Mcl-1 and Bcl-xL Cooperatively Maintain Integrity of Hepatocytes in Developing and Adult Murine Liver

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Anti-apoptotic members of the Bcl-2 family, including Bcl-2, Bcl-xL, Mcl-1, Bcl-w and Bfl-1, inhibit the mitochondrial pathway of apoptosis. Bcl-xL and Mcl-1 are constitutively expressed in the liver. Although previous research established Bcl-xL as a critical apoptosis antagonist in differentiated hepatocytes, the significance of Mcl-1 in the liver, especially in conjunction with Bcl-xL, has not been clear. To examine this question, we generated hepatocyte-specific Mcl-1–deficient mice by crossing *mcl-1*$^{flox/flox}$ mice and *AlbCre* mice and further crossed them with *bcl-x*$^{flox/flox}$ mice, giving Mcl-1/Bcl-xL–deficient mice. The *mcl-1*$^{flox/flox}$ *AlbCre* mice showed spontaneous apoptosis of hepatocytes after birth, as evidenced by elevated levels of serum alanine aminotransferase (ALT) and caspase-3/7 activity and an increased number of terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate nick-end labeling (TUNEL)-positive cells in the liver; these phenotypes were very close to those previously found in hepatocyte-specific Bcl-xL–deficient mice. Although *mcl-1*$^{flox/+}$ *AlbCre* mice did not display apoptosis, their susceptibility to Fas-mediated liver injury significantly increased. Further crossing of Mcl-1 mice with Bcl-xL mice showed that *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre* mice also showed spontaneous hepatocyte apoptosis similar to Bcl-xL–deficient or Mcl-1–deficient mice. In contrast, *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/+}$ *AlbCre*, *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/flox}$ *AlbCre*, and *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/flox}$ *AlbCre* mice displayed a decreased number of hepatocytes and a reduced volume of the liver on day 18.5 of embryogenesis and rapidly died within 1 day after birth, developing hepatic failure evidenced by increased levels of blood ammonia and bilirubin. *Conclusion:* Mcl-1 is critical for blocking apoptosis in adult liver and, in the absence of Bcl-xL, is essential for normal liver development. Mcl-1 and Bcl-xL are two major anti-apoptotic Bcl-2 family proteins expressed in the liver and cooperatively control hepatic integrity during liver development and in adult liver homeostasis in a gene dose-dependent manner. (HEPATOLOGY 2009;50:1217-1226.)

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Abbreviations: ALT, alanine aminotransferase; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; TNF-α, tumor necrosis factor alpha; TUNEL, terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate nick-end labeling.

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The mitochondrial pathway of apoptosis is regulated by the Bcl-2 family proteins.[1,2] They are functionally divided into two basic groups: pro-apoptotic and anti-apoptotic members. Pro-apoptotic members are further divided into multi-domain members, such as Bax and Bak, and BH3-only proteins. Bax/Bak triggers release from mitochondria of cytochrome c, presumably by forming pores at the mitochondrial outer membrane. Cytochrome c released into the cytosol activates multiple caspases, which cut a variety of cellular substrates and dismantle the cell.[3] The release of Bax/Bak–mediated cytochrome c is considered to be a point of no return and a commitment to cell death.[4] Killing by BH3-only proteins, such as Bid, Bim, or Puma, requires Bax or Bak, placing them upstream of Bak/Bax activation. BH3-only proteins are transcriptionally or posttranslationally activated by a variety of cellular stresses. They are considered to be sensors that transmit apoptotic stimuli to mitochondria. Anti-apoptotic members, including Bcl-2, Bcl-xL, Mcl-1, Bcl-w, and Bfl-1, inhibit the mitochon-

drial pathway of apoptosis either by directly blocking Bak/Bax activation or by sequestering BH3-only proteins from Bak or Bax.

Mcl-1 has increasingly attracted attention because of its role in liver disease. Several reports have shown that Mcl-1 is overexpressed in a subset of human hepatocellular carcinomas and provides apoptosis resistance.[5-7] The multi-kinase inhibitor sorafenib, which was recently approved by the Food and Drug Administration as a chemotherapeutic agent for hepatocellular carcinoma,[8] is capable of down-regulating Mcl-1 expression and producing apoptosis in hepatoma cells.[9] Cycloxygenase 2 or hepatocyte growth factor up-regulates Mcl-1 expression in hepatocytes and improves Fas-mediated liver injury.[10,11] Recently, enforced expression of Mcl-1 was reported to reduce liver injury induced by anti-Fas injection in mice.[12] However, little is known about the physiologic significance of Mcl-1 in hepatocytes.

We previously reported that hepatocyte-specific Bcl-xL knockout mice were born and grew up but developed spontaneous hepatocyte apoptosis, identifying Bcl-xL as a critical apoptosis antagonist in hepatocytes.[13] This raises a question of whether other anti-apoptotic Bcl-2 family members, such as Mcl-1, have a significant role in regulating hepatocyte apoptosis and what the relationship is among those molecules. To this end, in the current study, we generated hepatocyte-specific Mcl-1 knockout as well as Bcl-xL/Mcl-1 double knockout mice and found that, like Bcl-xL, Mcl-1 is critical for maintaining hepatocyte integrity in adult liver, but not essential for liver development. However, both deficiencies cause a severe defect in liver development and lethality during the early neonatal period because of severe hepatic failure. The current study identifies Bcl-xL and Mcl-1 as two major anti-apoptotic Bcl-2 family proteins in the liver and demonstrates their gene dose–dependent effects for controlling hepatic integrity.

Materials and Methods

Mice. Mice carrying the *mcl-1* gene encoding amino acids 1 through 179 flanked by 2 *lox*P (*mcl-1*$^{flox/flox}$) were provided by Dr. You-Wen He of Duke University.[14] Mice carrying a *bcl-x* gene with two *lox*P sequencers at the promoter region and a second intron (*bcl-x*$^{flox/flox}$) were described previously.[15] Heterozygous AlbCre transgenic mice expressing Cre recombinase gene under the promoter of the albumin gene were described previously.[13] We generated hepatocyte-specific Mcl-1 knockout mice (*mcl-1*$^{flox/floox}$ *AlbCre*) by mating *mcl-1*$^{flox/flox}$ and *AlbCre* mice. We then used these knockout mice to generate hepatocyte-specific Bcl-xL/Mcl-1 knockout mice (*bcl-x*$^{flox/flox}$ *mcl-1*$^{fl/oxflox}$ *AlbCre*) by mating them with *bcl-x*$^{flox/flox}$ mice. Traditional Bid knockout mice were described previously.[16] They were maintained in a specific pathogen–free facility and treated with humane care under approval from the Animal Care and Use Committee of Osaka University Medical School.

Genotyping. Genomic DNA was extracted from the tail and subjected for polymerase chain reaction (PCR) for genotyping mice. The primers used were as follows: 5′-GCCACCTCATCAGTCGGG-3′ and 5′-TCAGAAGCCGCAATATCCCC-3′ for the *bcl-x* allele; 5′-GGTTCCCTGTCTCCTTACTTACTGTAG-3′ and 5′-CTCCTAACCACTGTTCCTGACATCC-3′ for the *mcl-1* allele; 5′-GCGGTCTGGCAGTAAAAACTATC-3′, 5′-GTGAAACAGCATTGCTGTCACTT-3′, 5′CTAGGCCACAGAATTGAAAGATCT-3′ 5′-GTAGGTGGAAATTCTAGCATCATCC-3′ for the *AlbCre* allele; 5′-CCGAAA TGTCCCATAAGAG-3′, 5′-GAGATGGACCACAACATC-3′, and 5′ TGCTACTTCCATTTGTCACGTCCT-3′ for the *bid* allele. PCR products were electrophoretically separated using 2% agarose gels. The expected sizes of the PCR products were as follows: 165 bp for the wild-type *bcl-x* allele, 195 bp for the floxed *bcl-x* allele, 200 bp for the wild-type *mcl-1* allele, 300 bp for the floxed *mcl-1* allele, 130 bp for the wild-type *bid* allele, and 350 bp for the *bid* knockout allele. *AlbCre*-negative mice showed a 350-bp band, and heterozygous *AlbCre* mice showed 100-bp and 350-bp double bands.

Apoptosis Assay. To measure serum ALT level and caspase-3/7 activity, blood was collected from the inferior vena cava of mice and centrifuged. Serum was stored at −20°C until use. Serum ALT levels were measured by a standard method at Oriental Kobo Life Science Laboratory (Nagahama, Japan), and serum caspase-3/7 activity was measured by a luminescent substrate assay for caspase-3 and caspase-7 (Caspase-Glo assay, Promega, Tokyo, Japan). For histological analysis, livers were formalin-fixed, embedded in paraffin, and thin sliced. The liver sections were stained with hematoxylin-eosin. To detect cells with oligonucleosomal DNA breaks, the sections were also subjected to terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate nick-end labeling (TUNEL) staining, according to a previously reported procedure.[17] For Fas-stimulating study, anti-Fas antibody (Jo2 clone) (PharMingen, San Diego, CA) was intraperitoneally injected into mice 3 hours before sacrifice.

Western Blot Analysis. Approximately 25 mg liver tissues was lysed with a lysis buffer (1% NP-40, 0.5%

sodium deoxycholate, 0.1% sodium dodecyl sulfate and 1× protein inhibitor cocktail (Nacalai tesque, Kyoto, Japan), phosphate-buffered saline; pH 7.4). After incubation on ice for 15 minutes, the lysate was centrifuged at 10,000*g* for 15 minutes at 4°C. The protein content of the supernatants was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Equal amounts of protein were electrophoretically separated by sodium dodecyl sulfate polyacrylamide gels (8% or 12%) and transferred onto polyvinylidene fluoride membrane. 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-Bax antibody (Cell Signaling Technology, Beverly, MA), anti-Bid antibody (Cell Signaling Technology), anti-albumin antibody (Affinity Bioreagents, Golden, CO), and anti–beta actin antibody (Sigma-Aldrich, Saint Louis, MO). Detection of immunolabeled proteins was performed using a chemiluminescent substrate (Pierce).

Neonate Analysis. Neonatal mice delivered by cesarean section were suckled by a surrogate mother and sacrificed at 10 hours after birth. Blood from the neonatal mice was centrifuged, and the plasma was stored at −20°C until use. The levels of total bilirubin and ammonia were measured by Van den Bergh reaction and a standard enzymatic procedure, respectively, at Oriental Kobo Life Science Laboratory.

Real-Time Reverse-Transcription PCR. Total RNA was prepared from liver tissue using RNeasy kit (QIAGEN, Tokyo, Japan). For complementary DNA synthesis, 1 μg total RNA was reverse-transcribed using the High Capacity RNA-to-DNA Master Mix (Applied Biosystems, Foster City, CA). Complementary DNA, equivalent to 40 ng RNA, was used as a template for real-time reverse-transcription PCR (RT-PCR) using an Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems). The messenger RNA expressions of tumor necrosis factor alpha (TNF-α), collagen-alpha1(I), and transthyretin were measured using TaqMan Gene Expression Assays (Assay ID: Mm00443260_g1, Mm00801666_g1, and Mm00443267_m1, respectively), and were corrected with the quantified expression level of beta-actin messenger RNA measured using TaqMan Gene Expression Assays (Assay ID: Mm02619580_g1).

Statistical Analysis. Data are presented as mean ± standard deviation. Comparisons between two groups were performed by unpaired *t* test. Multiple comparisons were performed by analysis of variance followed by Scheffe *post hoc* correction. $P < 0.05$ was considered statistically significant.

Results

Hepatocyte-Specific Mcl-1 Deficiency Leads to Spontaneous Hepatocyte Apoptosis in the Adult Liver. To generate hepatocyte-specific Mcl-1–deficient mice, floxed *mcl-1* mice were crossed with heterozygous *AlbCre* mice. After *mcl-1*$^{flox/+}$ *AlbCre* mice were mated with *mcl-1*$^{flox/+}$ mice, and offspring were screened for genotyping and Mcl-1 expression. *mcl-1*$^{flox/flox}$ *AlbCre* mice were born and grew up. Their expression in the liver of Mcl-1 was greatly reduced compared with that of wild-type mice (Fig. 1A). The levels of Bcl-xL expression did not change in *mcl-1*$^{flox/flox}$ *AlbCre* liver. Bcl-xL and Mcl-1 proteins migrated as typical doublet bands of which the biochemical nature had been previously determined.[18] The trace amount of Mcl-1 expression found in the knockout liver may have been attributable to expression in nonparenchymal cells, as previously observed in hepatocyte-specific Bcl-xL–deficient mice.[13]

To investigate the significance of Mcl-1 in the liver, mice were sacrificed 6 weeks after birth and subjected to analysis of serum ALT levels and caspase-3/7 activity as well as liver histology and TUNEL staining. *mcl-1*$^{flox/flox}$ *AlbCre* mice displayed significantly higher levels of serum ALT than control mice (*AlbCre*-negative or *mcl-1*$^{+/+}$ *AlbCre* mice) (Fig. 1B). Hepatocytes with typical apoptosis morphology such as cellular shrinkage and nuclear condensation were frequently observed in the liver sections of *mcl-1*$^{flox/flox}$ *AlbCre* mice (Fig. 1C). Consistently, the number of cells with TUNEL positivity, a hallmark of apoptotic cell death, in the liver was significantly higher in *mcl-1*$^{flox/flox}$ *AlbCre* mice than in control mice (Fig. 1C). Activity of caspase-3/7, executioners of apoptosis, was significantly higher in circulation of *mcl-1*$^{flox/flox}$ *AlbCre* mice than in control mice, which might reflect activation of those proteases in the knockout liver (Fig. 1D). Bax expression was clearly increased in *mcl-1*$^{flox/flox}$ *AlbCre* mice, suggesting Bax activation being involved in the apoptosis in *mcl-1*$^{flox/flox}$ *AlbCre* mice (Fig. 1A). Furthermore, the expression of TNF-α and collagen-alpha1(I) was significantly increased in the *mcl-1*$^{flox/flox}$ *AlbCre* liver compared with the wild-type liver, as found in the Bcl-xL knockout liver (Fig. 1E). Taken together, hepatocyte-specific Mcl-1 knockout mice developed spontaneous apoptosis leading to sterile inflammation and fibrotic response in the liver, like hepatocyte-specific Bcl-xL knockout mice.[13]

Heterozygous Deletion of the mcl-1 Gene Does Not Produce Apoptosis But Increases the Susceptibility to Fas Stimulation. Although the levels of Mcl-1 expression were significantly decreased in *mcl-1*$^{flox/+}$ *AlbCre* liver (Fig. 1A, Supporting Fig. 1), *mcl-1*$^{flox/+}$ *AlbCre* mice did not have apoptosis phenotypes in the liver (Fig. 1B-D).

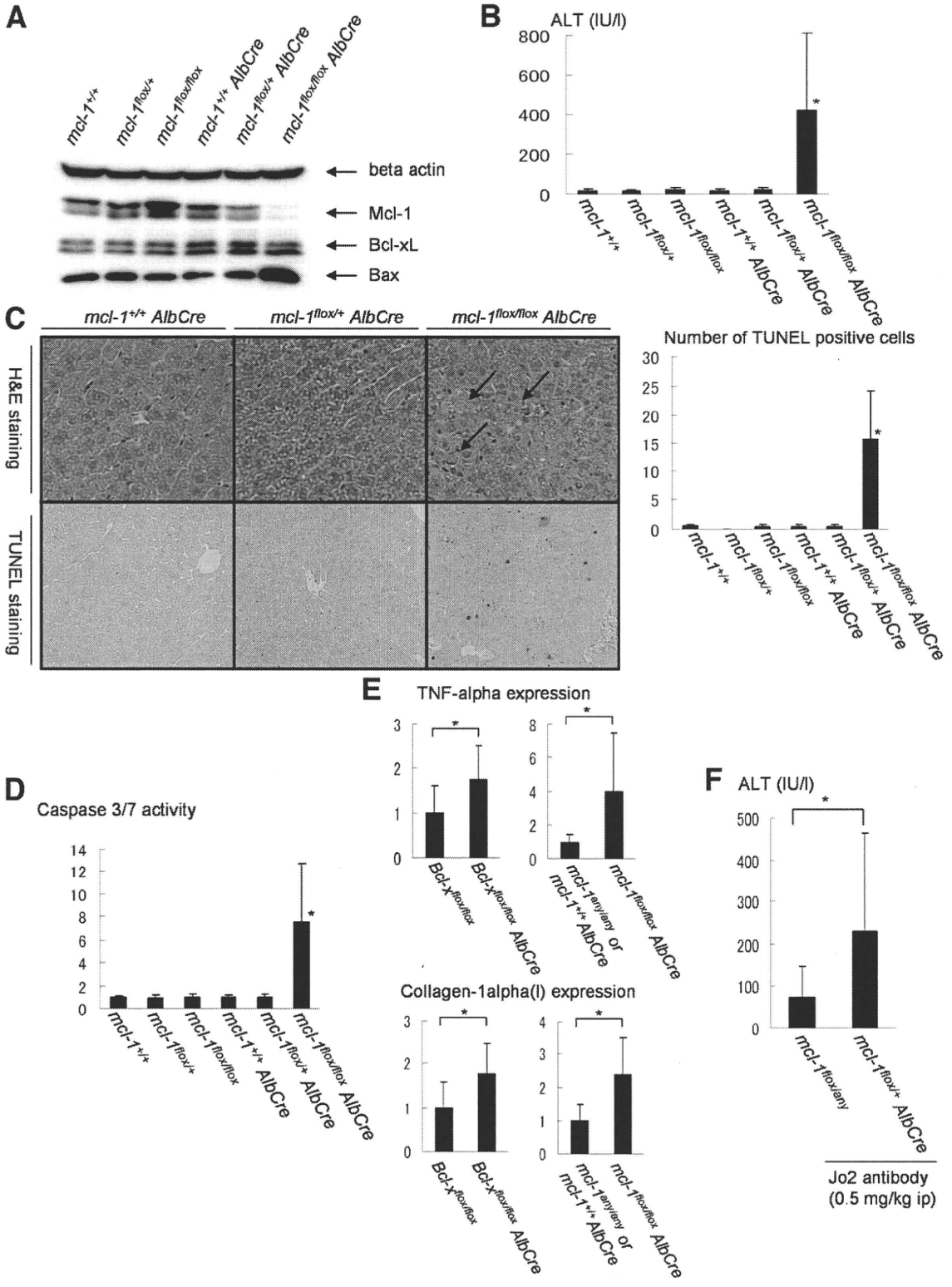

Fig. 1. Hepatocyte-specific Mcl-1 knockout mice. Offspring from mating of *mcl-1*$^{flox/+}$ *AlbCre* mice and *mcl-1*$^{flox/+}$ mice were sacrificed at the age of 6 weeks. (A) Western blot of whole liver lysate for the expression of Bcl-xL, Mcl-1, and Bax. (B) Serum ALT levels. N = 15 mice for each group. *$P < 0.05$ versus the other five groups. (C) Left panel shows hematoxylin-eosin and TUNEL staining of the liver section. Arrow indicates typical apoptotic cells. Right panel shows statistics of TUNEL-positive cells. The number of TUNEL-positive cells was determined in a defined area. N = 5 mice for each group. *$P < 0.05$ versus the other five groups. (D) Serum levels of caspase-3/7 activity. The levels were normalized to *mcl-1*$^{+/+}$ *AlbCre* *(−)* mice. N = 15 mice for each group. *P < 0.05 versus the other five groups. (E) Real-time RT-PCR analysis for TNF-α and collagen-1alpha(1) expression. *$P < 0.05$. N = 12 or 9. The levels were normalized to the wild-type mice. (F) Serum ALT levels of Fas-stimulated mice. The *mcl-1*$^{flox/+}$ *AlbCre* mice and *mcl-1*$^{flox/+ \text{ or } flox}$ mice were sacrificed 3 hours after intraperitoneal injection of 0.5 mg/kg Jo2 antibody. *$P < 0.05$. N = 13 or 7.

Therefore, we examined the susceptibility to Fas stimulation in these mice. We injected anti-Fas antibody into *mcl-1*$^{flox/+}$ *AlbCre* mice and *mcl-1*$^{flox/+\ or\ flox}$ mice and measured the levels of their serum ALT. *mcl-1*$^{flox/+}$ *AlbCre* mice displayed significantly higher levels of serum ALT than control mice (Fig. 1F). These findings suggest that haplo-deficiency of Mcl-1 does not produce apoptosis in a physiological setting but clearly reduces apoptosis resistance under pathological conditions.

Involvement of Bid in Apoptosis Caused by Mcl-1 Deficiency. BH3-only proteins regulate life and death balance by interacting with core Bcl-2 family members. The hepatocyte is a so-called type 2 cell, which requires Bid as a sensor for Fas-mediated apoptotic stresses.[19] In addition, it has been reported that the caspase-8/Bid pathway is involved in a variety of liver pathological conditions.[16,20] To examine the possibility of Bid being involved in hepatocyte apoptosis caused by Mcl-1 deficiency, we crossed hepatocyte-specific Mcl-1 knockout mice with Bid knockout mice. Offspring form mating of *bid*$^{+/-}$ *mcl-1*$^{flox/flox}$ *AlbCre* mice with *bid*$^{+/-}$ *mcl-1*$^{flox/flox}$ mice were sacrificed at 6 weeks after birth and subjected to analysis of apoptosis phenotypes. Mice with each genotype grew up, and, as expected, the levels of Bid and/or Mcl-1 expression in the liver were correspondingly reduced with their genotypes (Fig. 2A). The levels of serum ALT were significantly lower in *bid*$^{-/-}$ *mcl-1*$^{flox/flox}$ *AlbCre* mice than in *bid*$^{+/+}$ *mcl-1*$^{flox/flox}$ *AlbCre* mice (Fig. 2B). The results indicate that Bid was involved in hepatocyte apoptosis found in Mcl-1 knockout mice.

Combined Deficiency of Mcl-1 and Bcl-xL in Hepatocytes Causes Lethality. Phenotypes observed in hepatocyte-specific Mcl-1 knockout mice were very similar to those in hepatocyte-specific Bcl-xL knockout mice.[13] These results indicated that Bcl-xL and Mcl-1 share similar anti-apoptotic functions but do not compensate for the loss of each other. To examine whether their expression and function are completely nonredundant or just partially so, we generated hepatocyte-specific Bcl-xL/Mcl-1 double-knockout mice.

The *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre* mice were mated with *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/flox}$ mice, and genotypes of the offspring were screened at 3 weeks after birth. *AlbCre*-negative and *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre* mice were born and grew up, but not *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/+}$ *AlbCre*, *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/flox}$ *AlbCre*, and *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/flox}$ *AlbCre* mice (Table 1). The lack of Bcl-xL and Mcl-1 caused a more severe phenotype than either knockout, suggesting that they partially compensate for the loss of each other at least from the viewpoint of maintaining normal development.

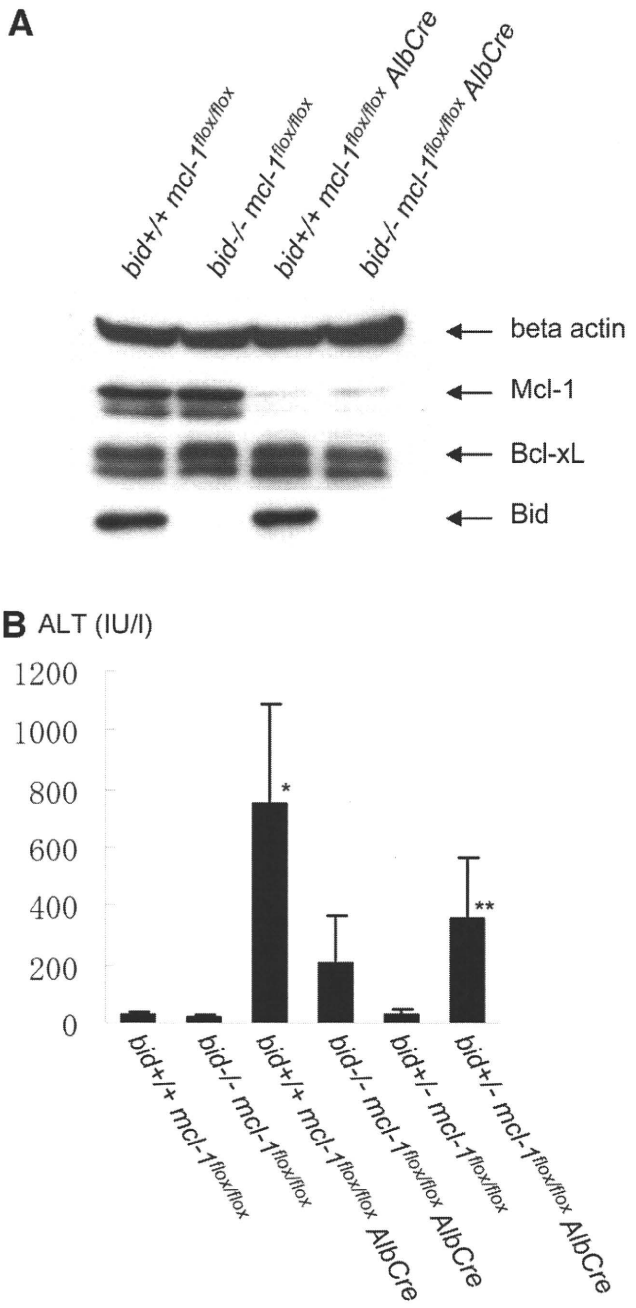

Fig. 2. Mcl-1/Bid double-knockout mice. Offspring from mating of *bid*$^{+/-}$ *mcl-1*$^{flox/flox}$ *AlbCre* mice with *bid*$^{+/-}$ *mcl-1*$^{flox/flox}$ mice were sacrificed at 6 weeks after birth. (A) Western blot of whole liver lysate for the expression of Mcl-1, Bcl-xL, and Bid. (B) Serum ALT levels. N = 12 mice for each group. *$P < 0.05$ versus the other five groups; **$P < 0.05$ versus the *AlbCre*-negative groups and the *bid*$^{+/+}$ *mcl-1*$^{flox/flox}$ *AlbCre* group.

Mice Lacking Single Alleles for Both Bcl-xL and Mcl-1 Develop Spontaneous Apoptosis in the Adult Liver Similar to Bcl-xL or Mcl-1 Knockout Mice. Offspring from mating of *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre and bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/flox}$ were sacrificed at 6 weeks after birth and subjected to analysis of Bcl-xL/Mcl-1 expression and

Table 1. Genotyping of Offspring Obtained by Crossing *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre* Mice and *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/flox}$ Mice

AlbCre	bcl-x	mcl-1	ED18.5	3 Weeks
−	flox/+	flox/+	4	14
−	flox/flox	flox/+	6	17
−	flox/+	flox/flox	12	17
−	flox/flox	flox/flox	7	17
+	flox/+	flox/+	11	22
+	flox/flox	flox/+	8	0
+	flox/+	flox/flox	9	0
+	flox/flox	flox/flox	10	0
	Total		67	87

ED, embryonic day.

Note that each genotype is expected to account for one-eighth of the offspring from this mating.

apoptosis phenotypes. As expected, *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre* liver expressed reduced levels of expression for both Bcl-xL and Mcl-1 (Fig. 3A). Interestingly, *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre* mice developed spontaneous hepatocyte apoptosis as evidenced by an increase in serum ALT levels and caspase-3/7 activity (Fig. 3B,C). In agreement with this, hepatocytes with typical apoptotic morphology and positive for TUNEL staining were found scattered in the liver lobules in these mice (Fig. 3D,E). Furthermore, *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre* mice showed higher expression of TNF-α than wild-type mice (Fig. 3F). The phenotypes were very similar to hepatocyte-specific Bcl-xL or Mcl-1knockout mice.

Hepatocyte-Specific Mcl-1/Bcl-xL-Deficient Mice Show Impaired Development of the Liver and Liver Failure During the Neonatal Period. To examine the impact of Bcl-xL/Mcl-1deficiency at an earlier time point, offspring obtained from crossing *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre* mice and *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/flox}$ mice were analyzed on gestational day 18.5. Live-obtained embryo followed expected Mendelian frequencies (Table 1). Overall, they looked normal, and their body weight did not differ among genotypes (Fig. 4A,B). However, the livers obtained from live pups with genotype of *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/+}$ *AlbCre*, *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/flox}$ *AlbCre,* or *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/flox}$ *AlbCre* were clearly smaller. The ratios of liver weight to body weight were significantly lower in those pups than in *AlbCre*-negative or *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre* pups (Fig. 4C). The ratios of liver weight to body weight were also examined in *mcl-1*$^{flox/flox}$ with *AlbCre* or without *AlbCre* mice, and there was no significant difference between the two (6.0 ± 0.8 versus 5.5 ± 0.9, $N = 5$, $P = 0.34$), excluding the possibility that Mcl-1 knockout itself affects the liver size at this time point. Histological analysis revealed that there were a number of hepatocytes with rectangular morphology and hematopoietic cells in the developing liver of the *AlbCre*-negative pups (Fig. 4D). Whereas the number of rectangular hepatocytes in *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre* livers was similar to that in the *AlbCre*-negative livers, it was lower in *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/+}$ *AlbCre*, *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/flox}$ *AlbCre,* and *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/flox}$ *AlbCre* livers. Rectangular cells were rarely observed in *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/flox}$ *AlbCre* livers. Furthermore, the expression of albumin and transthyretin was examined in the liver as a marker for hepatocyte differentiation.[21] Consistent with histological findings, both expressions were gradually reduced from the *AlbCre*-negative livers to the *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/flox}$ *AlbCre* livers (Fig. 4E,F).

We noticed that offspring obtained from crossing *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre* mice and *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/flox}$ mice frequently died within 1 day after birth. To examine the cause of the early neonatal death, offspring were sacrificed at 10 hours after birth. They were divided into two groups according to the data shown in Table 1: expected survivors including *AlbCre*-negative and *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre* pups, and expected nonsurvivors including *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/+}$ *AlbCre*, *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/flox}$ *AlbCre*, and *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/flox}$ *AlbCre* pups. The levels of total bilirubin and ammonia in circulation were determined and compared between the groups. Both blood bilirubin levels and ammonia levels were significantly higher in the expected nonsurvivors than in the expected survivors (Fig. 5A,B). These results suggested that *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/+}$ *AlbCre*, *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/flox}$ *AlbCre*, and *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/flox}$ *AlbCre* mice died quickly after birth because of hepatic failure, in agreement with the findings of impaired liver development.

Discussion

Five members of the anti-apoptotic Bcl-2 family have been found: Bcl-2, Bcl-xL, Bcl-w, Bfl-1, and Mcl-1. Traditional knockout of Bcl-2, a prototype of this family, displays growth retardation, hair color abnormality, lymphocyte decrease, and polycystic kidney.[22,23] In agreement with the finding that Bcl-2 is not expressed in hepatocytes,[13] these mice did not show any liver phenotypes. Similarly, Bcl-w[24,25] or Bfl-1 knockout mice[26] were generated but no liver phenotypes have been reported. Traditional knockout of Bcl-xL or Mcl-1 caused more severe phenotypes. Deletion of the *bcl-x* gene resulted in embryonic lethality because of abnormal neuronal development and hematopoiesis.[27] The *mcl-1* knockout embryo fails to be implanted *in utero*.[28] Thus, study of traditional knockout mice could not reveal the significance of Bcl-xL or Mcl-1 in the liver.

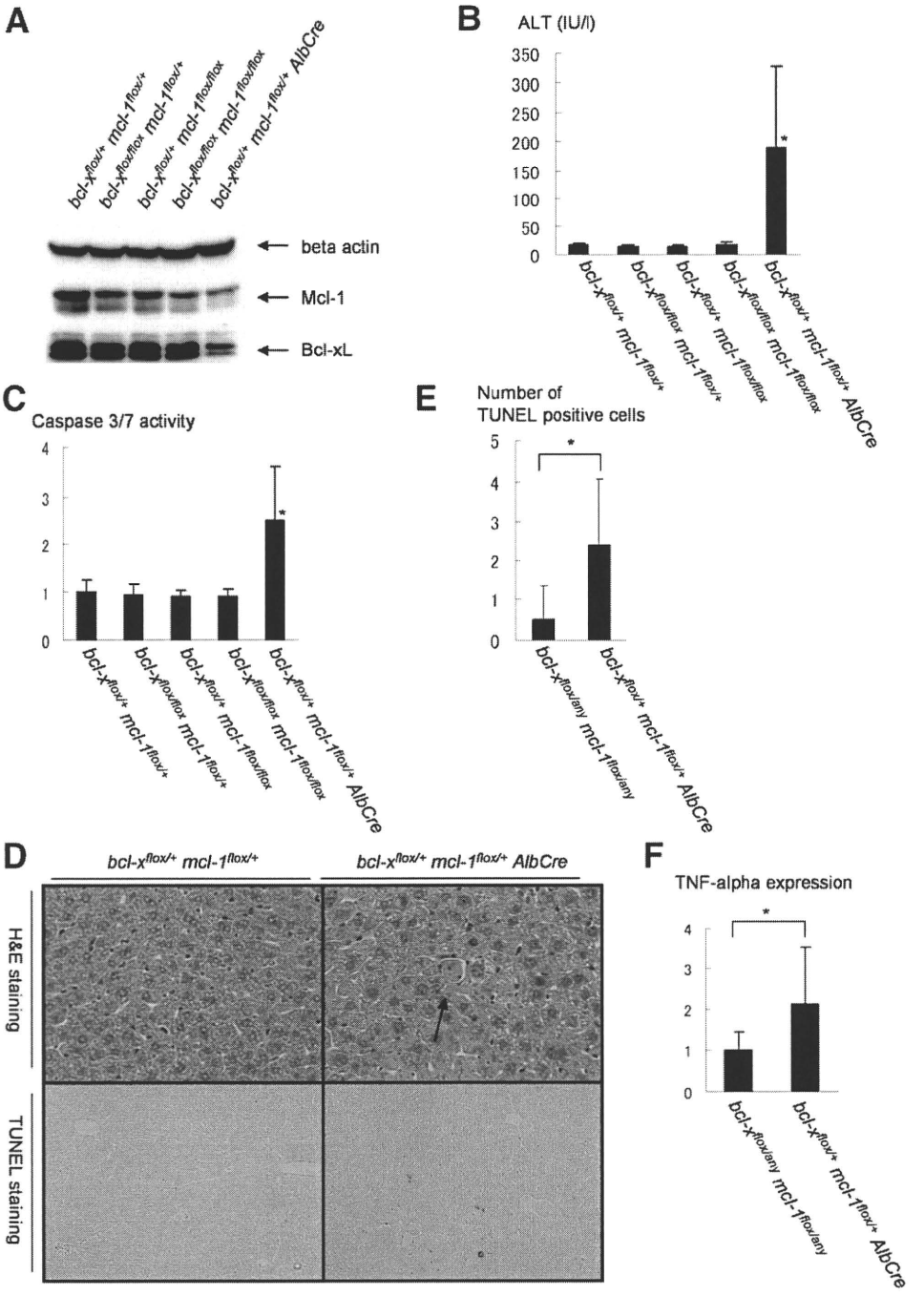

Fig. 3. Hepatocyte-specific Bcl-xL/Mcl-1-deficient mice. Offspring from mating *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre* mice and *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/flox}$ mice were sacrificed at the age of 6 weeks. (A) Western blot of whole liver lysate for the expression of Bcl-xL and Mcl-1. (B) Serum ALT levels. N = 9 mice for each group. *$P < 0.05$ versus the other five groups. (C) Serum levels of caspase-3/7 activity. The levels were normalized to *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ mice. N = 9 mice for each group. *$P < 0.05$ versus the other five groups. (D) Hematoxylin-eosin and TUNEL staining of the liver sections for *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre* mice. Findings for *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ mice are shown as a control. (E) Statistics of TUNEL-positive cells. The number of TUNEL-positive cells was determined in a defined area. N = 5 or 6. *$P < 0.05$. (F) RT-PCR analysis for TNF-α expression. The levels were normalized to the group of *bcl-x*$^{flox/+ \text{ or } flox}$ *mcl-1*$^{flox/+ \text{ or } flox}$. *$P < 0.05$. N = 9.

We previously reported that hepatocyte-specific knockout of Bcl-xL caused spontaneous apoptosis in hepatocytes after birth and established that Bcl-xL is critically important for the integrity of hepatocytes.[13] The current study demonstrated that Mcl-1 plays an anti-apoptotic role in differentiated hepatocytes similar to that of Bcl-xL. During the preparation of this manuscript, a report by Vick et al.[29] appeared on the Web, demonstrating a similar apoptosis phenotype in mice with specific knockout of the *mcl-1* gene in hepatocytes. Our findings

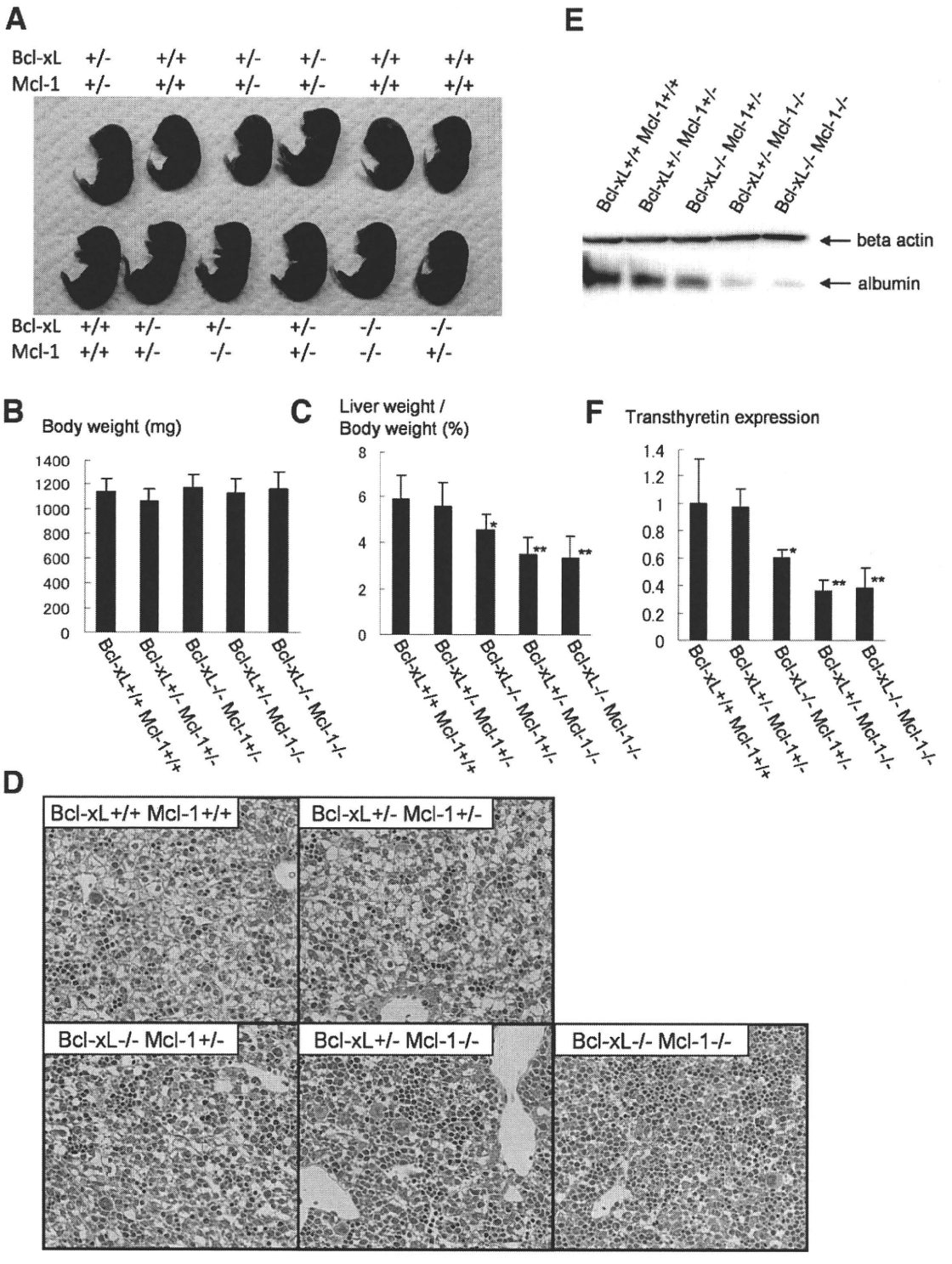

Fig. 4. Hepatocyte-specific Bcl-xL/Mcl-1-deficient embryos. Offspring from mating *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre* mice and *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/flox}$ mice were sacrificed on day 18.5 of gestation. Mice were classified into five groups. The *bcl-x*$^{flox/+ \text{ or } flox}$ *mcl-1*$^{flox/+ \text{ or } flox}$ are indicated by Bcl-xL +/+ Mcl-1 +/+; *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre* are indicated by Bcl-xL +/− Mcl-1 +/−; *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/+}$ *AlbCre* are indicated by Bcl-xL −/− Mcl-1 +/−; *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/flox}$ *AlbCre* are indicated by Bcl-xL +/− Mcl-1 −/−; *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/flox}$ *AlbCre* are indicated by Bcl-xL −/− Mcl-1 −/ −. The numbers of embryos analyzed were 30 for Bcl-xL +/+ Mcl-1 +/+, 11 for Bcl-xL +/− Mcl-1 +/−, 8 for Bcl-xL −/− Mcl-1 +/−, 9 for Bcl-xL +/− Mcl-1 −/−, and 10 for Bcl-xL −/− Mcl-1 −/−. (A) Gross appearance of embryos. Representative photo for a litter is shown. (B) Body weight. (C) The ratios of liver weight to body weight. *$P < 0.05$ versus Bcl-xL +/+ Mcl-1 +/+; **$P < 0.05$ versus Bcl-xL +/+ Mcl-1 +/+ and Bcl-xL +/− Mcl-1 +/−. (D) Hematoxylin-eosin staining of the liver sections. (E) Western blot of whole-liver lysate for albumin expression. (F) Real-time RT-PCR analysis for transthyretin expression. The levels were normalized to the group of Bcl-xL +/+ Mcl-1 +/+. *$P < 0.05$ versus Bcl-xL +/+ Mcl-1 +/+; **$P < 0.05$ versus Bcl-xL +/+ Mcl-1 +/+ and Bcl-xL +/− Mcl-1 +/−.

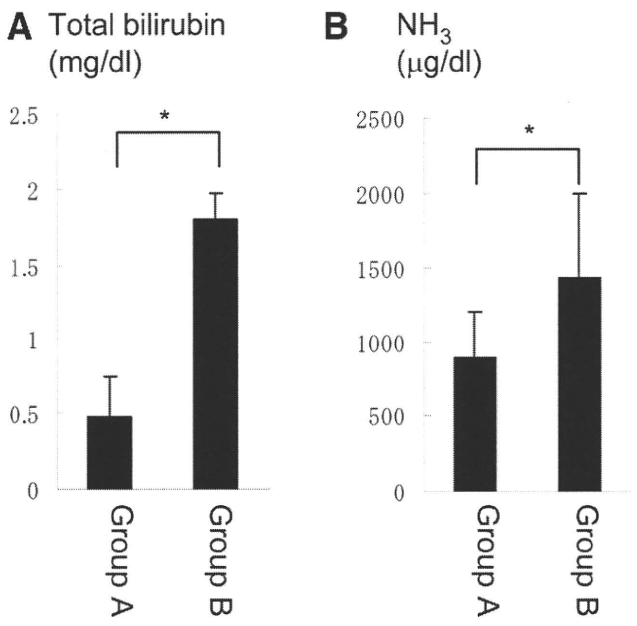

Fig. 5. Plasma biochemistry of hepatocyte-specific Bcl-xL/Mcl-1–deficient neonates 10 hours after birth. Group A (N = 13) was defined as expected survivors including *AlbCre*-negative mice and *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/+}$ *AlbCre* mice. Group B (N = 6) was defined as expected nonsurvivors including *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/+}$ *AlbCre*, *bcl-x*$^{flox/+}$ *mcl-1*$^{flox/flox}$ *AlbCre*, *bcl-x*$^{flox/flox}$ *mcl-1*$^{flox/flox}$ *AlbCre*. (A) Plasma total bilirubin levels. *$P < 0.05$. (B) Plasma ammonia levels in both groups. *$P < 0.05$.

are in agreement with theirs and further provide evidence that deletion of a single allele for the *mcl-1* gene fails to produce apoptosis phenotypes under physiological conditions, as observed in knockout of the *bcl-x* gene.[13] Mcl-1 heterozygous disrupted mice did not produce apoptosis at least until 16 weeks of age (our unpublished data). It was demonstrated that hepatocyte-specific Mcl-1 knockout mice showed higher levels of liver injury than control mice on anti-Fas antibody injection.[29] However, because mice lacking both *mcl-1* alleles possess preexisting liver injury, it would be difficult to exactly compare liver injury after anti-Fas antibody injection and to conclude whether decreased Mcl-1 expression actually increases the susceptibility to Fas. In the current study, we took advantage of Mcl-1 heterozygous disrupted mice to address this point. They showed significantly higher levels of liver injury after Fas stimulation than wild-type mice, formally proving the significance of Mcl-1 expression under pathological conditions. Furthermore, our data on Mcl-1/Bid–deficient mice implies that the Bid pathway is involved in generating apoptosis found in Mcl-1 knockout mice. Because Bid mediates a variety of cellular stresses in hepatocytes upstream of Mcl-1,[30,31] it will be interesting in future study to determine what stresses generate hepatocyte apoptosis in Mcl-1 knockout mice.

Bcl-xL and Mcl-1 share similar structures and functions.[1] The observations that either deficiency similarly leads to spontaneous hepatocyte apoptosis imply that they play a non-redundant role in maintaining the integrity of hepatocytes in the adult liver. To further understand the relationship of both molecules, we generated hepatocyte-specific Bcl-xL/Mcl-1 knockout mice. Interestingly, mice lacking single alleles for both genes (*bcl-x*$^{+/-}$ *mcl-1*$^{+/-}$) induced spontaneous hepatocyte apoptosis that could not be distinguished from that found in Bcl-xL or Mcl-1 knockout mice. This indicates that, whereas knockout of a single allele of the *bcl-x* or *mcl-1* gene did not produce apoptosis, knockout of two alleles of any combination among both genes was sufficient to produce hepatocyte apoptosis. This finding suggests that both molecules are not independently but rather interdependently required for ensuring integrity of differentiated hepatocytes.

Bcl-xL/Mcl-1–deficient mice as well as mice only having a single allele of either *bcl-x* or *mcl-1* gene displayed a decreased number of hepatocytes and reduced liver size on day 18.5 of gestation and appeared to develop lethal liver failure within 1 day after birth. Because the liver contains a large number of hematopoietic cells during development (Fig. 4D), it is very difficult to determine the expression levels of Bcl-xL or Mcl-1 specifically in hepatocytes in each knockout mouse. Liver development begins on day 8.5 of gestation in the mouse when the liver primordium is delineated from the endoderm.[32] The albumin promoter, which is active in both hepatoblasts and hepatocytes, shows a 20-fold increase in transcriptional activity from day 9.5 to day 12.5 of gestation. The level of albumin then continues to increase as the liver develops simultaneously with the biliary tree and the hepatic bile duct being formed.[33] Thus, the target genes could probably be successfully deleted during embryogenesis in the *AlbCre* recombination system. The observation that Bcl-xL/Mcl-1–deficient mice developed severer phenotypes than Bcl-xL–deficient or Mcl-1–deficient mice supports the idea that Cre-mediated deletion of the target genes actually took place during embryogenesis in our model. In contrast to the knockout of two alleles, knockout of three alleles and more of the *bcl-x* and *mcl-1* genes induced lethal neonatal hepatic failure. Thus, hepatocyte integrity appeared to be strictly controlled by Bcl-xL and Mcl-1 in a gene dose–dependent manner.

Hepatocyte-specific deficiency of both Bcl-xL and Mcl-1 led to significant reduction of liver volume because of impaired hepatocyte development. However, overall, mice with these phenotypes were capable of developing normally until birth and rapidly developed liver failure and died within 1 day after birth. This finding suggests that differentiated hepatocytes are critically required for maintaining host homeostasis after birth but not during embryogenesis. The placenta

plays an important role in nutritional support and detoxification of the embryo. Our data imply that it could probably compensate for most functions of the liver cells during embryogenesis, whereas the liver would turn to the critical organ that is essential for maintaining host homeostasis after birth. Bcl-xL/Mcl-1 knockout mice provide interesting implications for the difference in the impact of differentiated hepatocytes between embryogenesis and the early neonatal period.

In conclusion, Mcl-1 and Bcl-xL are two major Bcl-2 family proteins inhibiting hepatocyte apoptosis. Together with previous work on traditional knockout mice, our data imply that other members, if any, could not compensate for their functions. Mcl-1 and Bcl-xL cooperatively maintain hepatocyte integrity during liver development and in adult liver homeostasis, and their effects are gene-dose dependent. Recent studies also have established that Mcl-1[5-7] and Bcl-xL[18,34] are frequently overexpressed and confer resistance to apoptosis in hepatocellular carcinoma. Therefore, Mcl-1 and Bcl-xL are important apoptosis antagonists in a variety of pathophysiological conditions of the liver.

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Review

Adipocytokines and liver disease

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Adipose tissue is a massive source of bioactive substances known as adipocytokines, including tumor necrosis factor (TNF)-α, resistin, leptin, and adiponectin. Recent advances in medical research view obesity as a chronic low-grade inflammatory state. Hypertrophied adipocytes in obesity release chemokines that induce macrophage accumulation in adipose tissue. Accumulated macrophages in obese adipose tissue produce proinflammatory cytokines and nitric oxide, and these inflammatory changes induce adipocytokine dysregulation. The latter is characterized by a decrease in insulin-sensitizing and anti-inflammatory adipocytokines, and an increase in proinflammatory adipocytokines. Adipocytokine dysregulation induces obesity-related metabolic disorders, the so-called metabolic syndrome. Metabolic syndrome is a cluster of metabolic abnormalities, including diabetes mellitus, hypertension, hyperlipidemia, and nonalcoholic steatohepatitis (NASH). Recent studies have revealed that obesity is an independent risk factor for chronic liver diseases, such as NASH, alcoholic liver disease, chronic hepatitis C, and hepatocellular carcinoma. A common mechanism underlying these hepatic clinical states is thought to be adipocytokine dysregulation. In this review, we discuss the association of adipocytokines, especially leptin, adiponectin, TNF-α, and resistin, with liver diseases.

Key words: nonalcoholic steatohepatitis (NASH), chronic hepatitis C, obesity, adipocytokine, adiponectin, leptin, TNF-α

Introduction

Adipose tissue is an energy-storing organ that produces and secretes several bioactive substances[1,2] known as adipocytokines,[3] such as adiponectin,[4] leptin,[5] resistin,[6] plasminogen activator inhibitor 1 (PAI-1),[3] and tumor necrosis factor α (TNF-α).[7] Recent studies have suggested that obesity is a state of chronic, low-grade inflammation that contributes to insulin resistance and type 2 diabetes.[8,9] Hypertrophied adipocytes in obesity release chemokines, which recruit macrophages, especially in visceral adipose tissue. Adipose tissue macrophages produce nitric oxide (NO) and inflammatory cytokines such as TNF-α, interleukin (IL)-6, and IL-1β. These inflammatory changes in adipose tissue induce adipocytokine dysregulation: a decrease in insulin-sensitizing and anti-inflammatory adipocytokines such as adiponectin, and an increase in proinflammatory adipocytokines involved in insulin resistance such as TNF-α, interleukins, and resistin[10,11] (Fig. 1). Furthermore, adipocytokine dysregulation is thought to play a crucial role in metabolic syndrome.[12]

Hepatic cirrhosis is six times more prevalent in obese individuals than in the general population,[13,14] and obesity is an independent risk factor for severity of liver fibrosis in nonalcoholic steatohepatitis (NASH), alcohol-induced liver disease, chronic hepatitis C (CHC), and hepatocellular carcinoma (HCC).[14–20] Recently, several studies have reported that adipocytokine dysregulation affects the pathological state of liver diseases.[21–34] For example, serum leptin and TNF-α levels were significantly higher, and adiponectin levels were significantly lower, in patients with NASH than in controls.[22] In this review, we describe the important roles of adipocytokines in liver diseases.

Leptin

Leptin is a 167-amino acid secreted protein encoded by the *ob* gene, and was identified by positional cloning in the *ob/ob* mouse as a key molecule in the regulation of body weight and energy balance.[35] Leptin is produced

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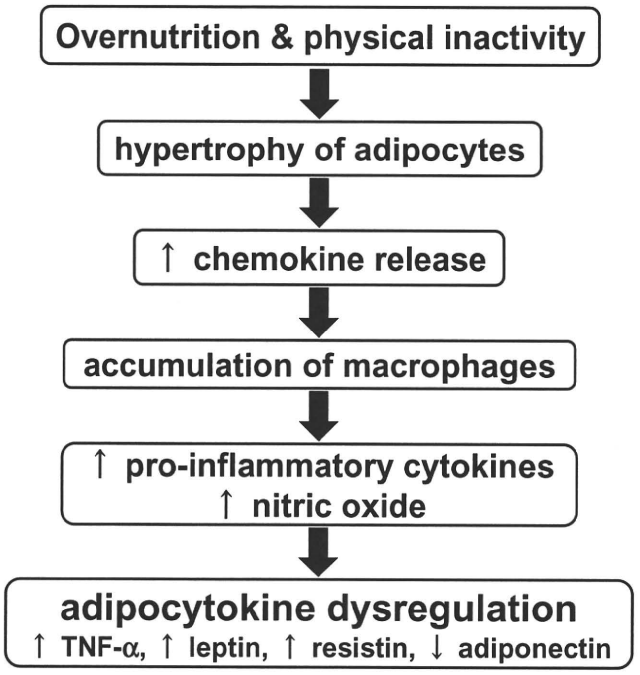

Fig. 1. Current hypothesis regarding the mechanism of adipocytokine dysregulation. *TNF-α*, tumor necrosis factor α

mainly by adipocytes. The expression of leptin in adipocytes is transcriptionally regulated, and is determined mainly by the status of energy stores in white adipose tissue and the size of adipocytes.[5] However, recent studies have confirmed that leptin is also expressed in other tissues such as skeletal muscles, stomach, ovaries, and liver.[36] Leptin plays a key role in the regulation of appetite and body weight. It also acts on the hypothalamus, altering energy intake by decreasing appetite and increasing energy expenditure via sympathetic stimulation of several tissues.[37] Mutations in the leptin gene cause obesity in rodents and human.[35,38]

Serum leptin concentrations correlate well with body weight and body fat mass, and are higher in women than in men even after adjustment for age and body mass index.[39]

Leptin resistance

Circulating leptin levels are elevated in obese subjects, but these subjects are resistant to the action of leptin. Leptin acts by binding to its receptor, Ob-R, and its gene is alternatively spliced into several isoforms. One of the splice variants, Ob-Re, is a soluble leptin receptor and binds to leptin to form a leptin–Ob-Re complex.[40] The complex formation can delay leptin clearance and thereby increase the availability of bioactive leptin.[41] In obese individuals, the serum free leptin level is high, and the Ob-Re level is low, resulting in a low leptin–Ob-Re complex level. A low serum Ob-Re level and low leptin–Ob-Re complex level can be markers of leptin resistance. However, excess Ob-Re is likely to inhibit free leptin function, because the complex cannot activate the transmembrane leptin receptor Ob-Rb.[40] Other groups have argued that diet-induced obesity causes downregulation of Ob-Rb and results in impairment of leptin signaling.[42,43]

Recent studies indicate that leptin can inhibit the orexigenic peptides (neuropeptide Y, agouti-related peptide) and stimulate the secretion of anorexigenic peptide (α-melanocyte-stimulating hormone) from arcuate melanocortin neurons of lean mice.[44] However, leptin failed to modulate the secretion of these peptides in high-fat-diet-induced obese (DIO) mice. Such resistance to leptin is due to increased levels of suppressor of cytokine-signaling protein 3 (a negative regulator of leptin signal transduction) in the arcuate nucleus in the hypothalamus of DIO mice. A reduction in diet fat content resulted in recovery of leptin responsiveness in DIO mice.

Leptin and liver diseases

In animal models, leptin prevents lipid accumulation in nonadipose tissues, such as skeletal muscles, pancreas, and liver, a concept referred to as lipotoxicity.[23] In the liver, leptin achieves its antilipogenic effects by lowering the expression of sterol regulatory element binding protein 1 (SREBP-1).[45] Patients with severe lipodystrophy present with hepatic steatosis and hepatocellular ballooning injury, similar to that seen in NASH, and recombinant leptin significantly reduces serum levels of triglycerides, transaminases, and liver fat content and improves hepatomegaly in these patients.[46]

Leptin injections in mice treated with carbon tetrachloride increased the expression levels of procollagen-I, transforming growth factor β1 (TGF-β1), and smooth muscle actin, a marker of activated hepatic stellate cells (HSCs), and eventually resulted in tissue fibrosis.[47] In the liver, leptin directly promotes fibrogenesis by stimulating the production of tissue inhibitor of metalloproteinase 1 via the Janus kinase/signal transducer and activator of transcription pathway in activated HSCs, which are a central player in liver fibrosis.[48] Moreover, leptin is described as a potent mitogen for HSCs and an inhibitor of apoptosis of HSCs through extracellular signal-regulated kinase (ERK) and the Akt-dependent pathway.[49] Activated HSCs acquire the ability of secrete leptin and are thought to further promote liver fibrosis.[50] In addition, leptin increases the expression of TGF-β1 in sinusoidal endothelial cells and Kupffer cells. In Zucker (*fa/fa*) rats, a naturally occurring functional leptin receptor-deficient animal, thioacetamide-induced hepatic fibrosis was prevented almost completely and induction of TGF-β1 and activation of HSCs were

abolished.[50] Considered together, the above results indicate that leptin and its functional receptor play a pivotal role in profibrogenic responses in the liver.

High serum leptin concentrations are present in cirrhosis patients.[30,34] However, despite high serum leptin concentrations in nonalcoholic fatty liver disease (NAFLD) patients, there is no relationship between leptin and the severity of hepatic fibrosis.[51] Moreover, leptin levels were initially found to be significantly higher in NASH patients than in controls matched for sex and body mass index (BMI), and correlated with the severity of hepatic steatosis but not with the severity of inflammation or fibrosis.[23] In another study, serum leptin levels and leptin receptor mRNA expression levels in the liver were not significantly different between patients with NASH and those with simple steatosis.[52] The relationship between serum leptin concentrations and the severity of liver fibrosis remains to be investigated.

Serum leptin levels were higher in CHC patients than controls,[53] and associated with the severity of fibrosis.[54] Serum leptin levels correlated with hepatic steatosis in patients infected with hepatitis C virus genotype 3 but not genotype 1.[55] In contrast, another study revealed that leptin levels do not correlate with fibrosis or severity of steatosis in CHC patients.[56] Considered together, these studies indicate that while serum leptin levels may be elevated in CHC patients, there is rather a conflicting relationship between serum leptin levels and liver histology in such patients.

There is a close relationship between BMI and a high mortality rate due to digestive cancers;[19] especially, obesity and HCC represent a particularly high risk.[20,57] The high plasma leptin levels in obesity may contribute to this phenomenon. Leptin acts as mitogen on many cell types in vitro, including HCC cells, via the ERK/mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (PI3K)/Akt pathway, and may facilitate progression to liver cancer in vivo.[58,59]

These findings suggest that leptin plays important roles in liver diseases such as attenuating hepatic steatosis, exacerbating liver fibrosis, and possibly promoting HCC growth. Further research needs to be conducted on the precise role of leptin in liver diseases.

Adiponectin

Adiponectin is an adipocyte-specific 28-kDa secreted protein expressed exclusively in adipose tissue.[4] However, recent studies have indicated that adiponectin is also produced by organs other than adipose tissue, such as bone marrow,[60] fetal tissue,[61] cardiomyocytes,[62] and hepatic endothelial cells.[31,63] However, the major source of plasma adiponectin in adults is adipocytes.

The protein contains a signal sequence and a collagen-repeat domain at the N terminus, and a C1q-like globular domain at the C terminus.[64] Adiponectin is present in a wide range of multimer complexes in plasma and assembles via its collagen domain into adiponectin trimers (low molecular weight), hexamers (middle molecular weight), and 12- to 18-mers [high molecular weight (HMW)].[64–66] The HMW forms appear to be responsible for insulin sensitivity and the anti-inflammatory effects of adiponectin.[65,67] Hydroxylation and glycosylation of the lysine residues within the collagen domain are critically involved in the regulation of HMW adiponectin formation.[68] The full-length adiponectin protein is proteolytically cleaved, with a smaller form including the globular domain, although in very small amounts.[69]

Adiponectin protein is present at high levels (range, 3–30 μg/ml) in plasma, accounting for about 0.01% of total plasma protein. Surprisingly, the plasma adiponectin level is inversely correlated with BMI in spite of its restricted expression in adipose tissue.[66] Especially, the plasma adiponectin level is low in subjects with visceral fat accumulation. Hypoadiponectinemia has been demonstrated to be independently associated with metabolic syndrome, including type 2 diabetes,[70] hypertension,[71] atherosclerosis,[72] and NASH.[25] Weight reduction results in a significant elevation of plasma adiponectin levels in humans.[70]

Why are plasma adiponectin levels low in obese subjects? While the exact mechanism is unknown, several theories have been postulated. TNF-α, one of the insulin resistance inducible factors, is upregulated in obese subjects, and suppresses the expression and plasma levels of adiponectin at the transcriptional level.[72] Production of reactive oxygen species (ROS) is selectively increased in adipose tissue of obese mice, accompanied by augmented expression of NADPH oxidase. Production of adiponectin is downregulated by elevated oxidative stress in adipose tissue. NADPH oxidase inhibitor reduces ROS production and increases adiponectin production in adipose tissue.[73] The frequency of a missense mutation at position 164 in the globular domain [Ile-164The (I164T)] is significantly higher in patients with type 2 diabetes and coronary artery disease than in normal control subjects.[74,75] Subjects with this mutation had significantly lower plasma adiponectin levels than those without it.

Adiponectin receptor

Two receptors for adiponectin, AdipoR1 and AdipoR2, have been cloned.[76] These adiponectin receptors are considered to contain seven transmembrane domains, despite being structurally and functionally distinct from G protein-coupled receptors. AdipoR1 is ubiquitously

expressed and is abundantly expressed in skeletal muscle, whereas AdipoR2 is most abundantly expressed in the liver. AdipoR1 and AdipoR2 serve as receptors for globular and full-length adiponectin and activate adenosine monophosphate-activated protein kinase (AMPK), peroxisome proliferator-activated receptor-α (PPAR-α), and p38 MAPK signaling pathways.[77] Disruption of these receptors abolished adiponectin binding and its actions.[78] Insulin reduces the expression of AdipoR1 and AdipoR2 via a PI3K/Forkhead boxO1-dependent pathway in cultured hepatocytes or myocytes.[28,79]

T-cadherin serves as a receptor for the hexameric and HMW forms of adiponectin.[80] Because T-cadherin is a glycosylphosphatidylinositol-anchored extracellular protein, it may act as a coreceptor for an unidentified signaling receptor.

Adiponectin and NAFLD

Obesity is an independent risk factor in the development of NASH[21–23] and HCC,[19,34] and patients with NASH who progress to liver cirrhosis are at increased risk of HCC.[81] In the two-hit theory of NASH pathogenesis, the first hit of hepatic steatosis is followed by the second hit of oxidative injury, leading to inflammation and progression to fibrosis and HCC.[81,82] In the following paragraphs, we focus on the roles of adiponectin in the two-hit theory of NASH pathogenesis.

Adiponectin and hepatic steatosis

We found that hepatic steatosis, induced by a choline-deficient L-amino acid-defined (CDAA) diet, is more severe in adiponectin knockout mice than in wild-type mice.[83] The CDAA diet was used to induce a nutritional animal model of NASH.[84] Overexpression of adiponectin protein by adenovirus vector resulted in attenuation of hepatic steatosis. The lack of adiponectin in these mice enhanced the expression of two rate-limiting enzymes in fatty acid synthesis, acetyl-CoA carboxylase (ACC) and fatty acid synthase. Adiponectin is also known to stimulate mitochondrial β-oxidation by activation of AMPK and PPAR-α.[65,69] Activated PPAR-α upregulates carnitine palmitoyltransferase (CPT)-1, a rate-limiting enzyme in fatty acid oxidation. In addition, activated AMPK phosphorylates ACC and attenuates the activity of ACC. Inactivation of ACC leads to a decrease in the concentration of its product, malonyl-CoA (a potent inhibitor of CPT-1), and induces fatty acid oxidation in the liver. Moreover, adiponectin downregulates SREBP-1c, a master regulator of fatty acid synthesis.[85] Thus, adiponectin increases β-oxidation of free fatty acids and decreases de novo free fatty acids production within hepatocytes.[69,86] These effects protect hepatocytes against triglyceride accumulation. The hypoadiponectinemia in obese individuals could exacerbate hepatic steatosis, the first hit in NASH, through the absence of these effects of adiponectin.

Adiponectin and inflammation

Adiponectin at physiological concentrations attenuates the attachment of monocytes to endothelial cells by reducing TNF-α-induced expression of adhesion molecules such as vascular cell adhesion molecule 1, endothelial-leukocyte adhesion molecule-1 (E-selectin), and intercellular cell adhesion molecule 1.[72] Nuclear transcription factor, nuclear factor κB (NFκB), induces the expression of cytokines and adhesion molecules in the inflammatory process. Adiponectin suppresses TNF-α-induced NFκB activation and blocks TNF-α release in endothelial cells.[87]

C-reactive protein (CRP) is a potent marker of systemic inflammation, and its plasma level correlates negatively and significantly with adiponectin levels in humans.[88,89] CRP levels correlate with body weight and percentage of body fat,[90] and CRP mRNA is expressed in human adipose tissue and its levels correlate inversely with adiponectin gene expression in human adipose tissue.[88]

Patients with NASH are at increased risk of small intestinal bacterial overgrowth,[91] and lipopolysaccharide (LPS) is involved in the pathogenesis of NASH.[92,93] In a mouse model of LPS-induced acute hepatitis, we found that adiponectin protected against hepatic injury through inhibition of production of the proinflammatory cytokine TNF-α and induction of the anti-inflammatory cytokine IL-10 in Kupffer cells.[94] Lack of adiponectin accelerated LPS-induced liver injury, and the survival rate of adiponectin knockout mice after LPS administration was significantly lower than that of wild-type mice. Pretreatment of these mice with adiponectin reduced LPS-induced TNF-α production, and increased IL-10 production by Kupffer cells. These findings are in agreement with another study in which pretreatment of KK-Ay obese mice with adiponectin protected them from LPS-induced hepatic injury through modulation of TNF-α.[95] Other investigators have shown that adiponectin also suppresses macrophage function[96] and induces anti-inflammatory cytokines, such as IL-10 and IL-1RA, in human leukocytes.[97,98] In addition, adiponectin alleviates experimental T-cell-mediated hepatitis induced by concanavalin A in mice, and protects primary hepatocytes from TNF-α-induced death.[99] These results suggest that hypoadiponectinemia in obese subjects can thus lead to enhanced sensitivity of Kupffer cells to proinflammatory mediators such as LPS.

A recent study reported that adiponectin promotes clearance of early apoptotic cells by macrophages through a receptor-dependent pathway involving calre-

ticulin.[100] This novel function of adiponectin is similar to that of surfactant proteins and C1q, which serve as anti-inflammatory molecules by promoting the clearance of apoptotic cell debris.[101]

Adiponectin and oxidative stress

Obesity is regarded as a chronic inflammatory state. The associated hepatic lipid overload induces impairment of mitochondrial β oxidation, which may lead to the formation of ROS and lipid peroxidation products. ROS and lipid peroxidation in turn upregulate a series of proinflammatory cytokines,[102] causing further mitochondrial dysfunction and oxidative stress, thus contributing to the progression of liver injury.

Recent studies indicate that adiponectin can suppress oxidative stress,[103,104] and that systemic oxidative stress, as measured by urinary 8-epi-prostaglandin F2α, correlates strongly with hypoadiponectinemia.[105] Studies from our laboratories showed enhanced oxidative stress in adiponectin knockout mice in a CDAA diet-induced NASH mouse model.[83] Accumulating evidence suggests that alcohol-mediated upregulation of CYP2E1 may initiate lipid peroxidation via production of ROS.[106] CYP2E1 is upregulated in human liver in NASH,[107] and in a rodent model of NASH.[108,109] In our CDAA diet-induced NASH mouse model, CYP2E1 was induced in adiponectin knockout mice and adiponectin overexpression downregulated CYP2E1.[83] Moreover, thiobarbituric acid reactive substance, a marker of oxidative stress, and 8-hydroxydeoxyguanosine, a marker of oxidative DNA damage, were also increased in the livers of adiponectin knockout mice. Adiponectin knockout mice also showed significantly enhanced hepatic tumor formation compared with wild-type mice. Thus, adiponectin deficiency might enhance the level of oxidative stress through induction of CYP2E1 in the liver, allowing progression of liver injury in adiponectin-deficient mice.

TNF-α plays crucial roles in NASH progression,[110,111] and adiponectin suppresses TNF-α production.[96] In our study, we found significantly elevated serum levels of TNF-α in adiponectin knockout mice,[83] which may play a role in the progression of hepatopathology in adiponectin knockout mouse liver.

Adiponectin and fibrosis

In a clinical study of NASH patients, the fibrosis stage correlated significantly with low serum adiponectin levels.[29] We reported previously that adiponectin attenuates carbon tetrachloride-induced liver fibrosis.[112] Adiponectin suppressed the proliferation and migration of activated HSCs, which play central roles in liver fibrosis, and attenuated the effect of TGF-β1 on the expression of fibrogenic genes, and on nuclear translocation of Smad2 in HSCs. Other groups have reported that adiponectin induces apoptosis of activated HSCs,[113] and activated AMPK, which modulates the activated HSC phenotype.[114,115] These findings indicate that adiponectin has antifibrogenic properties in liver diseases through the suppression of activated HSC proliferation and fibrogenic function.

Inflammation and fibrosis of the liver have recently been reported in individuals with nonalcoholic fatty liver, which is frequently associated with obesity and type-2 diabetes. A considerable number of these patients develop liver cirrhosis, a clinical entity termed NASH. In addition, epidemiological studies have shown that obesity, which is associated with hypoadiponectinemia, is a risk factor for the development of liver fibrosis in patients with NASH, alcoholic liver disease, and CHC.[22–33] These results indicate that hypoadiponectinemia may be one reason obese patients are at high risk for development of liver cirrhosis.

Thus, adiponectin attenuates inflammation, oxidative stress, and proinflammatory cytokine production, which are considered the second hit in NASH. Moreover, adiponectin ameliorates liver fibrosis via suppression of activated HSC function, and might decelerate the progression of hepatocarcinogenesis via suppression of oxidative stress (Fig. 2).

Adiponectin and clinical studies in liver diseases

Adiponectin and NAFLD

In a study of 80 NASH patients, hypoadiponectinemia was independently associated with NASH and with more severe hepatic steatosis and necroinflammation.[25] Other reports have also indicated that plasma adiponectin levels are lower in NASH patients than in patients with simple steatosis.[26,27] Interestingly, the major hepatic adiponectin receptor AdipoR2 is underexpressed in fatty liver and in NASH patients, and *AdipoR2* gene expression correlates inversely with the severity of liver fibrosis.[27,28] After adjustment for age, sex, and BMI, plasma levels of adiponectin correlated inversely with the alanine transaminase level.[86] Musso et al.[29] reported that hypoadiponectinemia is a feature of NASH and may play a pathogenetic role in hepatic necroinflammation and fibrosis, independent of insulin resistance, visceral fat accumulation, serum TNF-α level, or dietary intake. In contrast, another study demonstrated the presence of hypoadiponectinemia in NAFLD patients but failed to find differences in the serum adiponectin concentration between patients with NASH and those with simple steatosis, and concluded that adiponectin concentration correlated inversely with insulin resistance.[116] Considered together, these results suggest that hypoadiponectinemia and underexpression of hepatic adiponectin receptor may play important roles in the clinical progression of NASH.

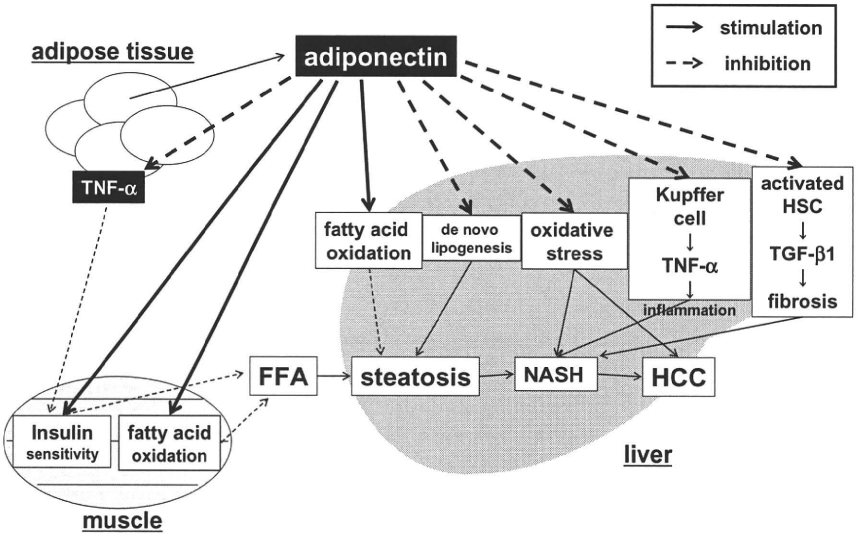

Fig. 2. Roles of adiponectin in NASH development. *Solid arrows*, stimulatory effects; *dotted arrows*, inhibitory effects. *NASH*, nonalcoholic steatohepatitis; *FFA*, free fatty acid; *HCC*, hepatocellular carcinoma; *HSC*, hepatic stellate cell; *TGF*-β1, transforming growth factor β1

Adiponectin and viral hepatitis

Currently, there is a great interest in the role of adiponectin in CHC. Serum adiponectin levels are higher in CHC patients than in those with chronic hepatitis B.[117] In CHC patients, hypoadiponectinemia correlates significantly with steatosis but not with the severity of fibrosis.[31–33] Hepatic steatosis is a common histological finding in CHC, with an incidence of 305–70%.[118,119] Hepatic steatosis in CHC correlates with progression of liver fibrosis,[17,120] and is a risk factor for HCC.[121] In addition, a recent report indicated that low serum adiponectin is an independent predictor of nonvirological response to interferon therapy in CHC patients.[33]

On the other hand, hyperadiponectinemia has been described in cirrhosis patients.[122,123] The liver is the main organ of adiponectin metabolism, and biliary secretion is involved in adiponectin clearance; accordingly, elevated plasma concentrations of adiponectin in advanced cirrhosis are due to decreased metabolism and biliary secretion of adiponectin.

Adiponectin and cancer

Recent studies showed that plasma adiponectin levels are inversely correlated with the risk of cancers.[124] In clinical studies, hypoadiponectinemia is correlated with colorectal cancer,[125,126] gastric cancer,[127] prostate cancer,[128] endometrial cancer,[129] and breast cancer.[130] In addition, in vitro studies revealed that adiponectin protein inhibits cell proliferation in cells lines originating from various types of cancer, including prostate cancer, HCC, breast cancer, leukemia, and esophageal cancer.[96,131–133] These studies emphasize the potential role of hypoadiponectinemia as a risk factor for various cancers.

Pharmacological and dietary interventions

The above findings suggest that hypoadiponectinemia in obese people may be an important risk factor for clinical progression of chronic liver diseases, and that upregulation of adiponectin signaling might be useful therapeutically by increasing plasma adiponectin levels or development of adiponectin receptor agonists. However, considering the high plasma levels of adiponectin, direct administration of adiponectin protein to individuals with liver diseases might not be a good strategy because of difficulties in maintaining high plasma concentrations. Thiazolidinediones elevate the promoter activity of adiponectin and increase the plasma concentration of adiponectin.[134] Adiponectin promoter has a functional PPAR-responsive element (PPRE) site.[135] Not only PPARγ but also PPARα ligands increase the expression of adiponectin through a PPRE site located in its promoter region.[136] Furthermore, PPARα agonist increases the expression of both AdipoR1 and AdipoR2 in adipocytes and macrophages.[137] Pioglitazone increases the ratio of HMW adiponectin/total adiponectin and increases the hepatic sensitivity to insulin.[138] These findings indicate that dual activation of PPARγ and PPARα intensifies adiponectin actions by increasing plasma levels of adiponectin, especially HMW adiponectin, and by increasing adiponectin receptors.

Blockade of the renin–angiotensin system (RAS) with angiotensin-converting enzyme inhibitors or angiotensin receptor blocker increases adiponectin concentrations.[139,140] However, the precise mechanism of increased plasma adiponectin level by RAS blockade remains incompletely unclear.

The effects of diet on plasma adiponectin concentration were recently reported. Dietary soy protein, lin-

oleic acid, and oolong tea increase plasma adiponectin levels in rodents and humans.[141–143] The molecular mechanism of these dietary effects on adiponectin levels remains unclear.

A recent study suggested that osmotin, a member of the PR-5 family of plant defense proteins, is a ligand for the yeast homolog of adiponectin receptor and has functional similarity to adiponectin.[144] Osmotin is abundant in plant tissues (seeds, fruits, vegetables) and is extremely stable; it remains active even when in contact with human digestive or respiratory systems.[144] Further research into similarities in adiponectin and osmotin functions may facilitate the development of potential adiponectin receptor agonists.

Collectively, in addition to weight reduction, the aforementioned pharmacological and dietary interventions can improve hypoadiponectinemia, and might be useful therapeutic approaches to attenuate metabolic syndrome.

TNF-α

TNF-α is a proinflammatory cytokine that was originally found to induce necrosis of tumors after acute bacterial infection. Its first link to obesity, insulin resistance, and chronic inflammation was made in a research paper that described significant elevation of TNF-α in adipose tissue of genetically obese mice (*db/db* mice).[145]

The adipose tissue of obese individuals is characterized by increased infiltration of macrophages and hypertrophied adipocytes.[10] Hypertrophied adipocytes release large quantities of free fatty acid (FFA) via macrophage-induced adipocyte lipolysis. FFA serves as a naturally occurring ligand for Toll-like receptor (TLR) 4.[146,147] FFA increases thereby the production of TNF-α in macrophages through the TLR4/ NFκB pathway. Thus, a vicious cycle is established.

Kupffer cells are the main producer of TNF-α in the liver, and LPS-induced activation of these cells enhances their production of TNF-α. In animal models, activation of Kupffer cells leads to induction of the TNF-α/TNF receptor signaling pathway, which is critically involved in the pathogenesis of liver fibrosis in NASH.[110] In addition, *ob/ob* mice, a model for NAFLD, overexpress TNF-α.[92] Treatment with anti-TNF antibody reduced the activity of Jun N-terminal kinase, which promotes insulin resistance, and decreased the DNA binding activity of NFκB, which accelerates inflammation, with a resultant improvement of NAFLD in *ob/ob* mice.

In human, TNF-α levels are increased significantly in simple steatosis and NASH, and correlate with hepatic fibrosis in NASH.[26] The gene expression of TNF-α and its receptor are significantly elevated in hepatic and adipose tissues of NASH patients.[21] Recently, a histologic scoring system, the NAFLD activity score (NAS), has been proposed that can assist in the diagnosis of NAFLD and may be useful for assessing the response to therapy.[148] Serum TNF-α levels significantly correlated with NAS score.[149] In Japanese NAFLD patients, polymorphisms in the TNF-α promoter region and serum level of soluble TNF receptor 2 significantly correlated with progression of NAFLD.[150] Moreover, administration of pentoxifylline, a TNF-α inhibitor, improved aminotransferase levels and the insulin resistance index assessed by homeostatic metabolic assessment (HOMA-IR) in NASH patients.[151,152]

Considered collectively, the above data from animal and human studies suggest that TNF-α plays important roles in the progression of NAFLD, including hepatic inflammation and fibrogenesis.

Resistin

Resistin is a member of the resistin-like molecule family of cysteine-rich secretory 12-kDa proteins. In mice, the expression of resistin is restricted to adipose tissue, and the expression of resistin is downregulated by thiazolidinedione and fasting.[6] Resistin leads to insulin resistance, and hyperresistinemia increases blood glucose and insulin levels in mice.[153] Resistin overexpression induces dyslipidemia characterized by high serum total cholesterol and triglyceride levels, and reduces high-density lipoprotein cholesterol concentration, which is commonly seen in metabolic syndrome.[154]

In humans, resistin expression in adipose tissue is very low, but it is mainly found in bone marrow and in macrophages.[155,156] Serum resistin concentrations are elevated in patients with NAFLD. Increased resistin levels correlate with histological severity of liver disease but not with insulin resistance.[24] Serum resistin levels are higher in obese than in lean individuals, but when adjusted for BMI, resistin levels do not correlate with insulin resistance.[157] The exact role of resistin in obesity and insulin resistance in humans remains elusive. More research is needed to clarify the role of resistin in humans.

Conclusions

We have summarized the recent advances in our understanding of the role of adipocytokines in liver diseases. Adipocytes produce and secrete various adipocytokines to control the functions of other organs, including liver. Production and secretion of adipocytokines are dynamically regulated by nutritional status. Overeating and physical inactivity results in obesity with visceral fat

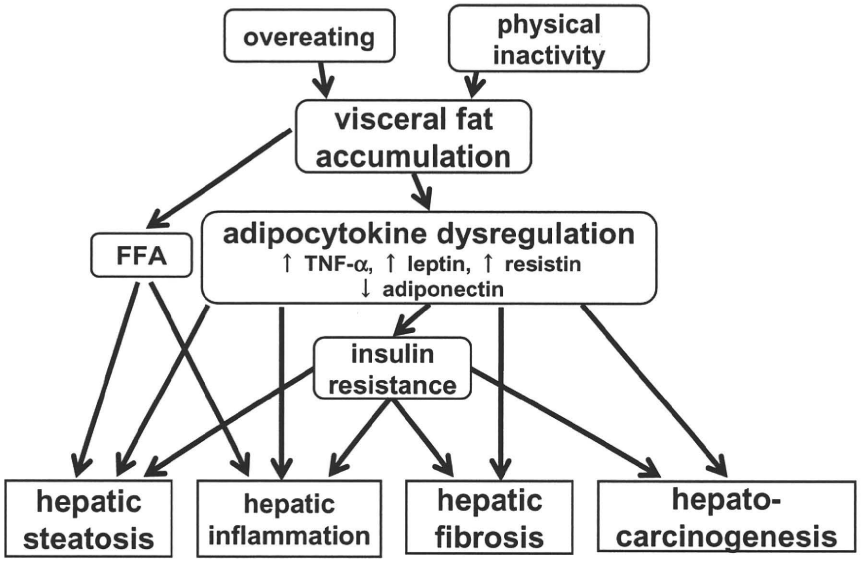

Fig. 3. Current hypothesis regarding the association between adipocytokines and liver diseases. Arrows, stimulatory effects

accumulation, a state of chronic low-grade inflammation. The inflammatory changes in obese adipose tissue induce adipocytokine dysregulation: an increase in offensive adipocytokines, TNF-α, IL-6, and resistin, and a decrease in the defensive adipocytokine adiponectin. Increased serum levels of TNF-α, resistin, and leptin, which are usually observed in obese subjects, may enhance steatosis, inflammation, fibrogenesis, or hepatocarcinogenesis in the liver. In addition, hypoadiponectinemia seems to enhance hepatic steatosis, inflammation, and fibrosis, as well as hepatocarcinogenesis (Fig. 3). Attenuation of proinflammatory adipocytokines or augmentation of the function of adiponectin might be an effective therapy for metabolic syndrome. Further clinical and experimental research should elucidate the relationship between adipocytokines and liver diseases.

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