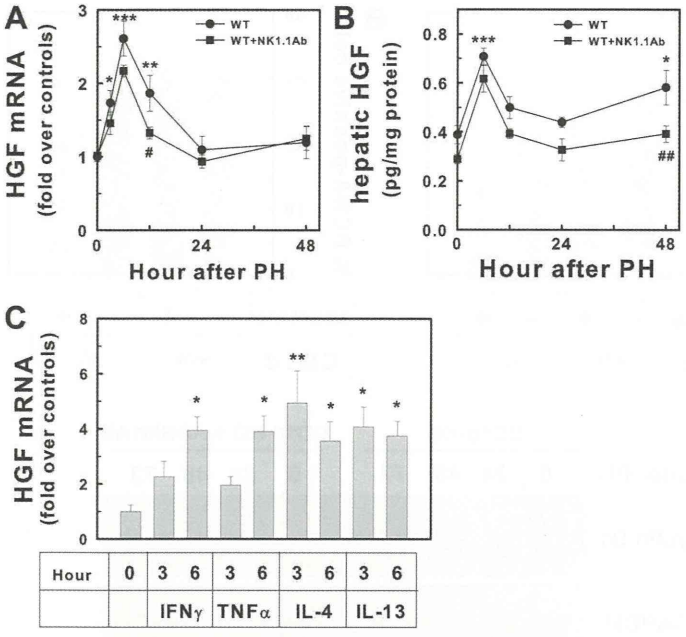

Fig. 5. Expression of hepatocyte growth factor (HGF) following PH in mice pretreated with an anti-NK1.1 antibody. Steady-state mRNA and protein levels for HGF in the liver were measured by real-time RT-PCR and ELISA, respectively. Average values of HGF mRNA levels (*A*) and HGF protein levels (*B*) in WT (●) and anti-NK1.1 antibody-treated WT (■) are plotted ($n = 5$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. WT before PH; $\#P < 0.05$; $\#\#P < 0.01$ vs. WT at the same time point). Hepatic stellate cell-T6 cells were incubated with IFN-γ, TNF-α, IL-4, or IL-13 (10 ng/ml each) for 3–6 h, and mRNA levels for HGF were quantified using real-time RT-PCR. Average mRNA levels from 5 separate dishes are plotted (*C*; $^{*}P < 0.05$; $^{**}P < 0.01$ vs. controls).

NK1.1 antibody blunts cytokines/growth factors triggering regeneration and hepatocyte proliferation.

Pretreatment with the antiasialo GM1 antibody to CD1d-KO mice impairs liver regeneration. To confirm whether depletion of both NK and NKT cells causes impaired regeneration, we used CD1d-KO mice, which lack CD1d-restricted NKT cells systemically, in combination with antiasialo GM1 antibody. Antiasialo GM1 antibody is well known to deplete NK cells specifically (13), and depletion of NK cells using this antibody has been shown to enhance the regenerating process following PH (28). Here, we applied this antibody to both WT and CD1d-KO mice, 24 h prior to PH, and observed the regenerating process (Fig. 6). WT mice given an antiasialo GM1 antibody showed almost normal uptake of BrdU and PCNA expression in hepatocytes, 48 h after PH. Similarly, CD1d-KO mice, which lack NKT cells, also demonstrated normal BrdU uptake and PCNA expression. In sharp contrast, CD1d-KO mice pretreated with an antiasialo GM1 antibody showed significant decreases in both BrdU uptake and PCNA expression, 48 h after PH (Fig. 6, *A* and *B*). Furthermore, hepatic expression of cyclin D1 was largely blunted in CD1d-KO given an antiasialo GM1 antibody (Fig. 6, *C* and *D*), the pattern being quite similar to WT mice pretreated with an anti-NK1.1 antibody (Fig. 2*A*). Collectively, depletion of NK and NKT cells by two different approaches resulted in impaired liver regeneration after PH, supporting the hypothesis that NK and NKT cells cooperatively promote normal regenerative responses in the liver.

DISCUSSION

Here, we demonstrated that depletion of both NK and NKT cells by an anti-NK1.1 antibody impairs liver regeneration following PH (Figs. 1 and 2). The mechanisms underlying this phenomenon appear to be the downregulation of regeneration-triggering cytokine responses involving TNF-α, IL-6, and the JAK-STAT signaling pathway and induction of HGF following PH (Figs. 3–5). Moreover, pretreatment with the antiasialo GM1 antibody to CD1d-KO mice, which results in the depletion of both NK and NKT cells, also exhibited poor regeneration after PH (Fig. 6). These observations obviously excluded a possibility of an antibody-specific artifact, confirming the fact that liver regeneration is indeed impaired through depletion of these two innate immune cells. Given the previous findings that depletion of NK cells enhances liver regeneration (28), our findings are quite striking because NK cell depletion in the absence of NKT cells paradoxically inhibits regenerative responses after PH.

The downregulation in TNF-α and IL-6 in anti-NK1.1 antibody-pretreated mice after PH (Fig. 3, *C–E*) suggested that NK and NKT cells participate in the production of these cytokines in corporation with Kupffer cells and other cytokine-producing cells. Furthermore, pretreatment with the anti-NK1.1 antibody almost completely abolished induction of IL-4 following PH (Fig. 3*B*). Since IL-4 has been demonstrated to control IL-6 production following PH, in cooperation with the complement system (4), abrogation of IL-4 explains, in part, the mechanism of retarded hepatic regeneration caused by anti-NK1.1 antibody. Another possibility is that depletion of NK and NKT cells alters the immune microenvironment, thus preventing activation of Kupffer cells in the early stage of regeneration. Anyway, blunting expression of TNF-α, which triggers regenerative responses following PH, seems to be quite important in the mechanisms of impaired regeneration in the absence of hepatic NK and NKT cells. Indeed, we observed the blunting induction of HGF following PH in anti-NK1.1 antibody-treated mice (Fig. 5, *A* and *B*), where induction of HGF by NKT cell-derived cytokines in HSCs most likely plays a role (Fig. 5*C*).

Here, in this study, mice pretreated with an anti-NK1.1 antibody showed blunted induction of IL-6 (Fig. 3, *D* and *E*) and subsequent activation of the JAK-STAT pathway (Fig. 4), clearly indicating that downregulation of this signaling pathway causes poor regenerative responses following PH. These findings are coincident with the lines of evidence that these factors promote liver regeneration through investigations of KO animals (29). In terms of IL-6 and the JAK-STAT pathways, however, there are some controversial observations; prolonged, enhanced activation of STAT3 reciprocally inhibits regenerative responses (15, 18, 30). Indeed, recently, we reported that KK-A^{y} mice, which spontaneously develop steatohepatitis with metabolic syndrome-like phenotypes, showed poor liver regeneration following PH, where augmented activation of STAT3 with a delayed peak was observed (2). This phenomenon is most likely due to tremendous overexpression of IL-6 and leptin, which share the same JAK-STAT signaling. Interestingly, KK-A^{y} mice also demonstrated depletion of hepatic NKT cells; however, the mechanism underlying the impaired regeneration seems to be different from the observations in this study, especially with respect to IL-6 and the

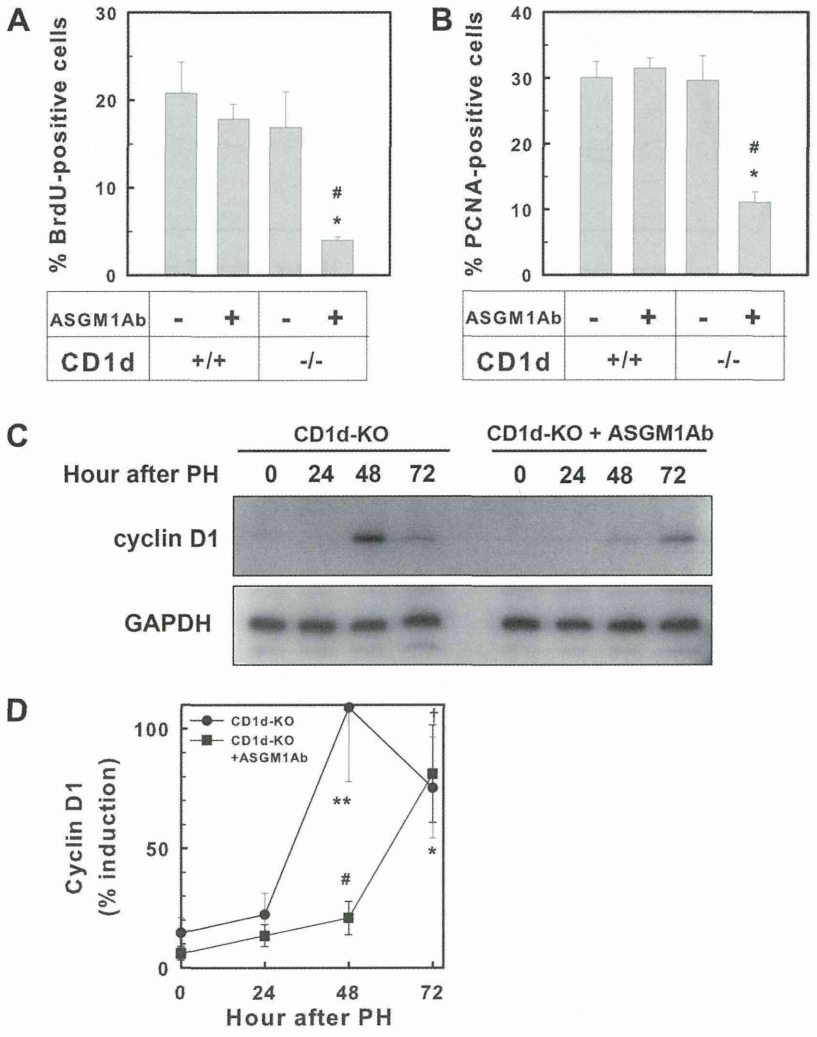

Fig. 6. Anti-asialo ganglio-*N*-tetraosylceramide (GM1) antibody (ASGM1Ab) impairs liver regeneration following PH in CD1d-knockout (KO) mice. WT and CD1d-KO mice were given a single injection of ASGM1Ab (200 μg/body), 24 h before PH. The average percentages of BrdU-positive hepatocytes (*A*) and PCNA-positive hepatocytes (*B*), 48 h after PH, are plotted ($n = 5$; $^*P < 0.05$ vs. WT without ASGM1Ab; $\#P < 0.05$ vs. CD1d-KO without ASGM1Ab). Hepatic expression of cyclin D1 was detected by Western blotting (*C*). Densitometrical data (*D*) for CD1d-KO (●) and CD1d-KO pretreated with ASGM1Ab (■) are shown ($n = 5$; $^*P < 0.05$; $^{**}P < 0.01$ vs. CD1d-KO before PH; $\dagger P < 0.05$ vs. ASGM1Ab-treated CD1d-KO before PH; $\#P < 0.05$ vs. WT at the same time point).

JAK-STAT pathway. Nonetheless, NK and NKT cells most likely play a pivotal role in regulation of IL-6 and the JAK-STAT pathway, thereby modulating regenerating responses in the liver.

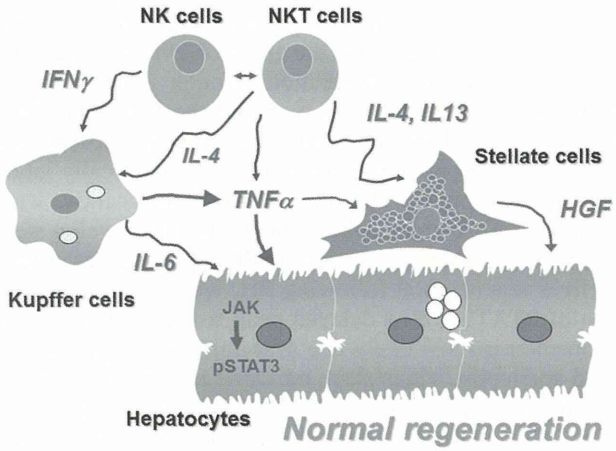

Fig. 7. Working hypothesis: natural killer (NK) and NKT cells contribute to the normal regenerative responses in the liver.

In conclusion, our findings in the present study clearly indicated that depletion of both NKT and NK cells by two different ways results in impaired liver regeneration. The role of NK cells in hepatic regeneration appears to be paradoxical in the presence or absence of NKT cells, and this phenomenon cannot be explained simply by the secretion of IFN-γ. Rather, these two innate immune cells most likely upregulate TNF-α, IL-6, and the JAK-STAT pathway and HGF in a coordinate fashion, thus promoting normal regenerative responses in the liver (Fig. 7).

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DISCLOSURES

All authors have no conflict of interest in terms of this study.

AUTHOR CONTRIBUTIONS

Author contributions: K.I. conception and design of research; S.H., K.A., S.I., H.Y., and T.A. performed experiments; S.H., K.I., K.K., and S.Y. analyzed data; S.H., K.I., K.T., K.K., and S.Y. interpreted results of experiments; S.H. and K.I. prepared figures; S.H. and K.I. drafted manuscript; K.I. and S.W. edited and revised manuscript; S.H., K.I., K.T., K.A., S.I., H.Y., T.A., K.K., S.Y., and S.W. approved final version of manuscript.

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Original Article

Abnormality of autophagic function and cathepsin expression in the liver from patients with non-alcoholic fatty liver disease

Yuka Fukuo,[1] Shunhei Yamashina,[1] Hiroshi Sonoue,[2] Atsushi Arakawa,[2] Eisuke Nakadera,[1] Tomonori Aoyama,[1] Akira Uchiyama,[1] Kazuyoshi Kon,[1] Kenichi Ikejima[1] and Sumio Watanabe[1]

Departments of [1]Gastroenterology and [2]Human Pathology, Juntendo University School of Medicine, Tokyo, Japan

Aim: Recent evidences indicate that hepatic steatosis suppresses autophagic proteolysis. The present study evaluated the correlation between autophagic function and cathepsin expression in the liver from patients with non-alcoholic fatty liver disease (NAFLD).

Methods: Liver biopsy specimens were obtained from patients with chronic liver diseases (chronic hepatitis C [CHC; $n = 20$], chronic hepatitis B [CHB; $n = 16$], primary biliary cirrhosis [PBC; $n = 23$], NAFLD [$n = 22$] and control [$n = 14$]). The number of autophagic vesicles in hepatocytes was counted by using transmission electron microscopy. Expression of cathepsin B, D, L and p62 in the liver section was analyzed by immunohistochemical staining. The histological severity of NAFLD is assessed by NAFLD activity score (NAS).

Results: The number of autophagic vesicles in hepatocytes was significantly increased in both CHC and NAFLD groups, but not CHB and PBC, more than control. Although hepatocytes with aggregation of p62 were observed in less than 15% of CHC, p62 aggregation was detected in approximately 65% of NAFLD. Cathepsin B, D and L expression was significantly suppressed in the liver from NAFLD patients. Suppression of cathepsin B, D and L expression was not observed in CHB, CHC and PBC. In NAFLD patients, p62 aggregation was correlated with serum alanine aminotransferase value and inflammatory activity by NAS.

Conclusion: These results indicate that a decrease in hepatic cathepsin expression in NAFLD is associated with autophagic dysfunction. Hepatic inflammation correlates with autophagic dysfunction in NAFLD. These findings indicate that the suppression of autophagic proteolysis by hepatic steatosis is involved in the pathogenesis of NAFLD.

Key words: autophagy, inflammation, p62, steatosis

INTRODUCTION

NON-ALCOHOLIC FATTY LIVER disease (NAFLD) is associated with the metabolic disorders such as abdominal obesity and insulin resistance. Approximately 10–20% of NAFLD progresses from relatively benign simple steatosis to non-alcoholic steatohepatitis (NASH) which can progress to cirrhosis and hepatocellular carcinoma.[1,2] The "two hit theory" is widely accepted to explain the pathogenesis of NAFLD and NASH.[3] In this theory, the first hit consists of the accumulation of fatty acids/triglycerides (TG) in the liver, while the second hit involves oxidative stress, mitochondrial dysfunction and inflammation, which ultimately cause liver damage. The formation of hepatic inclusion of Mallory–Denk bodies is one of the histological features of NASH. It was shown that autophagic deficiency causes cellular inclusion in experiments using *Atg7* conditional knockout mice.[4] Moreover, it was reported that autophagy and lipid metabolism are interrelated and autophagic dysfunction in obese mice enhances endoplasmic reticulum (ER) stress and causes hepatic insulin resistance.[5,6] These findings suggest that autophagic dysfunction plays a pivotal role in the progression of NAFLD.

Macroautophagy, which is a catabolic process, degrades long-lived proteins and cellular organelles such as mitochondria and ER for an alternative energy source during nutrient deprivation.[7,8] Induction of

Correspondence: Dr Shunhei Yamashina, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan. Email: syamashi@juntendo.ac.jp
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autophagy has been shown to be regulated by mammalian target of rapamycin (mTOR).[9,10] During starvation, withdrawal of growth factor or an inhibition of mTOR rapidly leads to formation of a small vesicle called the isolation membrane. The isolation membrane elongates and subsequently encloses cytoplasmic proteins, which results in the formation of a double-membrane structure known as autophagosomes.[11,12] Autophagosomes are fused with lysosomes to degrade the enclosed material by taking advantage of the lysosomal proteinases such as the cystein proteinase cathepsin.[13] Importantly, autophagy is essential for maintaining cellular homeostasis, generating intracellular nutrient and removing the misfolded proteins and damaged cellular organelles.

p62/SQSTM1 is a stress-inducible intracellular protein that is involved in cell survival and death signaling. Recently, p62 has been identified as a component of inclusion bodies in various liver diseases such as hepatocellular carcinoma, alcoholic liver disease and non-alcoholic steatohepatitis. Moreover, it was recognized that p62 is one of the selective substrates for autophagy. This protein is recruited into the autophagosome and directly interacts with autophagosome membrane protein LC3. Subsequently, p62 is incorporated into the autophagosome and degraded.[4] Thus, impaired autophagy causes accumulation of p62. Recent evidence disclosed data that the accumulation of p62 due to autophagy deficiency plays a pivotal role in hepatoma development via persistent activation of Nrf2.[14,15]

Hepatic autophagy can contribute to the development of not only NAFLD but also various liver diseases.[11] Hepatitis C virus (HCV) and hepatitis B virus (HBV) utilize autophagy for their replication and entry into cells.[16–19] Previous experiments revealed that the infection of HCV accelerates the rate of autophagy.[19] Furthermore, it was disclosed that suppression of autophagy occurs during the replication of both HBV and HCV.[17,20] The pathogenesis of progressive bile duct loss in primary biliary cirrhosis (PBC) is likely to connect to biliary epithelial senescence by autophagic abnormality.[21]

In this study, we evaluated that autophagic function in NAFLD patients in comparison with other chronic liver diseases and identified the clinical features influencing the autophagic function in the liver.

METHODS

Patients and clinical assessment

INCLUDED IN THIS study were samples from 22 NAFLD, 23 PBC, 16 chronic hepatitis B (CHB) patients and 20 with chronic hepatitis C (CHC) patients who underwent liver biopsy between 2007 and 2012 at Juntendo University. Patients with liver cirrhosis diagnosed by ultrasonographic examination and liver histological evaluation were excluded. Fourteen patients with liver metastatic tumors without markers of infection for HBV or HCV or for HIV were used as controls. Liver samples of normal tissue excised from areas surrounding tumor tissue were collected from a resected hepatic lobe with a metastatic tumor. All patients gave their informed consent before liver biopsy.

A part of liver tissue specimens from patients with chronic liver diseases were fixed for histological evaluation, immunohistochemical staining and electron microscopic analysis. The clinical data collected from patients at liver biopsy is as follows: sex, age, blood platelet level (Plt), serum levels of alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (γ-GTP), total cholesterol (T-cho), TG and glucose (Glu). This study was approved by the ethics committee at Juntendo University School of Medicine, and full written informed consent was obtained from each patient undergoing any procedure. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee.

Histological analysis

Tissue specimens from all patients and controls were fixed in formalin-fixed paraffin-embedded tissue. Sections, 5 μm thick, were cut from paraffin blocks and stained with hematoxylin–eosin and Azan–Mallory staining for histological evaluation. Liver biopsy specimens were analyzed by two experienced pathologists who were blinded to the clinical and laboratory data. A diagnosis of NAFLD/NASH and histological parameters were scored on a graded scale according to NASH Clinical Research Network Scoring System. Steatosis (0–3), lobular inflammation (0–3) and hepatocyte ballooning (0–2) were scored to evaluate severity of NAFLD using NAFLD activity score (NAS). Fibrotic stage was scored according to NAS: stage 1, zone 3 perisinusoidal only (1a, 1b) or portal/periportal only (1c); stage 2, zone 3 perisinusoidal and portal fibrosis; stage 3, bridging fibrosis; and stage 4, cirrhosis.

Electron microscopic analysis

A portion of each liver specimen from patients in the study population was immersed in fixative containing 3% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4, at 4°C for 24 h, and then post-fixed in 1% osmium tetroxide in 0.1 M PBS for 2 h. After a brief

wash with PBS, tissues were dehydrated with alcohol and embedded in EPON Resin 813(Momentive Specialty Chemicals, Columbus, OH, USA). After staining with lead citrate and uranium acetate, ultrathin sections of liver were prepared and viewed by transmission electron microscopy. For morphometric analysis, a minimum of five random fields per patient were taken and examined in blinded manner at ×7000 magnification. Electron microscopy findings were assessed by two readers unaware of clinical and laboratory data. The term "autophagic vesicle" refers to an autophagosome or autolysosome.

Immunohistochemical staining

Immunohistochemical staining was performed using polyclonal antibody to SQSTM1 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), monoclonal antibody to anti-cathepsin B (1:500; R&D Systems Europe, Abingdon, UK), anti-cathepsin D (1:500; R&D Systems Europe) and anti-cathepsin L (1:1000; Abcam, Cambridge, UK). Sections were cut into thicknesses of 4 μm and were deparaffinized in xylene and dehydrated in descending dilutions of ethanol. For antigen retrieval, slides of cathepsin L were treated in citrate buffer (pH 6.0) and slides of SQSTM1 were treated in TE buffer (pH 9.0 DAKO, Carpentaria, CA, USA) by boiling for 45 min. Endogenous peroxidase activity was blocked by 30 min of incubation with 0.3% hydrogen peroxidase in absolute solution. The sections of tissue stained for SQSTM1 and cathepsin L were incubated with primary antibody, followed by the dextran polymer method (Chem Mate Envision; DAKO). Sections stained for cathepsin B and D were incubated with primary antibody, followed by testing with Nichirei simple stain MAX-POG immune-peroxidase polymer (Nichirei Bioscience, Tokyo, Japan). Diaminobenzidine tetrahydrochloride was used to enhance the hydrogen peroxide stain and then, the sections were counterstained with Mayer hematoxylin.

Quantitative image analysis

Immunostained slides were analyzed at ×40 original magnification using the inverted microscope (Zeiss KS400, Oberkochen, Germany) to quantify the amount of staining for cathepsin B, D and L in the human liver tissue sections. Positive staining was calculated after identifying the stronger brown positive hepatocytes in a region excluding bile ducts and vessels. The intensity of the stain was calculated by masking out all non-sinusoidal areas and infiltrated inflammatory cells from the tissue section and calculating the integrated optical density of brown within the remaining area. Then, nuclei numbers of hepatocytes were counted to evaluate the number of hepatocytes. Total brownish areas were divided by nuclei numbers to calculate the average intensity of a hepatocyte in the tissue section.

Statistics

All data are presented as mean ± standard error. Quantitative variables were expressed as median with range, and categorical variables as absolute and relative frequencies. Comparisons between groups of quantitative and qualitative variables were performed using the ANOVA on ranks in analysis of the number of autophagosomes, p62 aggregation, and cathepsin B, D and L expression. Mann–Whitney *U*-test was performed to evaluate the relationship between p62 and laboratory data or NAS in NAFLD group. $P < 0.05$ was considered statistically significant. Multiple logistic regression analysis was used to test the correlation between the rate of patients with p62 aggregation and chronic liver diseases. This statistical analysis was performed using SPSS version 19.0 software (SPSS, Chicago, IL, USA).

RESULTS

Patients' characteristics

THE CHARACTERISTICS OF the 81 patients with chronic liver diseases are listed in Table 1. Only the CHB group was significantly younger than the control group. The serum ALT levels in all patients with chronic liver disease were higher than the control group. Serum ALP was significantly elevated in patients with only PBC (652.0 ± 65.4 IU/L). Serum γ-GTP was significantly elevated in patients with NAFLD (135.5 ± 24.1 IU/L) and PBC (293.1 ± 49.0 IU/L). There was no significant difference in Plt, serum T-cho, TG and Glu levels between all groups.

Autophagic vesicles and p62 were accumulated in hepatocytes from NAFLD patients

Double-membrane vesicles with the morphology of autophagosomes were identified in 4.0 ± 0.43 vacuoles/cells in control using electron microscopy. We found that the number of autophagic vesicles in CHC patients (12.8 ± 1.28/cell) and NAFLD (13.8 ± 1.44/cell) is threefold higher than controls (Fig. 1). Interestingly, the accumulation of autophagic vesicles in hepatocytes from NAFLD patients was similar to CHC patients. On the other hand, there were no significant differences in the number of autophagic vesicles among CHB, PBC and controls.

Table 1 Characteristics of patients

	Control ($n = 14$)	CHB ($n = 16$)	CHC ($n = 20$)	NAFLD ($n = 22$)	PBC ($n = 23$)
Male/female	9/5	15/1	13/7	11/11	4/19
Age (years)	63.0 ± 3.7	43.6 ± 3.0*	57.2 ± 1.9	49.1 ± 3.5	55.5 ± 2.5
Plt (10^9/L)	22.1 ± 2.4	19.7 ± 1.2	16.3 ± 1.4	21.9 ± 1.3	23.9 ± 1.3
ALT (IU/L)	16.6 ± 2.9	190.3 ± 65.0*	67.7 ± 10.2*	119.2 ± 22.3*	53.5 ± 6.6*
ALP (IU/L)	295.8 ± 47.6	250.3 ± 24.8	244.2 ± 15.7	249.5 ± 15.3	652.0 ± 65.4*
γ-GTP (IU/L)	56.9 ± 20.0	66.4 ± 13.4	63.8 ± 11.0	135.5 ± 24.1*	293.1 ± 49.0*
T-cho (mg/dL)	183.5 ± 11.7	177.8 ± 6.3	172.1 ± 8.0	211.9 ± 10.5	210.8 ± 6.0
TG (mg/dL)	97.1 ± 8.6	96.3 ± 15.9	109.3 ± 10.7	191.5 ± 33.4	139.6 ± 21.1
Glu (mg/dL)	99.1 ± 5.9	88.1 ± 2.5	97.2 ± 4.1	99.5 ± 4.3	94.4 ± 2.8

Data are mean (± standard error).
*$P < 0.05$ compared to control by ANOVA on ranks.
γ-GTP, γ-glutamyltranspeptidase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; CHB, chronic hepatitis B; CHC, chronic hepatitis C; Glu, glucose; Plt, platelets; T-cho, total cholesterol; TG, triglycerides.

Next, we performed immunohistochemical staining of p62 to evaluate the function of autophagy. Aggregation of p62 in hepatocytes was observed in approximately 68% of NAFLD patients, whereas the p62 expression was not identified in the control group (Fig. 2). Accumulation of p62 in the hepatocytes is detected in less than 25% of CHB, CHC and PBC patients. The association between the detection of p62 aggregation and NAFLD was confirmed by multiple logistic regression analysis; however, identification of p62 aggregation was not correlated with the other chronic hepatitis groups. Mallory–Denk bodies were not identified in hepatocytes with the aggregation of p62 (Fig. 2b).

Cathepsin expression is suppressed in hepatocytes from NAFLD patients

Immunohistochemical analysis of cathepsin was performed to evaluate if autophagic proteolysis is disturbed by the impairment of lysosomal enzyme activity. In control liver, cathepsin B expression was detected in cytoplasm of hepatocytes (Fig. 3a,b). Expression of cathepsin B in hepatocytes was also observed in the liver from patients with CHB, CHC and PBC, and there was no significant difference in comparison with the control group; however, cathepsin B expression was significantly suppressed in the liver from NAFLD patients by approximately 50% as compared to the control group. Expression of cathepsin D expression was also detected in cytoplasm of hepatocytes from control patients (Fig. 3a,c). Cathepsin D expression was significantly suppressed in the liver from NAFLD patients to approximately half of the control group. Expression of cathepsin D in CHB and PBC was almost equal to the control group; however, elevated expression of cathepsin D was observed in CHC. Cathepsin L was expressed in cytoplasm of hepatocytes from the control group. There was no significant difference of cathepsin L expression among CHB, CHC, PBC and control groups (Fig. 3a,d); however, cathepsin L expression was also significantly lower in the NAFLD group as seen with other cathepsin data in this study. Interestingly, the aggregation of p62 was correlated with the suppression of cathepsin B ($P = 0.016$), D ($P = 0.037$) and L ($P = 0.015$) expression individually.

Detection of p62 aggregation correlates with an increase in serum ALT and hepatic inflammation in NAFLD patients

The relationship between the detection of p62 aggregation in hepatocytes and laboratory values was assessed in NAFLD. There was no significant correlation between the aggregation of p62 detected in the liver section and the following variables: Plt, ALP, γ-GTP, T-cho, TG, Glu and type 4 collagen (Fig. 4a,c–h). Interestingly, serum ALT value correlated with p62 aggregation in NAFLD patients significantly (Fig. 4b).

Next, we evaluated a link between histological characteristics and the formation of p62 aggregation in NAFLD patients. Total score by NAS in NAFLD with p62 aggregation (5.4 ± 0.35) was significantly higher than NAFLD patients without p62 aggregation (3.8 ± 0.77) (Fig. 5a). Moreover, approximately 73.3% of NAFLD patients with NAS of 5 or more exhibited p62 aggregation in liver sections. Interestingly, the increase in lobular inflammation by NAS correlated with the

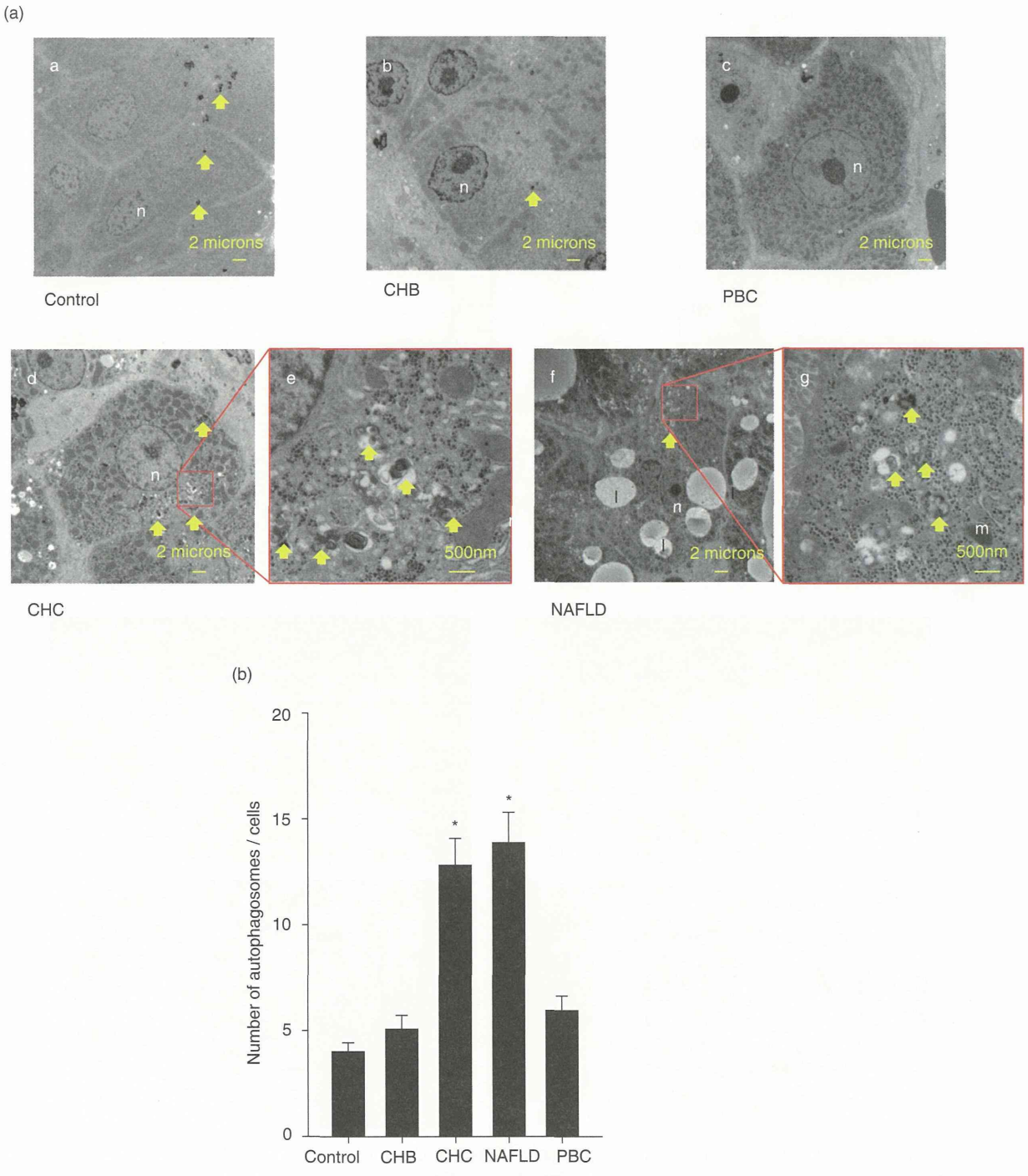

Figure 1 Autophagy level of hepatocytes in chronic liver diseases. (a) Electron micrographs showing ultrastructure of hepatocytes from chronic liver diseases. Arrows point to autophagic vesicles. (original magnification, ×3000) Partial view of hepatocyte containing an autophagic vesicle in CHC and NAFLD (original magnification, ×20 000). m, mitochondria; n, nucleus; l, lipid. (b) Number of autophagosomes in a hepatocyte was evaluated. Quantification of autophagic vesicles in hepatocytes was performed with 30 different areas of the cytoplasm of hepatocytes using electron micrographs. Numbers of autophagosomes were expressed as the mean ± standard error (*$P < 0.05$ compared to control, by ANOVA on ranks). CHB, chronic hepatitis B; CHC, chronic hepatitis C; NAFLD, non-alcoholic fatty liver disease; PBC, primary biliary cirrhosis.

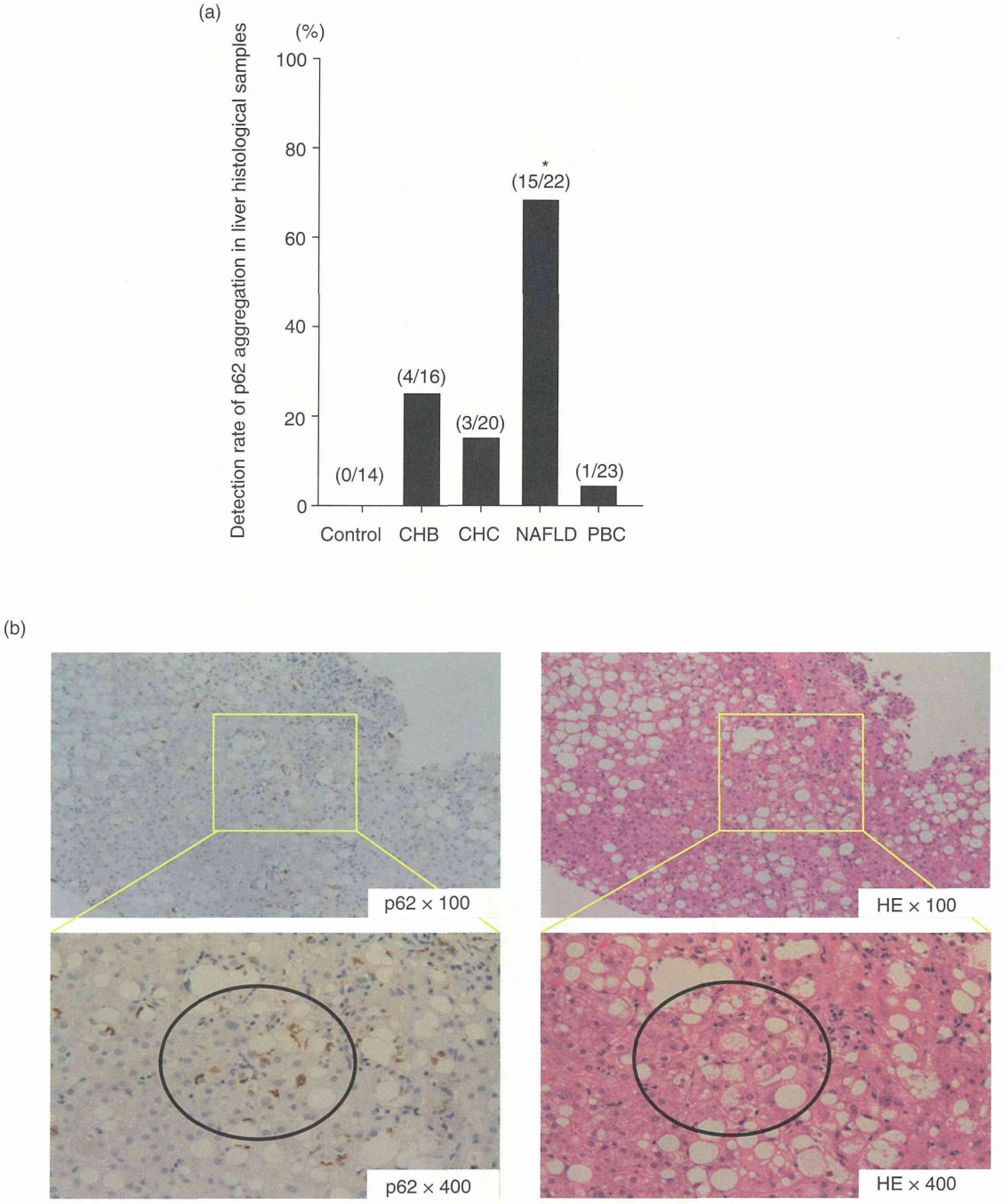

Figure 2 Detection of hepatocytes contained with the aggregation of p62 in chronic liver diseases. (a) Detection rate of hepatocytes which contained with p62 aggregation in each chronic liver disease. Data are presented as detection rate (%).*$P < 0.05$ with multiple logistic regression analysis. Fractions represent number of patients with p62 aggregation/total number of patients. (b) Representative images by immunohistochemical staining of p62 (left) and HE staining (right) in NAFLD. Upper panels are shown at ×100 magnification, lower panels are shown at ×400 magnification. Arrows point to the aggregation of p62 in hepatocytes in an NAFLD patient. CHB, chronic hepatitis B; CHC, chronic hepatitis C; HE, hematoxylin–eosin; NAFLD, non-alcoholic fatty liver disease; PBC, primary biliary cirrhosis.

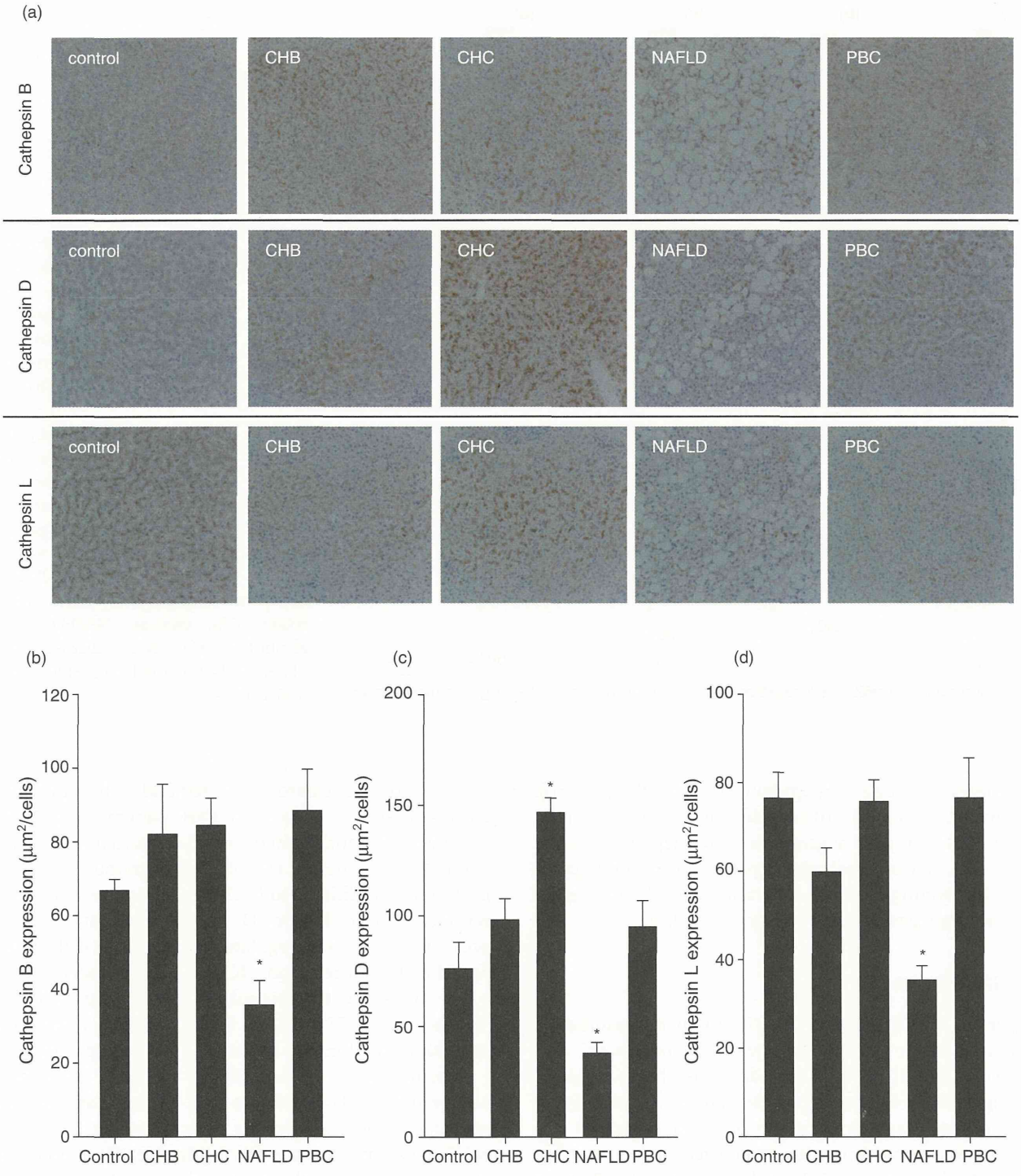

Figure 3 Expression level of cathepsin B, D and L in the liver from chronic liver diseases. (a) Upper lane is immunohistochemical analysis of cathepsin B, middle lane is cathepsin D and lower lane is cathepsin L expression in liver from chronic liver diseases: control, CHB, CHC, NAFLD and PBC (original magnification, ×200). (b) Quantification of cathepsin B, (c) cathepsin D, (d) cathepsin L, for expression in hepatocytes from chronic liver diseases. Data are presented as means ± standard error (*$P < 0.05$ compared to control by ANOVA on ranks). CHB, chronic hepatitis B; CHC, chronic hepatitis C; NAFLD, non-alcoholic fatty liver disease; PBC, primary biliary cirrhosis.