mcl-1* KO liver at 1 year of age stained with anti-8-OHdG. Representative images of liver sections stained with anti-8-OHdG (left) and statistics of the number of positive nuclei (N = 9 or 7 per group) (right). *$p < 0.05$.

explained by the difference in levels of hepatocyte apoptosis and serum ALT, which are higher in *Mcl-1* KO mice than in *Bcl-xL* KO mice of the same age [10,11].

Mcl-1 executes its anti-apoptotic function by either directly or indirectly inhibiting the pro-apoptotic functions of Bak and/or Bax [24]. In the present study, we have shown that deletion of the *bak*

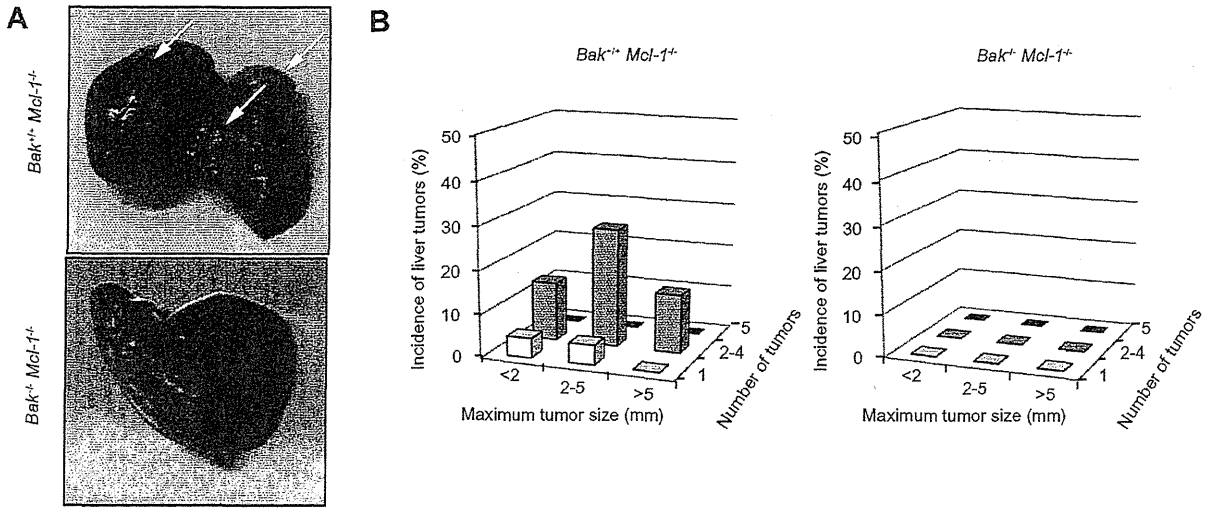

Fig. 5. Liver of aged *Bak/Mcl-1* double KO mice. (A and B) *Bak*-deficient *Mcl-1* KO mice ($Bak^{-/-}$ $Mcl\text{-}1^{-/-}$) (N = 7) and control *Mcl-1* KO mice ($Bak^{+/+}$ $Mcl\text{-}1^{-/-}$) (N = 22) were sacrificed at 1 year of age. (A) Representative macroscopic view of the livers with arrows indicating tumors. (B) Incidence of liver tumors separated by maximum tumor size and number of tumors.

gene resulted in a clear reduction in hepatocyte apoptosis in *Mcl-1* KO mice. Of importance is the finding that *bak* deletion leads to reduction of the liver regenerative response in *Mcl-1* KO mice. Bak is exclusively localized at the mitochondria in hepatocytes [25] and, upon exposure to apoptotic stimuli, undergoes oligomerization to form pores in the outer membrane of mitochondria, releasing cytochrome c, which in turn activates caspases. Since Bak is not involved in the activity of Mcl-1 in the nucleus, our present finding suggests that the regeneration observed in the *Mcl-1* KO liver is not due to loss of the Mcl-1 anti-proliferative effect but mainly to the compensatory regeneration of increased apoptosis. Most importantly, *bak* deletion clearly leads to reduced liver tumor incidence. This finding strongly suggests that the hepatocarcinogenesis observed in *Mcl-1* KO mice can be mostly ascribed to increased apoptosis in hepatocytes.

What does make hepatocytes undergo malignant transformation in the liver with increasing apoptosis? Regeneration is a physiological process in the liver like that in bone marrow or the intestine and compensatory liver regeneration itself is probably not sufficient to induce liver cancer [26]. The present study raised the possibility that TNF-α and oxidative stress are candidate factors responsible for the malignant transformation in the apoptosis-prone liver. TNF-α is reported to be a potent endogenous mutagen that promotes cellular transformation [20], and oxidative stress is reported to cause DNA damage leading to carcinogenesis [21]. Our results revealed that both TNF-α and oxidative stress were significantly increased in KO livers, and importantly, that inhibition of apoptosis by deletion of the *bak* gene reduced the levels of TNF-α and oxidative stress with a decrease in the tumorigenic rate. Some studies have shown that TNF-α induces oxidative stress in hepatocytes [27,28], while oxidative stress promotes production of inflammatory cytokines [29–31]. Taken together, oxidative stress and inflammatory cytokines may positively affect each other to turn healthy hepatocytes into malignant transformed hepatocytes in the liver of KO mice. Further studies are needed to examine the role of oxidative stress and inflammatory cytokines in apoptosis-induced hepatocarcinogenesis.

Apoptosis resistance has been established as a hallmark of cancer [32]. Indeed, accumulating evidence indicates that human HCC frequently overexpresses a variety of molecules which confer apoptosis resistance, such as anti-apoptotic Bcl-2 family proteins, Bcl-xL [33] and Mcl-1 [34,35]. Their overexpression was found to be associated with malignant phenotypes of tumors and poor prognosis of patients [36]. In the present study, tumors that developed in *Bcl-xL* or *Mcl-1* KO mice lacked expression of the respective proteins but reciprocally overexpressed Mcl-1 or Bcl-xL at high rates. We recently reported that conditional expression of Bcl-xL in tumor cells was translated into higher tumor growth in xenograft models [37], indicating that overexpression of anti-apoptotic Bcl-2 family proteins is important for tumor progression. Lack of Bcl-xL or Mcl-1 in hepatocytes generates persistent hepatocyte apoptosis leading to liver tumor development. On the other hand, reciprocal overexpression of Mcl-1 or Bcl-xL in the tumor of *Bcl-xL* or *Mcl-1* KO mice might be required for tumor progression.

Increasing evidence indicates that the serum level of ALT, a marker of hepatocyte apoptosis, is a risk factor for HCC in viral hepatitis [38] and non-alcoholic steatohepatitis [39]. A population-based study also revealed that elevated ALT levels raise the risk of liver cancer [40]. The present study provides evidence that spontaneous apoptosis in hepatocytes leads to liver cancer development and also offers genetic evidence that inhibition of apoptosis can help prevent liver cancer. Administration of caspase inhibitor was previously reported to lower serum ALT levels in patients with chronic hepatitis C [41]. It may be interesting and important, from a clinical point of view, to further determine whether pharmacological inhibition of apoptosis can be useful in preventing liver cancer development in *Bcl-xL* or *Mcl-1* KO mice.

Financial support

This work was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (to T. Tak.) and a Grant-in-Aid for Research

on Hepatitis from the Ministry of Health, Labour, and Welfare of Japan.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Acknowledgements

We sincerely thank Dr. You-Wen He (Department of Immunology, Duke University Medical Center, Durham, NC) for providing the *mcl-1* floxed mice and Dr. Lothar Hennighausen (Laboratory of Genetics and Physiology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institute of Health, Bethesda, MD) for providing the *Bcl-x* floxed mice.
This work was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (to T. Tak.) and Grant-in-Aid for Research on Hepatitis from the Ministry of Health, Labour and Welfare of Japan.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2012.01.027.

References

[1] Malhi H, Gores G. Cellular and molecular mechanisms of liver injury. Gastroenterology 2008;134:1641–1654.
[2] Hiramatsu N, Hayashi N, Katayama K, Mochizuki K, Kawanishi Y, Kasahara A, et al. Immunohistochemical detection of Fas antigen in liver tissue of patients with chronic hepatitis C. Hepatology 1994;19:1354–1359.
[3] Mochizuki K, Hayashi N, Hiramatsu N, Katayama K, Kawanishi Y, Kasahara A, et al. Fas antigen expression in liver tissues of patients with chronic hepatitis B. J Hepatol 1996;24:1–7.
[4] Feldstein A, Canbay A, Angulo P, Taniai M, Burgart L, Lindor K, et al. Hepatocyte apoptosis and fas expression are prominent features of human nonalcoholic steatohepatitis. Gastroenterology 2003;125:437–443.
[5] Kronenberger B, Wagner M, Herrmann E, Mihm U, Piiper A, Sarrazin C, et al. Apoptotic cytokeratin 18 neoepitopes in serum of patients with chronic hepatitis C. J Viral Hepat 2005;12:307–314.
[6] Papatheodoridis GV, Hadziyannis E, Tsochatzis E, Chrysanthos N, Georgiou A, Kafiri G, et al. Serum apoptotic caspase activity as a marker of severity in HBeAg-negative chronic hepatitis B virus infection. Gut 2008;57:500–506.
[7] Wieckowska A, Zein NN, Yerian LM, Lopez AR, McCullough AJ, Feldstein AE. In vivo assessment of liver cell apoptosis as a novel biomarker of disease severity in nonalcoholic fatty liver disease. Hepatology 2006;44:27–33.
[8] Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Ishibashi K, et al. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nat Med 1998;4:1065–1067.
[9] Pikarsky E, Porat RM, Stein I, Abramovitch R, Amit S, Kasem S, et al. NF-kappaB functions as a tumour promoter in inflammation-associated cancer. Nature 2004;431:461–466.
[10] Takehara T, Tatsumi T, Suzuki T, Rucker Er, Hennighausen L, Jinushi M, et al. Hepatocyte-specific disruption of Bcl-xL leads to continuous hepatocyte apoptosis and liver fibrotic responses. Gastroenterology 2004;127:1189–1197.
[11] Hikita H, Takehara T, Shimizu S, Kodama T, Li W, Miyagi T, et al. Mcl-1 and Bcl-xL cooperatively maintain integrity of hepatocytes in developing and adult murine liver. Hepatology 2009;50:1217–1226.
[12] Weber A, Boger R, Vick B, Urbanik T, Haybaeck J, Zoller S, et al. Hepatocyte-specific deletion of the antiapoptotic protein myeloid cell leukemia-1 triggers proliferation and hepatocarcinogenesis in mice. Hepatology 2010;51:1226–1236.
[13] Fujise K, Zhang D, Liu J, Yeh ET. Regulation of apoptosis and cell cycle progression by MCL1. Differential role of proliferating cell nuclear antigen. J Biol Chem 2000;275:39458–39465.
[14] Jamil S, Sobouti R, Hojabrpour P, Raj M, Kast J, Duronio V. A proteolytic fragment of Mcl-1 exhibits nuclear localization and regulates cell growth by interaction with Cdk1. Biochem J 2005;387:659–667.
[15] Pawlikowska P, Leray I, de Laval B, Guihard S, Kumar R, Rosselli F, et al. ATM-dependent expression of IEX-1 controls nuclear accumulation of Mcl-1 and the DNA damage response. Cell Death Differ 2010;17:1739–1750.
[16] Jamil S, Mojtabavi S, Hojabrpour P, Cheah S, Duronio V. An essential role for MCL-1 in ATR-mediated CHK1 phosphorylation. Mol Biol Cell 2008;19:3212–3220.
[17] Takehara T, Hayashi N, Tatsumi T, Kanto T, Mita E, Sasaki Y, et al. Interleukin 1beta protects mice from Fas-mediated hepatocyte apoptosis and death. Gastroenterology 1999;117:661–668.
[18] Ito Y, Sasaki Y, Horimoto M, Wada S, Tanaka Y, Kasahara A, et al. Activation of mitogen-activated protein kinases/extracellular signal-regulated kinases in human hepatocellular carcinoma. Hepatology 1998;27:951–958.
[19] Chen F, Beezhold K, Castranova V. JNK1, a potential therapeutic target for hepatocellular carcinoma. Biochim Biophys Acta 2009;1796:242–251.
[20] Yan B, Wang H, Rabbani ZN, Zhao Y, Li W, Yuan Y, et al. Tumor necrosis factor-alpha is a potent endogenous mutagen that promotes cellular transformation. Cancer Res 2006;66:11565–11570.
[21] Lonkar P, Dedon PC. Reactive species and DNA damage in chronic inflammation: reconciling chemical mechanisms and biological fates. Int J Cancer 2011;128:1999–2009.
[22] Motoyama N, Wang F, Roth KA, Sawa H, Nakayama K, Negishi I, et al. Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. Science 1995;267:1506–1510.
[23] Rinkenberger JL, Horning S, Klocke B, Roth K, Korsmeyer SJ. Mcl-1 deficiency results in peri-implantation embryonic lethality. Genes Dev 2000;14:23–27.
[24] Willis SN, Chen L, Dewson G, Wei A, Naik E, Fletcher JI, et al. Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. Genes Dev 2005;19:1294–1305.
[25] Hikita H, Takehara T, Kodama T, Shimizu S, Hosui A, Miyagi T, et al. BH3-only protein bid participates in the Bcl-2 network in healthy liver cells. Hepatology 2009;50:1972–1980.
[26] Aravalli RN, Steer CJ, Cressman EN. Molecular mechanisms of hepatocellular carcinoma. Hepatology 2008;48:2047–2063.
[27] Kamata H, Honda S, Maeda S, Chang L, Hirata H, Karin M. Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. Cell 2005;120:649–661.
[28] Schwabe RF, Brenner DA. Mechanisms of Liver Injury. I. TNF-alpha-induced liver injury: role of IKK, JNK, and ROS pathways. Am J Physiol Gastrointest Liver Physiol 2006;290:G583–G589.
[29] Bulua AC, Simon A, Maddipati R, Pelletier M, Park H, Kim KY, et al. Mitochondrial reactive oxygen species promote production of proinflammatory cytokines and are elevated in TNFR1-associated periodic syndrome (TRAPS). J Exp Med 2011;208:519–533.
[30] Nakahira K, Haspel JA, Rathinam VA, Lee SJ, Dolinay T, Lam HC, et al. Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. Nat Immunol 2011;12:222–230.
[31] Zhou R, Yazdi AS, Menu P, Tschopp J. A role for mitochondria in NLRP3 inflammasome activation. Nature 2011;469:221–225.
[32] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646–674.
[33] Takehara T, Liu X, Fujimoto J, Friedman S, Takahashi H. Expression and role of Bcl-xL in human hepatocellular carcinomas. Hepatology 2001;34:55–61.
[34] Fleischer B, Schulze-Bergkamen H, Schuchmann M, Weber A, Biesterfeld S, Müller M, et al. Mcl-1 is an anti-apoptotic factor for human hepatocellular carcinoma. Int J Oncol 2006;28:25–32.
[35] Sieghart W, Losert D, Strommer S, Cejka D, Schmid K, Rasoul-Rockenschaub S, et al. Mcl-1 overexpression in hepatocellular carcinoma: a potential target for antisense therapy. J Hepatol 2006;44:151–157.
[36] Watanabe J, Kushihata F, Honda K, Sugita A, Tateishi N, Mominoki K, et al. Prognostic significance of Bcl-xL in human hepatocellular carcinoma. Surgery 2004;135:604–612.
[37] Hikita H, Takehara T, Shimizu S, Kodama T, Shigekawa M, Iwase K, et al. The Bcl-xL inhibitor, ABT-737, efficiently induces apoptosis and suppresses

growth of hepatoma cells in combination with sorafenib. Hepatology 2010;52:1310–1321.
[38] Chen CF, Lee WC, Yang HI, Chang HC, Jen CL, Iloeje UH, et al. Changes in serum levels of HBV DNA and alanine aminotransferase determine risk for hepatocellular carcinoma. Gastroenterology 2011;141:1240–1248.
[39] Bhala N, Angulo P, van der Poorten D, Lee E, Hui JM, Saracco G, et al. The natural history of nonalcoholic fatty liver disease with advanced fibrosis or cirrhosis: an international collaborative study. Hepatology 2011;54:1208–1216.
[40] Ruhl CE, Everhart JE. Elevated serum alanine aminotransferase and gamma-glutamyltransferase and mortality in the United States population. Gastroenterology 2009;136:477–485, e411.
[41] Pockros P, Schiff E, Shiffman M, McHutchison J, Gish R, Afdhal N, et al. Oral IDN-6556, an antiapoptotic caspase inhibitor, may lower aminotransferase activity in patients with chronic hepatitis C. Hepatology 2007;46:324–329.

Cancer Immunol Immunother (2012) 61:1425–1432
DOI 10.1007/s00262-012-1208-6

ORIGINAL ARTICLE

Interleukin-1β enhances the production of soluble MICA in human hepatocellular carcinoma

Keisuke Kohga · Tomohide Tatsumi · Hinako Tsunematsu · Satoshi Aono · Satoshi Shimizu · Takahiro Kodama · Hayato Hikita · Masashi Yamamoto · Tsugiko Oze · Hiroshi Aketa · Atsushi Hosui · Takuya Miyagi · Hisashi Ishida · Naoki Hiramatsu · Tatsuya Kanto · Norio Hayashi · Tetsuo Takehara

Received: 15 July 2011 / Accepted: 17 January 2012 / Published online: 1 February 2012
© Springer-Verlag 2012

Abstract The production of soluble major histocompatibility complex class I–related chain A (MICA) is thought to antagonize NKG2D-mediated immunosurveillance. Interleukin-1β (IL-1β) is elevated in patients with chronic hepatitis C (CH), and this might contribute to the escape of hepatocellular carcinoma (HCC) cells from innate immunity. In this study, we investigated the immunoregulatory role of IL-1β in the production of soluble MICA of HCC cells. First, we investigated the correlation between the serum IL-1β levels and soluble MICA in CH patients. Serum IL-1β levels were associated with soluble MICA levels in CH patients. The serum IL-1β levels of CH patients with the HCC occurrence were significantly higher than those of CH patients without HCC. We next examined the MICA production of IL-1β-treated HCC cells. Addition of IL-1β resulted in significant increase in the production of soluble MICA in HepG2 and PLC/PRF/5 cells, human HCC cells. But soluble MICA was not detected in both non-treated and IL-1β-treated normal hepatocytes. Addition of IL-1β did not increase the expressions of membrane-bound MICA on HCC cells. These were observed similarly in various cancer cells including a gastric cancer (MKN1), two colon cancers (HCT116 and HT29) and a cervical cancer (HeLa). Addition of IL-1β also increased the expression of a disintegrin and metalloproteinase (ADAM)9 in HCC cells, and the knockdown of ADAM9 in IL-1β-treated HCC cells resulted in the decrease in the production of soluble MICA of HCC cells. These findings indicate that IL-1β might enhance the production of soluble MICA by activating ADAM9 in human HCC.

Keywords IL-1β · Hepatocellular carcinoma · Soluble MICA · ADAM9

Abbreviations

IL	Interleukin
HCC	Hepatocellular carcinoma
MICA	Major histocompatibility complex class I–related chain A
ADAM9	A disintegrin and metalloproteinase 9

Introduction

Interleukin-1β (IL-1β) is a proinflammatory cytokine with multiple biological effects [1]. Serum levels of IL-1β are elevated in patients infected with hepatitis C virus (HCV), suggesting the role of IL-1β in the inflammation of liver [2–4]. Several polymorphisms of the IL-1 gene have been reported to affect IL-1β production [5, 6]. A number of clinical studies suggested that polymorphisms of IL-1β gene are associated with diverse disease including cancer [5, 7]. IL-1β gene polymorphisms have also been reported to be associated with HCC in HCV- or HBV-infected patients [8–10]. While genetic studies have suggested an important role for IL-1β in cancer, direct evidence that IL-1β contributes to the pathogenesis of cancer has been lacking. Recently,

Keisuke Kohga and Tomohide Tatsumi have equally contributed to this work and share the first authorship.

K. Kohga · T. Tatsumi (✉) · H. Tsunematsu · S. Aono · S. Shimizu · T. Kodama · H. Hikita · M. Yamamoto · T. Oze · H. Aketa · A. Hosui · T. Miyagi · H. Ishida · N. Hiramatsu · T. Kanto · N. Hayashi · T. Takehara
Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan
e-mail: tatsumit@gh.med.osaka-u.ac.jp

N. Hayashi
Kansai-Rosai Hospital, Amagasaki, Hyogo 660-8511, Japan

Tu et al. [11] reported that stomach-specific expression of IL-1β in transgenic mice leads to spontaneous gastric inflammation and cancer that correlates with myeloid-derived suppressor cells to the stomach. However, no studies have been published on the direct effect of IL-1β on the HCC cells in patients infected with HCV.

MHC class I–related chain A (MICA), a ligand for NKG2D, is rarely expressed on normal cells, but frequently on tumor cells [12, 13]. The engagement of MICA and NKG2D strongly activates NK cells enhancing their cytolytic activity and cytokine production [14]. Thus, the MICA-NKG2D pathway is an important mechanism by which the host immune system recognizes and kills transformed cells [15]. In addition to those membrane-bound forms, MICA molecules are cleaved proteolytically from tumor cells and appear as soluble forms in the sera of patients with malignancy including HCC [16–18]. The release of soluble MICA/B from tumor cells is thought to antagonize NKG2D-mediated immunosurveillance. We previously demonstrated that a disintegrin and metalloproteinase (ADAM)9 protease plays essential roles in the shedding of MICA molecules on HCC cells [19]. However, the mechanism of regulating the production of soluble MICA in HCC cells remains to be elucidated.

In this study, we investigated the immunoregulatory role of IL-1β in the production of soluble MICA from HCC cells. Of importance is the discovery that the serum IL-1β levels in chronic hepatitis patients with the HCC occurrence were significantly higher than those without HCC occurrence and that IL-1β enhances the production of soluble MICA via activating ADAM9 in human HCC cells. The present study sheds light on previously unrecognized immunological effects of IL-1β on HCC cells.

Materials and methods

HCC cell lines and normal hepatocyte cultures

HepG2 and PLC/PRF/5, human HCC cell lines, were purchased from American Type Culture Collection (Rockville, MD) and were cultured with Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (GIBCO/Life Technologies, Grand Island, NY) in a humidified incubator at 5% CO_2 and 37°C. 2×10^5 HepG2 and PLC/PRF/5 cells or normal hepatocytes (ScienCell Research Laboratories, Carlsbad, CA) were cultured in 6-well tissue culture plates for 48 h in the presence or absence of human interleukin-1β (IL-1β) (50 ng/ml, PeproTech EC, London, UK), and the HCC cells were harvested and subjected to evaluating the expression of membrane-bound MICA and ADAM9 and the production of soluble MICA.

Flow cytometry

For the detection of membrane-bound MICA, HCC cells were incubated with anti-MICA-specific Ab (Santa Cruz Biotechnology, Santa Cruz, CA) and stained with Goat $F(ab')2$ fragment anti-mouse IgG($H + L$) – PE (Beckman Coulter, Fullerton, CA) as a secondary reagent. Flow cytometric analysis was performed using a FACScan flow cytometer (Becton–Dickinson, San Jose, CA).

Western blotting

The total cellular protein was electrophoretically separated by sodium dodecyl sulfate-12% polyacrylamide gels and transferred onto PVDF membrane. The membrane was blocked in Tris-buffered saline–Tween containing 5% skim milk for 1 h and then probed with anti-ADAM9 mAb (R&D Systems, Minneapolis, MN) at 4°C overnight. Horseradish peroxidase-conjugated anti-rabbit Ab and SuperSignal West Pico System (Pierce, Rockford, IL) were used for the detection of blots.

Real-time reverse transcription (RT) PCR

Total RNA was isolated using RNeasy Mini Kit (Qiagen K.K., Tokyo, Japan) and was reverse transcribed using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster city, CA). The mRNA levels were evaluated using ABI PRISM 7900 Sequence Detection System (Applied Biosystems). Ready-to-use assays (Applied Biosystems) were used for the quantification of ADAM9 (Hs00177638_m1), MICA (Hs00792195_m1) and β-actin (Hs99999903_m1) mRNAs according to the manufacturer's instructions. β-Actin mRNA from each sample was quantified as an endogenous control of internal RNA.

RNA silencing

The small interfering RNA (siRNA) method was used to knockdown ADAM9 as previously described [19]. At 24-h post-transfection, the cells were analyzed for specific depletion of the protein of ADAM9 by western blotting. The following siRNA were used: ADAM9, 5′-UGUCCAAAC ACAUUAAUCCCGCCUG-3′; an irrelevant siRNA as a control, 5′-UGUCGCACAAACACUUAACUCCCUG-3′.

ELISA

The sera from chronic hepatitis C patients ($N = 24$) with or without the occurrence of HCC were subjected to analysis of IL-1β and soluble MICA. Informed consent, under an Institutional Review Board–approved protocol, was obtained from all patients before sample acquisition.

The sera and the supernatants of cultured HCC cells were harvested, and the levels of IL-1β and soluble MICA were determined by human IL-1β ELISA set II (BD Biosciences, San Diego, CA) and DuoSet MICA eELISA kit (R&D Systems, Minneapolis, MN) in accordance with the manufacturer's instructions, respectively.

NK cell analysis

NK cells were isolated from human peripheral blood mononuclear cells by magnetic cell sorting using CD56 MicroBeads (Miltenyi Biotech, Auburn, CA) as previously described [19]. HepG2 and PLC/PRF/5 cells were treated with IL-1β (50 ng/ml) for 48 h. The cytolytic ability of NK cells against IL-1β-treated or non-treated HepG2 and PLC/PRF/5 cells was assessed by 4-hr ^{51}Cr-releasing assay as previously described [19].

Statistics

All values were expressed as the mean and SD. The statistical significance of differences between the groups was determined by applying Student's t test or two-sample t test with Welch correction after each group had been tested with equal variance and Fisher's exact probability test. We defined statistical significance as $p < 0.05$.

Results

Serum IL-1β levels were associated with soluble MICA in chronic liver disease patients

We first examined the IL-1β levels and soluble MICA levels of twenty-four chronic hepatitis C (CH) patients. Serum IL-1β levels in CH patients correlated with soluble MICA levels (Fig. 1a). We next examined the serum IL-1β levels of CH patients with or without the occurrence of HCC. We examined serum IL-1β levels of these 24 CH patients before HCC occurrence and followed these patients for 5 years. CH patients could be divided into two groups according to the occurrence of HCC (Table 1). As shown in Fig. 1b, the serum IL-1β levels of patients with the occurrence of HCC ($n = 11$) were significantly higher than those of patients without the occurrence of HCC ($n = 13$). These results suggested that the elevation of serum IL-1β levels might be associated with the occurrence of HCC in CH patients.

IL-1β increases the production of soluble MICA from HCC cells, but not from normal hepatocytes

We examined whether IL-1β treatment could induce MICA expressions on HCC cells (PLC/PRF/5 cells and HepG2 cells). Both PLC/PRF/5 cells and HepG2 cells were cultured for 48 h with IL-1β (50 ng/ml) and then subjected to analysis of the expression of membrane-bound MICA and mRNA of MICA. The expression of membrane-bound MICA of IL-1β-treated HCC cells was similar to that of non-treated HCC cells (Fig. 2a). IL-1β treatment induced significant increase of mRNA of MICA in PLC/PRF/5 cells, but this did not in HepG2 cells (Fig. 2b). We next examined the production of soluble MICA in the supernatants of the IL-1β-treated HCC cells. IL-1β treatment

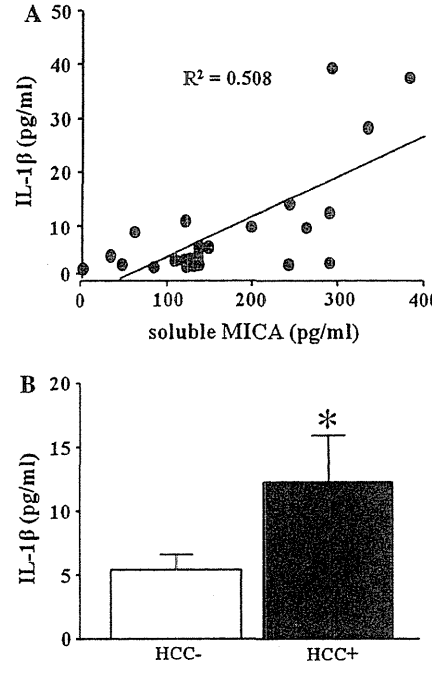

Fig. 1 The correlation between serum IL-1β and soluble MICA in patients with chronic liver disease and serum IL-1β levels in chronic liver disease patients with or without the HCC occurrence. **a** Correlation between serum IL-1β levels and soluble MICA levels in patients with chronic liver disease ($N = 24$). The serum IL-1β and soluble MICA were evaluated by specific ELISA, respectively. **b** Serum IL-1β levels in chronic hepatitis patients with HCC occurrence (HCC+, $N = 11$) or without HCC occurrence (HCC−, $N = 13$) were evaluated by specific ELISA. All patients were HCV-RNA-positive. $*p < 0.05$

Table 1 Clinical backgrounds

	HCC(+)	HCC(−)
Number	11	13
Age	61 ± 6	61 ± 8
Gender (M/F)	8/3	11/2
Platelet ($\times 10^4$/μl)	15 ± 5	14 ± 3
ALT (IU/l)	122 ± 109	89 ± 44

HCC(+) chronic hepatitis C patients with the occurrence of HCC, *HCC*(−) chronic hepatitis C patients without the occurrence of HCC, *M* male, *F* female, *ALT* alanine aminotransferase

resulted in the significant increase in the production of soluble MICA in both PLC/PRF/5 and HepG2 cells (Fig. 2c). These results demonstrated that the addition of IL-1β did not change the expression of membrane-bound MICA but resulted in significant increase in the production of soluble MICA in HCC cells. We also examined the effect of IL-1β on normal hepatocytes. As shown in Fig. 2d, normal hepatocytes did not produce soluble MICA and the addition of IL-1β did not result in its production.

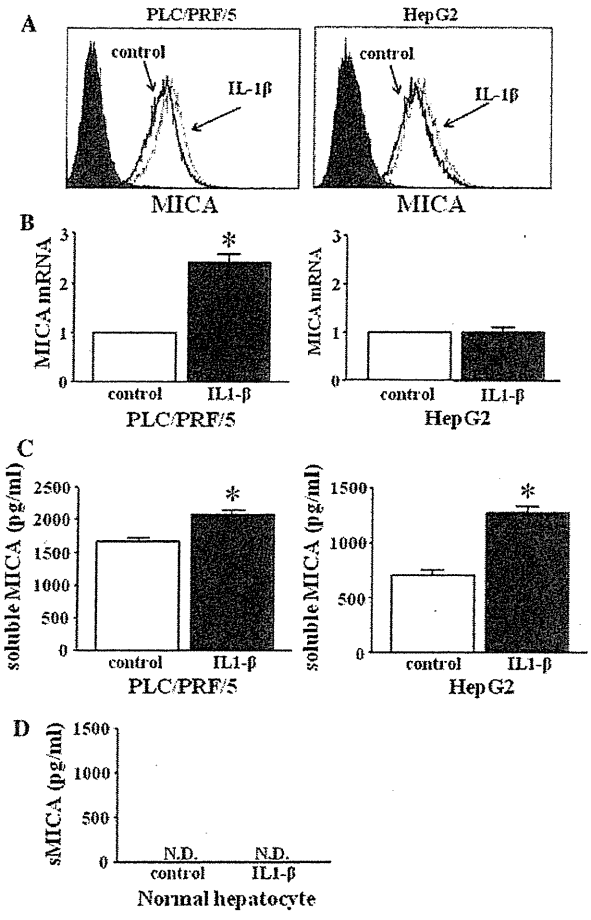

Fig. 2 Expression of membrane-bound MICA and the production of soluble MICA in IL-1β-treated HCC cells and normal hepatocytes. Both PLC/PRF/5 cells and HepG2 cells were treated in the presence or absence of IL-1β (50 ng/ml) for 48 h. The expression of membrane-bound MICA (**a**) and mRNA expression of MICA (**b**) in IL-1β-treated or non-treated HCC cells were evaluated by flow cytometry or real-time RT-PCR, respectively. *Black line histograms*, MICA staining of non-treated cells; *dotted line histograms*, MICA staining of IL-1β-treated cells; *shaded/black histograms*, control IgG isotype Ab staining. Similar results were obtained from two independent experiments. $*p < 0.05$. **c** We examined the production of soluble MICA on IL-1β-treated or non-treated HCC cells by specific ELISA. $*p < 0.05$. **d** Normal hepatocytes were treated in the presence or absence of IL-1β (50 ng/ml) for 48 h. The production of soluble MICA on IL-1β-treated or non-treated normal hepatocytes was examined by specific ELISA. *ND* not detected

These results demonstrated that IL-1β could induce the increase in the production of soluble MICA only from HCC cells, but not from normal hepatocytes.

IL-1β treatment increases the production of soluble MICA from various cancer cells

We also examined IL-1β-dependent MICA regulation on another cancer cells including a gastric cancer cell line (MKN1), colon cancer cell lines (HCT116, HT29) and a cervical cancer cell line (HeLa). The expressions of membrane-bound MICA on these cells did not change by the addition of IL-1β in all cancer cells. Interestingly, the addition of IL-1β resulted in significant increase in the production of soluble MICA in all cancer cells (Fig. 3).

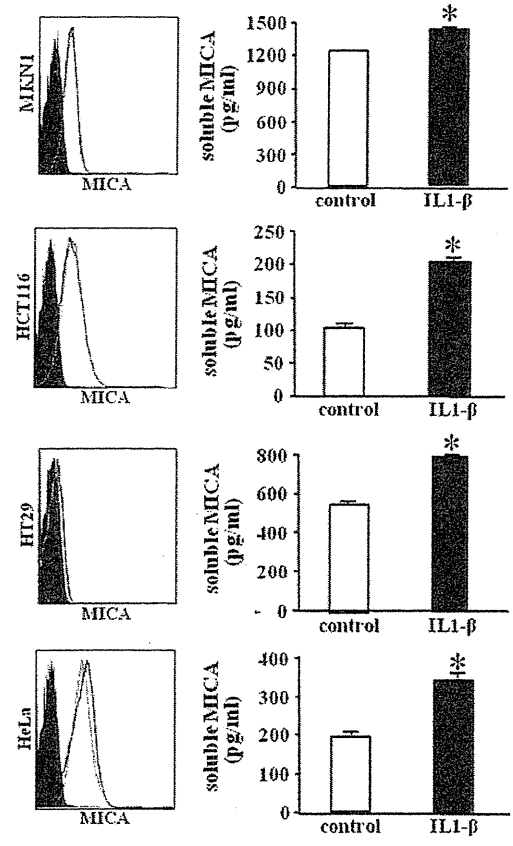

Fig. 3 Soluble MICA production of IL-1β-treated various cancer cells. Various cancer cells including MKN1, HCT116, HT29 and HeLa cells were treated in the presence or absence of IL-1β (50 ng/ml) for 48 h. Soluble MICA production of IL-1β-treated or non-treated various cancer cells was evaluated by specific ELISA (*right panel*). sMICA, soluble MICA. We also examined the expression of membrane-bound MICA on IL-1β-treated or non-treated various cancer cells by flow cytometry (*left panel*). *Black line histograms*, MICA staining of non-treated cells; *Gray line histograms*, MICA staining of IL-1β-treated cells; *shaded/black histograms*, control IgG isotype Ab staining. Similar results were obtained from two independent experiments. $*p < 0.05$

These results demonstrated that IL-1β could induce the increase in the production of soluble MICA not only from HCC cells but also from various cancer cells.

ADAM9 activated by IL-1β plays important roles in the production of soluble MICA from HCC cells

We next examined the mRNA of MICA and the production of soluble MICA in HCC cells treated with various doses of IL-1β. As shown in Fig. 4a, mRNA expression of MICA in IL-1β-treated PLC/PRF/5 cells significantly increased but that in HepG2 cells did not. The production of soluble MICA in IL-1β-treated PLC/PRF/5 cells significantly increased in a dose-dependent manner, and the production of soluble MICA significantly increased in 50 ng/ml IL-1β-treated HepG2 cells. Recently, members of the metzincin superfamily, such as ADAM proteins, have been reported to play essential roles in the proteolytic release of the ectodomain of transmembranous proteins, including MICA, from the cell surface [17, 20]. We previously reported that ADAM9 plays essential roles in MICA shedding in human HCC cells and that the activation of ADAM9 protease resulted in up-regulation of the production of soluble MICA from human HCC cells [19]. So we examined the involvement of ADAM9 in the up-regulation of soluble MICA production in IL-1β-treated HCC cells. As shown in Fig. 4b, mRNA levels of ADAM9 in IL-1β-treated PLC/PRF/5 cells significantly increased in a dose-dependent manner. mRNA of ADAM9 in IL-1β-treated HepG2 cells significantly increased in 10 ng/ml and 50 ng/ml IL-1β-treated HepG2 cells. The ADAM9 protein expression including both pro-form and active form also increased in IL-1β-treated HCC cells (Fig. 4c). To confirm the involvement of ADAM9 in IL-1β-treated HCC cells, we examined the soluble MICA production in IL-1β-treated ADAM9-knockdown (ADAM9KD) HCC cells. Both PLC/PRF/5 and HepG2 cells were transfected with ADAM9-siRNA or an irrelevant siRNA as a control. The expression of ADAM9 was clearly suppressed in PLC/PRF/5 cells and HepG2 cells at protein levels (Fig. 5a). In both PLC/PRF/5 and HepG2 cells transfected with control siRNA, the productions of soluble MICA in IL-1β-treated cells were significantly higher than those in non-treated HCC cells. In contrast, the production of soluble MICA in IL-1β-treated ADAM9KD-HepG2 cells was similar to that in non-treated ADAM9KD-HepG2 cells (Fig. 5b). The production of soluble MICA in IL-1β-treated ADAM9KD-PLC/PRF/5 cells also tended to decrease compared with that in non-treated ADAM9KD-PLC/PRF/5 cells (Fig. 5b). The decrease in soluble MICA production in ADAM9KD cells was different between PLC/PRF/5 cells and HepG2 cells. However, these results suggested at least that the increase in ADAM9 expression by IL-1β resulted in the increase in soluble MICA levels in IL-1β-treated HCC cells.

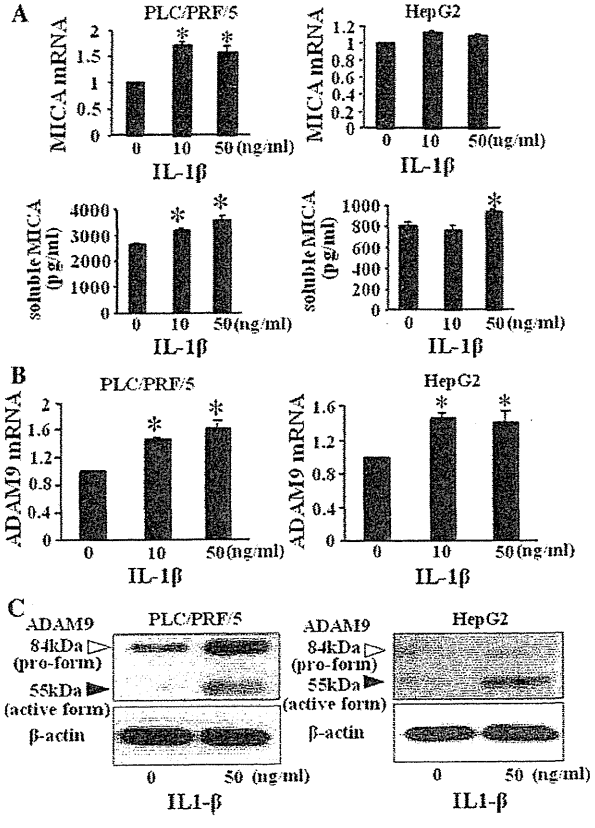

Fig. 4 IL-1β increased the ADAM9 expression of HCC cells and the production of soluble MICA. PLC/PRF/5 and HepG2 cells were cultured with 0, 10 and 50 ng/ml IL-1β for 48 h. **a** The production of soluble MICA from IL-1β-treated HCC cells was examined by specific ELISA, and mRNA levels of MICA of IL-1β-treated HCC cells were examined by real-time PCR. **b**, **c** mRNA and protein expression of ADAM9 by real-time RT-PCR (**b**) and western blotting (**c**), respectively. Representative results are shown. Similar results were obtained from 3 independent experiments. $*p < 0.05$

IL-1β-treated HCC cells are resistant to the cytolytic activity of NK cells

We next examined whether IL-1β could modify the NK sensitivity of human HCC cells. The cytolytic activities of NK cells against IL-1β-treated PLC/PRF/5 and IL-1β-treated HepG2 cells were lower than those against non-treated HCC cells (Fig. 5c). These results demonstrated that IL-1β treatment resulted in the increased resistance of HCC cells to NK cells.

Discussion

The liver contains a large compartment of innate immune cells (NK cells and NKT cells) and acquired immune cells

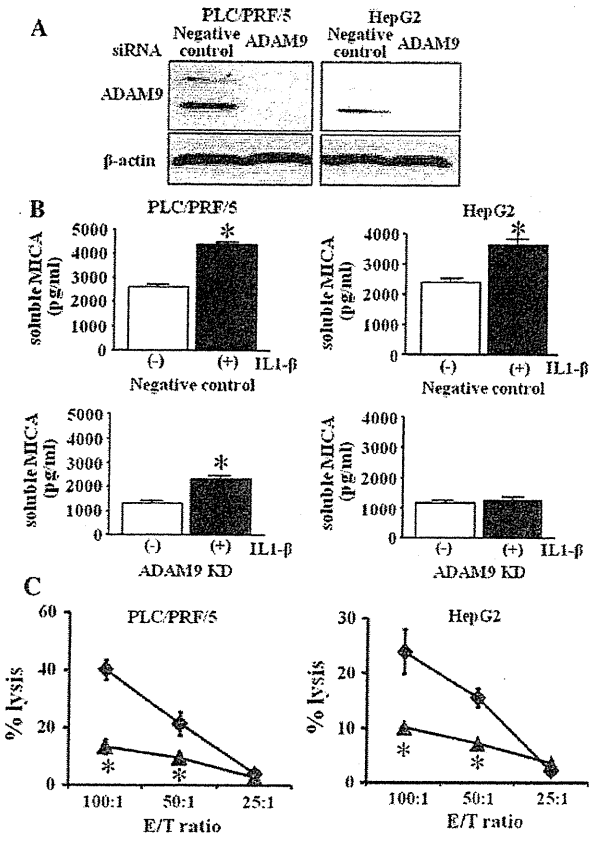

Fig. 5 The production of soluble MICA of ADAM9KD-HCC cells and the cytolytic activity against IL-1β-treated HCC cells. **a** Both HCC cells (PLC/PRF/5 and HepG2) were transfected with ADAM9 siRNA (ADAM9KD) or an irrelevant siRNA (negative control), and at 24-h post-transfection, the protein expression of ADAM9 was examined by western blotting. **b** Both HCC cells were cultured with (+) or without (−) 50 ng/ml IL-1β for 48 h. Soluble MICA production from ADAM9KD-HCC cells or negative control-HCC cells was evaluated by specific ELISA. $*p < 0.05$. Similar results were obtained from 3 independent experiments. **c** Both PLC/PRF/5 and HepG2 cells were cultured with or without IL-1β (50 ng/ml) for 48 h. The cytolytic activities of NK cells against IL-1β-treated or non-treated PLC/PRF/5 and HepG2 cells were evaluated by ^{51}Cr-releasing assay. Non-treated cells (*filled diamond*), IL-1β-treated cells (*filled triangle*). Representative results are shown. Similar results were obtained from three independent experiments. $*p < 0.05$ versus the cytolytic activity of non-treated cells. Similar results were obtained from 3 independent experiments

(T cells) [21, 22]. Recent study has demonstrated that innate immune system via NKG2D signal, expressing on NK cells, might play critical roles in tumor surveillance [23]. However, the escape mechanism of HCC cells from NK cells remains unclear. We previously demonstrated that membrane-bound MICA, activating molecule of NK cells, on HCC cells plays essential roles in the NK sensitivity of HCC cells [13, 24] and that the serum soluble MICA increase along the progression of chronic liver disease [18]. The production of soluble MICA in HCC patients is the highest compared with chronic hepatitis or liver cirrhosis patients without HCC [18]. These results suggest that unknown factors may accelerate the cleavage of MICA in HCC cells. IL-1β is produced mainly by local immune cells including activated Kupffer cells [25]. Because IL-1β increased in CH or LC patients [26–28], we focus on the possible role of IL-1β in the escape mechanism of HCC cells from NK cells.

Inflammatory cytokines including IL-1β and IL-6 increased in CH or LC patients [26–28], suggesting that both IL-1β and IL-6 might play roles in the HCC development. Recently high serum IL-6 level was an independent risk factor for HCC development in both chronic hepatitis C and B patients [29, 30], which suggested the possible roles of IL-6 in HCC development. However, the IL-1β levels in chronic liver disease, premalignant conditions, have been little reported. In this study, we demonstrated that serum IL-1β levels in chronic hepatitis C patients with HCC occurrence were significantly higher than those without HCC occurrence and that serum IL-1β levels correlated with soluble MICA which could inhibit NK activity. These results suggested that elevated IL-1β in CH patients might support the survival of HCC cells by changing local immunological environment.

MICA shedding is thought to be the principle mechanism by which tumor cells escape from NKG2D-mediated immunosurveillance [16]. In this study, we demonstrated that addition of IL-1β resulted in the increase in soluble MICA production from HCC cells. Interestingly, IL-1β treatment also resulted in the increase of soluble MICA in various cancer cells. Addition of other IL-1 family cytokines such as IL-1α, Il-18 and IL-33 did not result in the increase in soluble MICA production from both PLC/PRF/5 and HepG2 cells (Kohga, unpublished data). In addition to IL-1β, serum IL-6 and TNF-α are elevated in HCC patients. We compared IL-1β with IL-6 and TNF-α in the ability of the production of soluble MICA from HCC cells. IL-1β could increase the production of soluble MICA from HCC cells, but both IL-6 and TNF-α could not in PLC/PRF/5 cells and HepG2 cells. No synergistic effects of the combination of IL-1β, IL-6 and TNF-α were observed (Kohga, unpublished data). These results demonstrated that only IL-1β could induce the increase in the production of soluble MICA from HCC cells, suggesting that IL-1β might play an important role in the progression of HCC.

IL-1β treatment resulted in the increase in soluble MICA production but not the increase of mRNA in HepG2 cells. The production of soluble MICA depended on both the production of mRNA and the shedding of ADAM9. We previously demonstrated that ADAM9 plays an essential role in the shedding of MICA in HCC cells [19]. In the present study, we demonstrated that IL-1β treatment resulted in the increase in ADAM9 expression in HepG2

cells and that ADAM9 knockdown by siRNA resulted in the decrease in the production of soluble MICA from IL-1β-treated HepG2 cells. Our results suggested at least that the increase in ADAM9 might resulted in the increase in the shedding of soluble MICA in the IL-1β-treated HCC cells.

Recent studies have identified various metalloproteinases responsible for MICA/B cleavage in various cancers [31]. We previously found that ADAM9 plays critical roles in the shedding of MICA in human HCC. ADAM9 was directly associated with decreasing the expression of membrane-bound MICA and increasing the production of soluble MICA in human HCC [19]. Thus, it would be interesting to examine the activity of ADAM9 in IL-1β-treated HCC cells to understand how IL-1β regulates the production of soluble MICA from HCC cells. We demonstrated that IL-1β treatment could increase the mRNA and protein expression of ADAM9 in HCC cells and that ADAM9 knockdown in HCC cells resulted in decreasing of the soluble MICA production. These results suggested that ADAM9 played an important role in the increase in soluble MICA production from IL-1β-treated HCC cells. Both ADAMs and ADAMs with thrombospondin motifs (ADAMTS) are proteinases closely related to matrix metalloproteinases (MMPs). Structure of ADAMs and ADAMTS is highly conserved and involves metalloproteinase and disintegrin domains endowing them with features of both proteinases and adhesion molecules [32]. Several ADAMTSs including ADAMTS1 and ADAMTS9 were activated by IL-1β via NFATc1 transcription factor in chondrosarcoma [33, 34]. Although IL-1β may regulate such transcription factors in HCC cells, the detail mechanism of the activation of ADAM9 by IL-1β remains unclear. The concentration of IL-1β in our in vitro study was high compared with the serum IL-1β concentration level. However, the local IL-1β concentration in the liver tissues still remains unknown and may differ from the serum IL-1β concentration. Our in vitro study at least demonstrated that IL-1β could enhance the production of soluble MICA via up-regulating the expressions of ADAM9 in HCC cells, which might support the possible role of IL-1β in the survival of HCC cells.

Cai et al. [35] demonstrated that the numbers of CD56+ NK cells reduced in HCC tissues compared with healthy donors and CD56+ NK cells in HCC patients displayed impairments in cytotoxicity and IFN-γ production. This suggests that immunological microenvironment in liver tissues of CH patients might be favorable for the survival of HCC cells. We demonstrated that serum IL-1β levels correlated with soluble MICA in CH patients, which is consistent with our in vitro data. This suggests that the chronic elevation of IL-1β in CH patients might impair the function of NK cells by accelerating the production of soluble MICA. We also demonstrated that IL-1β treatment resulted in the inhibition of the cytolytic activity of NK cells against HCC cells. Intrahepatic activated macrophages and plasma cells could produce IL-1β inducing the inflammatory process in chronic liver disease [36]. If we could control the production of IL-1β with new reagents, it might be possible to develop a new therapeutic strategy against HCC. IL-1β receptor antagonist (IL-1RA) has been reported to apply clinically to the treatment of rheumatoid arthritis [37]. We believe the future clinical application of IL-1RA in HCC treatment as a new agent.

In spite of recent progress in the understanding of HCC, there remains to be unknown mechanism of the escape of HCC cells from innate immunity. We have shown here that ADAM9 was directly associated with increasing the production of soluble MICA in IL-1β-treated human HCC. These findings might indicate that IL-1β contributes to the survival of HCC cells by inhibiting innate immunity.

Acknowledgments This work was supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan and a Grant-in-Aid for Research on Hepatitis and BSE from the Ministry of Health, Labour and Welfare of Japan.

References

1. Dinarello CA (1996) Biologic basis for interleukin-1 in disease. Blood 87:2095–2147
2. Napoli J, Bishop GA, McGuinness PH, Painter DM, McCaughhan GW (1996) Progressive liver injury in chronic hepatitis C infection correlates with increased intrahepatic expression of Th1-associated cytokines. Hepatology 24:759–765
3. Powell EE, Edwards-Smith CJ, Hay JL, Clouston AD, Crawford DH, Shorthouse C, Purdie DM, Jonsson JR (2000) Host genetic factors influence disease progression in chronic hepatitis C. Hepatology 31:828–833
4. Tilg H, Wilmer A, Vogel W, Herold M, Nolchen B, Judmaier G, Huber C (1992) Serum levels of cytokines in chronic liver diseases. Gastroenterology 103:264–274
5. Bidwell J, Keen L, Gallagher G et al (1999) Cytokine gene polymorphism in human disease: on-line databases. Genes Immun 1:3–19
6. El-Omar EM, Carrington M, Chow WH et al (2000) Interleukin-1 polymorphisms associated with increased risk of gastric cancer. Nature 404:398–402
7. Howell WM, Calder PC, Grimble RF (2002) Gene polymorphism, inflammatory disease and cancer. Proc Nutr Soc 61:447–456
8. Wang Y, Kato N, Hoshida Y et al (2003) Interleukin-1β gene polymorphism associated with hepatocellular carcinoma in hepatitis C virus infection. Hepatology 37:65–71
9. Tanaka Y, Furuta T, Suzuki S, Orito E, Yeo AE, Hirashima N, Sugauchi F, Ueda R, Mizokami M (2003) Impact of interleukin-1β genetic polymorphism on the development of hepatitis C virus-related hepatocellular carcinoma in Japan. J Infec Dis 187:1822–1825
10. Hirankarn N, Kimkong I, Kummee P, Tangkijvanich P, Poovorawan Y (2006) Interleukin-1β gene polymorphism associated with hepatocellular carcinoma in hepatitis B virus infection. World J Gastroenterol 12:776–779

11. Tu S, Bhagat G, Cui G, Takaishi S, Kurt-Jones EA, Rickman B, Betz KS, Penz-Oesterreicher M, Bjorkdahl O, Fox JG, Wang TC (2008) Overexpression of interleukin-1β induces gastric inflammation and cancer and mobilizes myeloid-derived suppressor cells in mice. Cancer Cell 14:408–419
12. Groh V, Rhinehart R, Secrist H, Bauer S, Grabstein KH, Spies T (1999) Broad tumor-associated expression and recognition by tumor-derived $\gamma\delta$T cells of MICA and MICB. Proc Natl Acad Sci USA 96:6879–6884
13. Jinushi M, Takehara T, Tatsumi T et al (2003) Expression of MICA and MICB in human hepatocellular carcinomas and their regulation by retinoic acids. Int J Cancer 104:354–361
14. Ogasawara K, Lanier LL (2005) NKG2D in NK and T cell-mediated immunity. J Clin Immunol 25:534–540
15. Caudert JD, Held W (2006) The role of the NKG2D receptor for tumor immunity. Semin Cancer Biol 16:333–343
16. Groh V, Wu J, Yee C, Spies T (2002) Tumor-derived soluble MIC ligands impair expression of NKG2D and T cell activation. Nature 419:734–738
17. Salih HR, Rammensee HG, Steinle A (2002) Downregulation of MICA on human tumors by proteolytic shedding. J Immunol 169: 4098–4102
18. Kohga K, Takehara T, Tatsumi T et al (2008) Serum levels of soluble major histocompatibility complex (MHC) class I-related chain A in patients with chronic liver disease and changes during transcatheter arterial embolization for hepatocellular carcinoma. Cancer Sci 99:1643–1649
19. Kohga K, Takehara T, Tatsumi T, Ishida H, Miyagi T, Hosui A, Hayashi N (2010) Sorafenib inhibits the shedding of MICA on hepatocellular carcinoma cell by downregulating ADAM9. Hepatology 51:1264–1273
20. Holdenrieder S, Stieber P, Peterfi A, Nagel D, Steinle A, Salih HR (2006) Soluble MICA in malignant disease. Int J Cancer 118: 684–687
21. Doherty DG, O'Farrelly C (2000) Innate and adaptive lymphoid cells in human liver. Immunol Rev 174:5–20
22. Mehal WZ, Azzaroli F, Crispe IN (2001) Immunology of the healthy liver: old questions and new insights. Gatsroenterology 120:250–260
23. Guerra N, Tan YX, Joncker NT et al (2008) NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. Immunity 28:571–580
24. Kohga K, Takehara T, Tatsumi T, Miyagi T, Ishida H, Ohkawa K, Kanto T, Hiramatsu N, Hayashi N (2009) Anti-cancer chemotherapy inhibits MICA ectodomain shedding by downregulating ADAM10 expression in hepatocellular carcinoma. Cancer Res 69:8050–8057
25. Oyanagi Y, Takahashi T, Matsui S, Takahashi S, Boku S, Takahashi K, Furukawa K, Arai F, Asakura H (1999) Enhanced expression of interleukin-6 in chronic hepatitis C. Liver 19:464–472
26. Lapinski TW (2001) The levels of IL-1β, IL-4 and IL-6 in the serum and the liver tissue of chronic HCV-infected patients. Arch Immunol Ther Exp 49:311–316
27. Bortolami M, Kotsafti A, Cardin R, Farinati F (2008) Fas/FasL system, IL-1β expression and apoptosis in chronic HBV and HCV liver disease. J Viral Hepat 15:515–522
28. Migita K, Abiru S, Maeda Y et al (2005) Serum levels of interleukin-6 and its soluble receptors in patients with hepatitis C virus infection. Human Immunol 67:27–32
29. Nakagawa H, Maeda S, Yoshida H et al (2009) Serum IL-6 levels and the risk for hepatocarcinogenesis in chronic hepatitis C patients: an analysis based on gender difference. Int J Cancer 125:2264–2269
30. Wong VW, Yu J, Cheng AS et al (2009) High serum interleukin-6 level predicts future hepatocellular carcinoma development in patients with chronic hepatitis B. Int J Cancer 124:2766–2770
31. Waldhauer I, Goehlsdorf D, Gieseke F et al (2008) Tumor-associated MICA is shed by ADAM proteases. Cancer Res 68: 6368–6376
32. Seals DF, Courtneidge SA (2003) The ADAMs family of metalloproteases: multidomain proteins with multiple functions. Genes Dev 17:7–30
33. Yaykasli KO, Oohashi T, Hirohata S, Hatipoglu OF, Inagawa K, Demircan K, Ninomiya Y (2009) ADAMTS9 activation by interleukin 1 beta via NFATc1 in OUMS-27 chondrosarcoma cells and in human chondrocytes. Mol Cell Biochem 323:69–79
34. Kalinski T, Krueger S, Sel S, Wemer K, Ropke M, Roessner A (2007) ADAMTS1 is regulated by interleukin-1beta, not by hypoxia, in chondrosarcoma. Hum Pathol 38:86–94
35. Cai L, Zhang Z, Zhou L et al (2008) Functional impairment in circulating and intrahepatic NK cell and relative mechanism in hepatocellular carcinoma patients. Clin Immunol 129:428–437
36. Hassan G, Moreno S, Massimi M, Di Biagio P, Stefanini S (1997) Interleukin-1-producing plasma cells in close contact with hepatocytes in patients with chronic active hepatitis. J Hepatol 27:6–17
37. Gabby C, Lamacchia C, Palmer G (2010) IL-1 pathway in inflammation and human disease. Nat Rev Rheumatol 6:232–241