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Prostaglandin E2 receptor EP4 agonist induces Bcl-xL and independently activates proliferation signals in mouse primary hepatocytes

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Background. To improve the survival rate of fulminant hepatic failure (FHF), we examined the mechanism of the antiapoptotic effect, and the possible proliferative effect, of a specific agonist of prostaglandin E2 receptor EP4 (PGEP4-A) on mouse primary hepatocytes, as a candidate for a new therapeutic agent. ***Methods.*** The expression of four PGE2 receptor subtypes was detected by a reverse transcriptase polymerase chain reaction (PCR) method. Hepatocytes were stimulated with PGEP4-A, ONO-AE1-437, and changes in the expression levels of Bcl-xL and cyclin D1 and in the phosphorylation of epidermal growth factor receptor (EGF-R) and extracellular-signal related kinase (ERK) were examined by Western blot analysis. ***Results.*** Mouse primary hepatocytes constitutively expressed the mRNAs of all four PGE2 receptor subtypes, including that of PGEP4. PGEP4-A induced not only Bcl-xL protein expression (as we had previously demonstrated in HepG2 cells) but also induced cyclin D1 protein expression in mouse primary hepatocytes as well as the phosphorylation of EGF-R and ERK. The inhibition of ERK phosphorylation by a specific inhibitor, PD98059, did not affect the increase in Bcl-xL expression level. ***Conclusions.*** PGEP4-A may be a therapeutic agent for FHF because of its antiapoptotic and regenerative effects on hepatocytes.

Key words: hepatocyte apoptosis, proliferation, prostaglandin E2 receptor agonist, ERK, Bcl-xL

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Introduction

Fulminant hepatic failure (FHF) affects approximately 1000 patients per year in Japan and has a poor prognosis in the absence liver transplantation.[1] In most cases of acute hepatic injury, no specific treatment is necessary,[2] because the damage is rapidly and completely repaired by the vigorous proliferation of hepatocytes as reported previously.[3–6] However, when the hepatic damage is extensive, such as in FHF, hepatocyte regeneration is extremely poor and recovery from the damage is seriously impaired.[7] On the basis of these findings, uncontrollable and persistent hepatocyte apoptosis and impaired hepatocyte regeneration have been considered as the causes of the poor prognosis in FHF patients, and it is urgently required to develop new therapeutic approaches aimed at hepatocyte protection and regeneration.

Prostaglandins (PGs) E1 and E2 are well known to have a cytoprotective effect on various cell types.[8–11] Therefore, PGs E1 and E2 or their analogues have been considered as potential therapeutic agents for FHF and have been tentatively employed for patients with FHF,[12,13] although their clinical usefulness has not been demonstrated.[14] Since the time of the identification of four subtypes of PGE2 receptors; namely, PGE2-EP1, -EP2, -EP3, and -EP4, the effects of PGE2 have been studied in relation to individual receptors, and it was reported that stimulation through the EP2 and EP4 receptors (EP2-R and EP4-R) had anti-inflammatory effects on Kupffer cells.[15] Recently, we have demonstrated that a newly developed PGEP4-R agonist, ONO-4819, prevents liver damage in a rat acute hepatic failure model,[16] and that its active agent, ONO-AE1-437, inhibits Fas-agonist-induced hepatocyte apoptosis through the direct induction of the antiapoptotic Bcl-2 family proteins, Bcl-xL and Mcl-1, in HepG2 cells.[17]

It has also been reported that PGE2 phosphorylates and activates extracellular-signal related kinase (ERK) and p42/44 mitogen-activated protein kinase (MAPK)

through epidermal growth factor (EGF) receptor (EGF-R) activation in some cell lines.[18] This finding indicates that PGE2 can possibly accelerate the proliferation of epithelial cells.

On the basis of these findings, we hypothesized that PGE2 suppresses liver damage through not only the induction of antiapoptotic proteins but also through the promotion of cell proliferation. Therefore, we examined the expression of PGE2 receptors, the induction of Bcl-2 family proteins, and the activation of cell proliferation signals induced by PGE2R-A in primary mouse hepatocytes.

Materials and methods

Chemiscals

All chemicals were purchased from the indicated commercial sources, except for PGEP4-A (ONO-AE1-437), which was a kind gift from Ono Pharmaceutical (Osaka, Japan).

Animals and surgical procedure

The care and use of mice in our present experiments were reviewed and approved by the Institutional Animal Treatment Committee at Iwate Medical University. Seven-week-old male C57BL/6J mice (Clea Japan, Tokyo, Japan) were used in this study. With the animals under pentobarbiturate anesthesia, the liver was perfused with Ca^{2+}-free and Mg^{2+}-free Krebs-Ringer hydroxyethylpiperazine ethanesulfonic acid (HEPES) buffer (25 mM HEPES; Research Organics, Cleveland, OH, USA), 115 mM NaCl (Wako Chemicals, Osaka, Japan), 5 mM KCl (Wako Chemicals), 1 mM KH_2PO_4 (Wako Chemicals), 0.2 mM ethyleneglycoltetraacetic acid (EGTA; acid Sigma Chemicals, St. Louis, MO, USA; pH 7.4), and then with Ca^{2+}-free and Mg^{2+}-free Krebs-Ringer HEPES buffer (25 mM HEPES, 115 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 0.2 mM EGTA, 100 mM $CaCl_2$, pH 7.4), containing 0.075 units/ml collagenase D (Roche Dagnostics, Mannheim, Germany). After mechanical isolation, viable hepatocytes were purified by two steps of centrifugation at 50*g*, and resuspension, followed by 45% Percoll gradient centrifugation (Sigma Chemicals). For culture, hepatocytes were resuspended in a culture medium at 2.5×10^5 cells/ml. Flow cytometry of isolated cells, using a cell sorter (FACScan, CellQuest Software; Becton Dickinson, Heidelberg, Germany) and an anti-mouse CD-68 antibody (UK-Serotec, Oxford, UK) demonstrated that the purity of hepatocytes was 99.7% and the remaining Kupffer cells accounted for 0.3% of isolated cells, whereas the content of Kuppfer cells before purification by centrifugation and Percoll gradient centrifugation was approximately 2%.

Hepatocyte culture

Aliquots (10 ml) of a hepatocyte suspension were cultured in collagen-coated 10-cm-diameter tissue culture dishes (Asahi Tachno Glass, Funabashi, Japan) at 37°C under 5% CO_2/air. The culture medium was Waymouth's medium (Gibco BRL, Gaithesburg, MD, USA), containing 10% fetal bovine serum (Hyclone, Logan, UT, USA), 100 U/ml penicillin (Sigma Chemicals), 50 nM insulin (Sigma Chemicals), and 50 nM dexamethasone (Sigma Chemicals). After 2 h of incubation, the medium was replaced with fresh Waymouth's medium not containing fetal bovine serum, dexamethasone, or insulin. After an overnight incubation, the obtained hepatocytes were used for the experiments.

Hepatocyte treatment

Mouse primary hepatocytes were treated with 1 μM PGEP1-A with and without pretreatment with 50 μM PD98059 (Sigma Chemicals), an inhibitor of ERK phosphorylation. Serial changes in the protein levels of phosphorylated and total ERK, phosphorylated and total EGF-R, and Bcl-xL and cyclin D1 were determined by Western blot analysis.

Western blot analysis

Cells were rinsed and lysed with 500 μl of lysis buffer (150 mM NaCl, 50 mM HEPES [pH 7.5], 10% glycerol [Wako Chemicals]), 5 mM $MgSO_4$, 1 mM EGTA, 10 mM sodium orthovanadate (Wako Chemicals), 100 mM sodium fluoride (Wako Chemicals), 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma chemicals), 1% thioglycol (Wako Chemicals), 1% Triton X-100 (Sigma Chemicals) and a protease inhibitor cocktail (Sigma Chemicals). After centrifugation, cell lysates containing 50 μg of protein were denatured by boiling in Laemmli's sample buffer for 5 min; resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), using a 10% polyacrylamide gradient gel (Tefco, Tokyo, Japan); and subsequently transferred to nitrocellulose membranes (Osmonics, Gloucester, MA, USA). After blocking with Tris-based saline buffer containing 5% dry milk and 0.1% Tween 20 (Sigma Chemicals) for 1 h, the membranes were blotted with appropriate antibodies. The membranes were visualized using chemiluminescent substrate (PerkinElmer Life Science, Boston, MA, USA) and exposed to a Kodak BioMax Light film (Eastman Kodak, Rochester, NY, USA). The relative intensities of the signals were quantified with an image analyzer

(Molecular Imager FX, Quantity One; Nippon Bio-Rad Laboratories, Tokyo, Japan). The primary and secondary antibodies we used were as follows: purified mouse anti-Bcl-xL monoclonal antibody (PharMingen, San Diego, CA, USA), rabbit anti-EGF-R antibody (1005; Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-phospho-EGF-R antibody (Tyr1173; Santa Cruz Biotechnology), rabbit anti-ERK2 antibody (C-14; Santa Cruz Biotechnologhy), mouse monoclonal anti-phospho-ERK antibody (Tyr204; Santa Cruz Biotechnology), rabbit anti-cyclin D1 antibody (C-20; Santa Cruz Biotechnology), rabbit anti-actin antibody (C-11: Santa Cruz Biotechnology), goat anti-rabbit IgG conjugated with horseradish peroxidase (Cell Signaling Technology, Beverly, MA, USA), goat anti-mouse IgG conjugated with horseradish peroxidase (Cell Signaling Technology), and bovine anti-goat IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology).

Reverse transcriptase (RT)-polymerase chain reaction (PCR)

Total RNA was extracted from cultured hepatocytes, using a Qiagen RNeasy minikit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Reverse transcription was performed using 1 μg of total RNA with random primers in a final volume of 20 μl (Omniscript Reverse Transcriptase kit; Qiagen). The cDNA products were amplified by PCR, using one cycle of denaturation at 95°C for 2 min and 30 cycles, consisting of denaturation at 95°C for 1 min, primer annealing at 58°C for 1 min, and extension at 72°C for 1 min; an additional cycle at 95°C for 1 min, 60°C for 1 min, and 72°C for 7 min was performed. The specific oligonucleotide primers for each of the PGE2 receptor subtypes were as follows: EP1, 5′-GGGGGCTGGAACTCTA ACTC-3′ and 5′-CACTGTGCCGGGAACTACGC-3′; EP2, 5′-AGGACTTCGATGGCAGAGAGAC-3′ and 5′-CAGCCCCTTACACTTCTCCAATG-3′; EP3, GG TATGCCAGCCACAATGAAGAC-3′ and 5′-CAAG ATCTGGTTCAGCGAAGCC-5′; and EP4, 5′-TTCC GCTCGTGGTGCGAGTGTTC-3′ and 5′-GAGGTG GTGTCTGCTTGGGTCAG-5′. The primers for mouse CD68 were 5′-TTCTGCTGTGGAAATGCGC AAG-3′ and 5′-AGAGGGGCTGGTAGGTTGAT-3′. The RT-PCR products were separated on a 2% agarose gel, stained with ethidium bromide, and photographed using ultraviolet illumination.

Results

Mouse primary hepatocytes expressed all four PGE2 receptor subtypes

To examine the expression of PGE2 receptor subtypes on mouse hepatocytes, four PGE2 receptor subtypes, EP1, EP2, EP3, and EP4, and CD68 cDNA were searched in the same cDNA products as those generated by reverse transcription for total RNA extract from cultured mouse hepatocytes. The mRNAs for all four PGE2 receptor subtypes, but not CD68 mRNA, were detected, as shown in Fig. 1.

PGEP4-A induced Bcl-xL expression

To examine the effect of PGEP4-A on Bcl-xL induction, changes in Bcl-xL protein expression levels after ONO-AE1-437 treatment were determined by Western blot analysis. As shown in Fig. 2, the Bcl-xL expression level increased from 24 h after treatment with ONO-AE1-437 and peaked at 36 h.

PGEP4-A induced EGF-R and ERK2 phosphorylation and cyclin D1 expression

To determine whether PGEP4-A activated EGF-R, leading to MAPK signaling activation, changes in the phosphorylation of EGF-R and ERK2, and in the expression levels of cyclin D1, after ONO-Ae1-437 treatment were determined by Western blot analysis. As shown in Fig. 3a,b, EGF-R and ERK2 were transiently phosphorylated, peaking 20 min and 60 min after treatment, respectively. Following the phosphorylation of

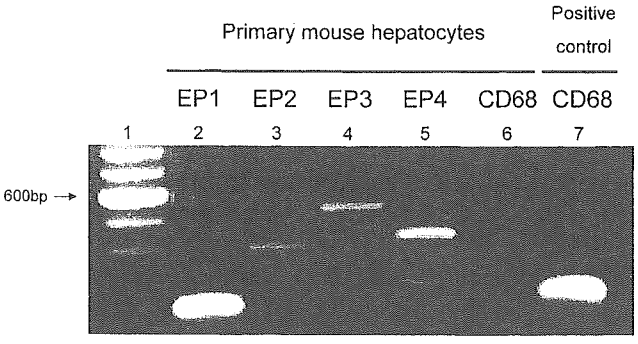

Fig. 1. Expression of prostaglandin (PG)E2 receptor subtypes on mouse primary hepatocytes. Reverse transcriptase (RT)-polymerase chain reaction (PCR) for four PGE2 receptor subtypes (*EP1*, *EP2*, *EP3*, *EP4*) and CD68. Using the cDNA solution described in the "Materials and Methods" section, PCR was performed for four PGE2 receptors and CD68 (*lanes 2–6*). For the positive control of CD68, cDNA prepared from nonpurified liver cells was used (*lane 7*)

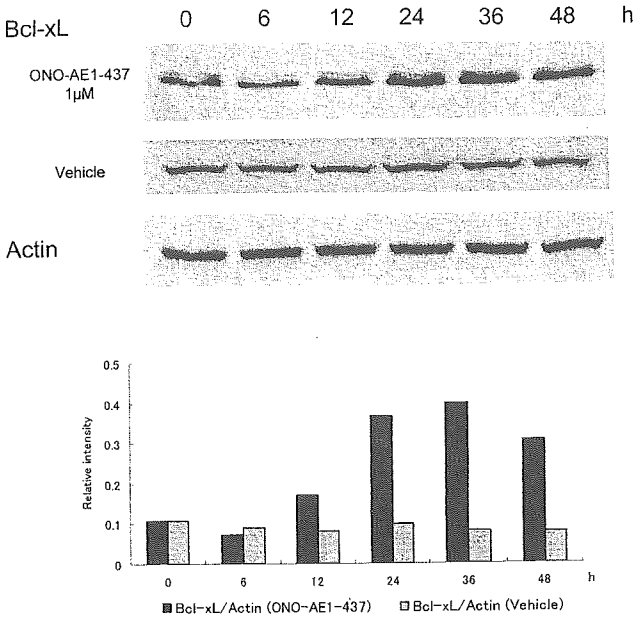

Fig. 2. PGE2 EP4 receptor PGEP4-A induces Bcl-xL expression in mouse primary hepatocytes. After treatment with 1 μM PGEP4-A (ONO-AE1-437) or vehicle (dimethyl sulfoxide), Western blot analysis of the cell lysate was performed for Bcl-xL, using anti-Bcl-xL antibody and anti-actin antibody. *Bar graph* represents the intensity of each Bcl-xL signal relative to that of the actin signal

EGF-R and ERK2, the expression level of cyclin D1 increased 24 to 48 h after ONO-AE1-437 treatment (Fig. 3c).

Inhibition of ERK phosphorylation did not affect the induction of Bcl-xL by PGEP4-A

To determine whether the activation of MAPK signaling by PGEP4-A affected the induction of Bcl-xL expression, changes in Bcl-xL expression levels after ONO-AE1-437 treatment were examined under the condition of inhibition of ERK2 phosphorylation by pretreatment with PD98059. As shown in Fig. 4, the increase in Bcl-xL expression level was not attenuated by pretreatment with PD98059, whereas the enhanced ERK2 phosphorylation induced by ONO-AE1-437 was completely suppressed (Fig. 3b).

Discussion

The principal findings of this study relate to the cellular mechanism by which PGEP4-A prevents hepatocyte apoptosis and, possibly, induces cell proliferation. These findings were as follows: (1) PGEP4-A induced an antiapoptotic Bcl-2 family protein, Bcl-xL; (2) PGEP4-A induced cyclin D1, ERK phosphorylation, and EGF-R phosphorylation; and (3) the suppression of ERK phosphorylation by PD98059 did not affect the induction of Bcl-xL by PGEP4-A. These findings suggest that PGEP4-A has the possibility of not only protecting hepatocytes from apoptosis but also of promoting hepatocyte proliferation.

The mitochondrial protection conferred by antiapoptotic Bcl-2 family proteins is crucial for cell survival against Fas-induced proapoptotic stimulation in so-called type I cells, such as hepatocytes.[19] We previously reported that PGEP4-A suppressed indomethacin-induced HepG2 cell apoptosis and that this effect of PGEP4-A on HepG2 cells depended on the induction of Bcl-xL.[17] These findings suggest that constitutive Bcl-xL expression is essential for the survival of HepG2 cells, and that PGEP4-A directly promotes Bcl-xL expression, findings which also suggest that HepG2 cells express PGEP4 receptor. On the basis of these in vitro findings, we concluded that the direct induction of Bcl-xL in hepatocytes was one of the mechanisms by which PGEP4-A suppresses liver injury in an in vivo acute hepatic failure model. In the present study, therefore, we examined whether primary hepatocytes also responded to PGEP4-A stimulation and expressed Bcl-xL.

The mechanism by which Bcl-xL is induced by PGs has not been elucidated, although it is well known that PG and cyclooxygenase-2 gene transfection promote Bcl-2 expression in some cell lines, such as colorectal cancer and cholangiocellular carcinoma cell lines.[20–22] Furthermore, Buchanan et al.[23] demonstrated that PGE2 activated phosphatidylinositol 3-kinase (PI3-K)/Akt through the transactivation of EGF-R in a human colorectal cancer cell line, LS147T cells. The activation of PI3-K suppresses bile acid-induced hepatocyte apoptosis upstream of the mitochondrial damage.[24] On the other hand, PGs promote the proliferation of epithelial cells. Pai et al.[18] reported that PGs induced EGF-R transactivation, ERK phosphorylation, and cell proliferation, leading to the promotion of gastrointestinal cancer cell growth. These findings suggest that the antiapoptotic effect of PGE2 acts through several pathways, such as PI3-K/Akt activation and cell proliferation, as well as via Bcl-2 family protein induction. On the basis of these findings, we examined the transactivation of EGF-R by PGEP4-A stimulation in primary hepatocytes, and the possible effect of MAPK signaling induced by PGEP4-A stimulation on Bcl-xL induction. We demonstrated the PGEP4-A induced the expression of cyclin D1 following the phosphorylation of EGF-R and ERK. This finding suggests that PGEP4-A promotes hepatocyte replication through EGF-R transactivation, as a growth factor, although further studies including those of dose dependence, are

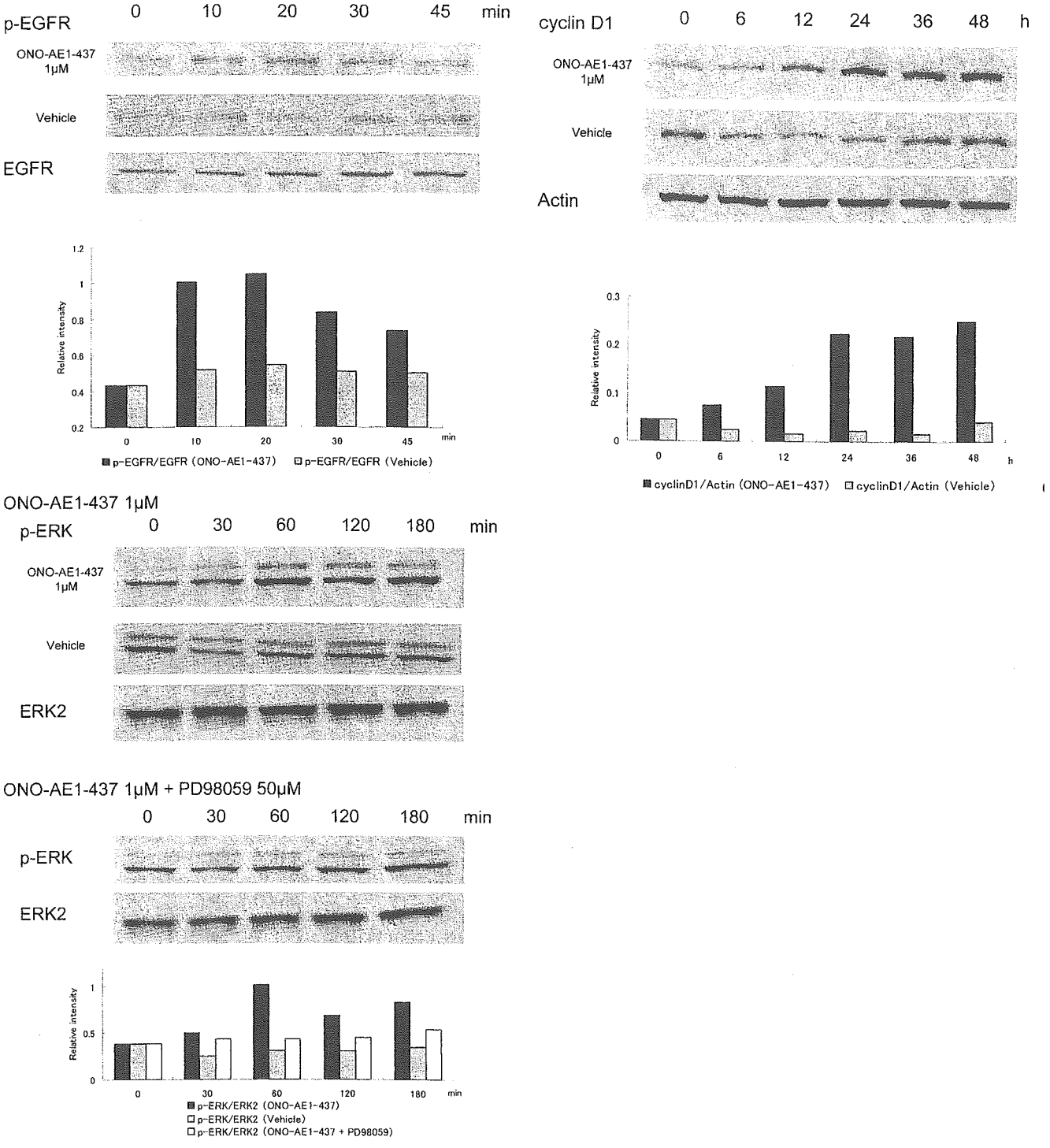

Fig. 3a–c. PGEP4-A promotes epidermal growth factor receptor (*EGF-R*) and extracellular-signal related kinase (*ERK*)2 phosphorylation and induces cyclin D1 expression in mouse primary hepatocytes. After treatment with 1 μM PGEP4-A (ONO-AE1-437) or vehicle (dimethyl sulfoxide), Western blot analysis of the cell lysate was performed, using anti-phospho-EGF-R (*p-EGFR*) antibody, and anti-EGF-R (*EGFR*) antibody (**a**), anti-phospho-ERK (*p-ERK*) antibody and anti-ERK2 (*ERK2*) antibody (**b**), and anti-cyclin D1 (*cyclin D1*) antibody (**c**). *Bar graphs* represent the intensity of each of the p-EGF-R, p-ERK, and cyclin D1 signals relative to that of the EGF-R, ERK, and actin signals, respectively

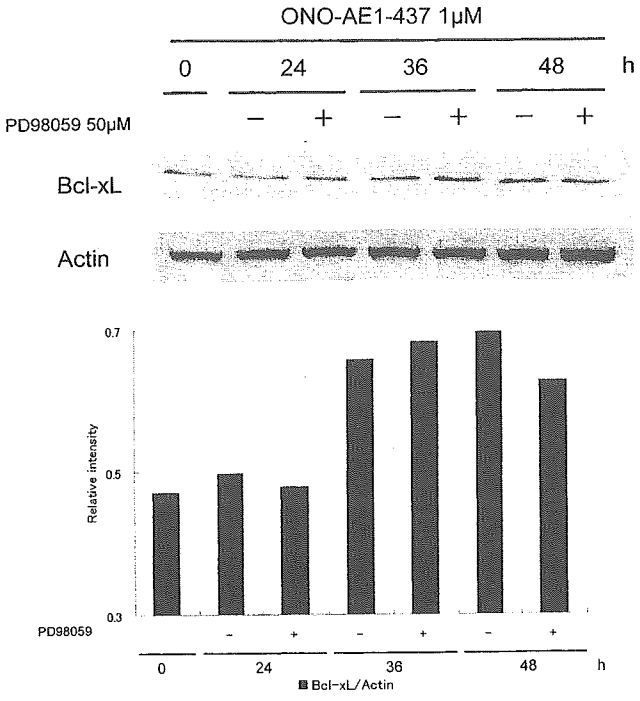

Fig. 4. Inhibition of ERK phosphorylation does not affect Bcl-xL expression induced by PGEP4-A. Hepatocytes were pretreated with 50μM PD98059 and subsequently treated with 1 μM PGEP4-A for an appropriate time. After treatment, Western blot analysis of the cell lysate was performed, as described in Fig. 2 legend. *Bar graph* represents the intensity of each Bcl-xL signal relative to that of the actin signal

required to specify the effects of PGEP4-A. Hashimoto et al.[25] have reported that PGs induce the proliferation of rat hepatocytes as a comitogenic factor of EGF or insulin through the PGE2 EP3 receptor.[18] Therefore, PGS, or their receptor agonists, may prevent hepatocyte death by promoting cell proliferation, as well as by inducing antiapoptotic Bcl-2 family protein.

In this study, the induction of Bcl-xL expression was not affected by the inhibition of enhanced ERK2 phosphorylation, although both processes were initiated by PGEP4-A stimulation. This indicates that PGEP4-A induces Bcl-xL expression independently of EGF signaling activation, at least in primary hepatocytes. We speculate, therefore, that the administration of a PG, or its receptor agonist, may represent a possible therapy for acute hepatic injury, although further studies using damaged or regenerating hepatocytes are require before proceeding to clinical application, considering the higher susceptibility of regenerating hepatocytes to proinflammatory cytokine-induced apoptosis.[26,27]

In conclusion, PGEP4-A is may be a new potential therapeutic agent for FHF, although further studies of the precise mechanisms underlying its antiapoptotic and proliferative effects, and studies of the conditions in which the agent is effective are required.

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HEPATOLOGY

Influence of load of hepatitis A virus on disease severity and its relationship with clinical manifestations in patients with hepatitis A

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Abstract

Background and Aim: The purpose of the present study was to investigate the influence of viral load on disease severity and analyze the possible relationship of the load of hepatitis A virus (HAV) with disease severity and laboratory findings.

Methods: Fifty-eight patients diagnosed with acute hepatitis A were used in the current study, of whom 12 patients progressed to severe acute hepatitis (s-AH) defined on the basis of a prothrombin time (PT) of <40% and 46 patients were diagnosed as having mild acute hepatitis (m-AH). The load of HAV was measured with real-time polymerase chain reaction.

Results: Peak viral load showed a significant correlation with alanine aminotransferase (ALT) ($r = 0.363$, $P = 0.0048$) and PT levels ($r = -0.330$, $P = 0.0110$). In terms of disease severity, there was a significant correlation with ALT ($r = 0.462$, $P = 0.0012$) and PT levels ($r = 0.400$, $P = 0.0059$) in the m-AH group, but not in the s-AH group. A significant positive correlation of peak viral load with the C-reactive protein level ($r = 0.270$, $P = 0.0400$) and a significant negative correlation of peak viral load with the platelet count ($r = -0.313$, $P = 0.0015$) was also found.

Conclusions: The load of HAV was closely correlated with liver damage and disease severity in m-AH, but not in s-AH. The load of HAV was also closely associated with the increase in C-reactive protein level and enhancement of thrombocytopenia.

Key words: acute hepatitis A, C-reactive protein, extrahepatic manifestation, real-time polymerase chain reaction, thrombocytopenia.

INTRODUCTION

Hepatitis A virus (HAV) is a 7.5-kb positive-strand RNA virus of the Picornaviridae family and the only member of the genus Hepatovirus. The viral genome is composed of three functional regions, termed P1, P2 and P3. The 5′ proximal region, P1, encodes four proteins, of which three (VP1, VP2 and VP3) form the viral capsid. The middle region, P2, contains the 2A, 2B and 2C genes. The 3′ proximal region, P3, encodes four proteins, namely, 3A, 3B, 3C and 3D. The HAV strains corresponding to only a single serotype have been classified into seven genotypes on the basis of a 168 nucleotide sequence at the viral protein 1/non-structural protein 2A (VP1/2A) junction.[1] Four of these genotypes, I, II, III and VII, are associated with human diseases. Genotypes I and III are further subdivided into subtypes A and B. Up to 80% of human strains belong to genotype I.

Hepatitis A is endemic in developing countries and most people in these countries are exposed during childhood. In contrast, the adult population in developed countries including Japan has a decreasing rate of exposure due to improvements in hygiene and sanitation. In young children the disease is often asymptomatic, whereas in older children and adults there might be a range of clinical manifestations from mild, anicteric infection to fulminant hepatic failure. The risk of fulminant hepatitis (FH) is high in patients that have an underlying chronic liver disease and are aged more than

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40 years.[2] Various extrahepatic manifestations, such as hemolysis, aplastic anemia, autoimmune thrombocytopenic purpura, pure red cell aplasia, cholecystitis, acute pancreatitis, acute renal failure, acute reactive arthritis, cryoglobulinemia, mononeuritis, mononeuritis multiplex, Guillain–Barré syndrome, postviral encephalitis and transverse myelitis, have been observed in patients with acute hepatitis A.[3–14]

With advances in virology it has been shown that nucleotide changes in the 5′ non-coding region (NCR) and amino acid changes in the 2B region of the HAV might be more frequent in FH.[15] It was also reported that nucleotide variations in the central part of 5′ NCR of the HAV might be one of the factors that determines disease severity.[16,17] However, the mechanism underlying the genetic changes of the HAV and the clinical status of the disease has been obscure. Although investigations on viral load during HAV infection are rare, it has recently been reported that low or undetectable HAV load is associated with FH and death.[18] In contrast, it has also been reported that the peak transaminase level correlates well with the peak load of the HAV in 11 patients with hepatitis A.[19] The disparity between these findings has not yet been resolved in current reports. Furthermore, no studies of correlation between the viral load and extrahepatic data have been undertaken.

In the present study, we measured the load of the HAV by real-time polymerase chain reaction (PCR) with two-step reverse transcription PCR (RT-PCR) in patients with hepatitis A to investigate its influence on disease severity or liver damage, and analyzed the possible relationship between the load of the HAV and laboratory findings.

METHODS

Patients

A total of 58 patients (24 men and 34 women; age, 23–69 years) diagnosed with acute hepatitis A (AH-A), on the basis of their positivity for the immunoglobulin M (IgM) anti-HAV antibody (IgM-HA) in conjunction with compatible symptoms and laboratory findings, were enrolled from March 1994 to November 2002 in the present study. Patients with AH-A were all admitted to the university hospitals of Iwate Medical University. They were all negative for the hepatitis B surface antigen (HBsAg), IgM antihepatitis B core antigen antibody (IgM-HBc), second generation antihepatitis C virus (HCV) antibody, IgM antihepatitis E virus (HEV) antibody, IgM antiherpes simplex virus antibody, IgM anti-Epstein–Barr virus antibody, IgM anticytomegalovirus antibody, antinuclear antibody and antimitochondrial antibody.

Of the 58 patients with AH-A, only one patient was considered as having FH: acute liver failure with less than 8 weeks between the onset of the illness and the development of encephalopathy. Patients with a prothrombin time (PT) of less than 40% of the control were diagnosed as having severe acute hepatitis (s-AH), including one patient with FH (Table 1), and others

Table 1 Characteristics of 12 patients with severe acute hepatitis A including fluminant hepatitis

Patient	Age	Gender	Average daily alcohol intake in ethanol/ No. years	Biological data on admission/at peak			Viral load	Genotype	Encephalopathy	Plasma exchange
				Bilirubin (mg/dL)	ALT (IU/L)	Prothrombin time (%)	log copies/mL			
1	55	M	114 g/30 years	5.5/13.6	4719/4719	14.0/14.0	3.13/3.13	1A	Grade 3	Done
2	41	F	21 g/15 years	2.4/7.6	11193/11193	40.0/40.0	4.49/4.49	1A	None	Not done
3	45	M	0 g	7.0/12.6	4741/4741	37.0/37.0	4.83/4.83	1A	None	Not done
4	46	F	0 g	9.6/19.9	1110/1110	36.0/36.0	2.92/2.92	1A	None	Not done
5	47	M	75 g/25 years	5.9/11.3	7156/7156	28.0/23.0	3.67/4.03	1A	None	Not done
6	47	F	0 g	4.3/4.7	2787/2787	27.0/27.0	5.03/5.15	1A	None	Not done
7	47	M	70 g/25 years	6.9/12.0	5546/5546	34.0/34.0	5.02/5.02	1A	None	Not done
8	49	M	25 g/30 years	4.0/9.0	7934/7934	11.0/11.0	0/4.29	1A	None	Not done
9	51	F	0 g	4.8/12.1	4658/4658	21.5/21.5	4.82/4.82	1A	None	Done
10	52	M	70 g/30 years	4.3/17.4	9110/9110	23.0/23.0	3.86/3.86	1A	None	Not done
11	56	F	0 g	7.9/14.0	5221/6780	33.0/33.0	5.41/5.41	1A	None	Not done
12	59	M	84 g/40 years	5.1/9.9	8134/8134	27.0/27.0	4.54/4.54	1A	None	Not done

ALT, alanine aminotransferase; F, female; M, male.

were diagnosed as having mild acute hepatitis (m-AH). Five patients had a history of alcohol intake of more than 50 g of ethanol daily for more than 20 years. Only one patient with FH among the 58 patients was a heavy drinker, drinking 114 g of ethanol daily for 30 years. Two patients including one with FH underwent plasma exchange. Furthermore, a non-FH patient who underwent plasma exchange had acute renal failure, therefore she underwent continuous hemofiltration. None of the patients died of acute liver failure or other complications during the study period.

The start of early symptoms, such as fever, anorexia, malaise, nausea, vomiting, jaundice and right hypochondrial discomfort, was defined as the clinical onset.

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethics committee of Iwate Medical University, and informed consent was obtained from each patient. Serum samples were obtained at more than two time points including the time of admission, except for a patient with FH, and stored at −20°C or below until assay. The serum samples after the plasma exchange were not used in the present study.

Clinical assessment

All patients enrolled in the present study had their detailed medical history taken, including general data such as age, gender, onset of illness, history of alcohol intake and the presence of encephalopathy. The following variables were assessed on admission and during hospitalization: white blood cell count, red blood cell count, hemoglobin level, hematocrit, platelet count, PT, fibrinogen level, antithrombin III (AT-III) level, fibrin degradation product (FDP) level, bilirubin level, aspartate aminotransferase (AST) level, alanine aminotransferase (ALT) level, blood urea nitrogen (BUN) level, creatinine level, C-reactive protein level and IgM level, and were serially measured at least once a week.

Detection of serum hepatitis A virus RNA by reverse transcription–polymerase chain reaction

Viral RNA was extracted from a 100 μL serum sample using SMITEST EX-R&D (Genome Science Laboratories, Fukushima, Japan) according to the manufacturer's instructions. Briefly, 100 μL of serum sample was incubated with 400 μL of working buffer to achieve virus lysis, and precipitated with 600 μL of isopropanol. The RNA pellet was washed twice with 70% ethanol and dissolved in 10 μL of distilled water containing 40 units of RNase inhibitor (TOYOBO, Co., Ltd, Osaka, Japan).

The RNA (10 μL) was heated at 100°C for 1 min, chilled rapidly on ice, and reverse transcribed to complementary DNA (cDNA) with 200 units of M-MLV reverse transcription (Invitrogen, Life Technologies, Tokyo, Japan) and 20 μmol of reverse primer HA-R1 (Table 2) in a 10 μL reaction mixture containing 5 μL of each dNTP (2 mmol), 5 μL of Taq buffer and 40 units of RNase inhibitor. After 60 min at 37°C, the reverse transcription reaction was stopped and cDNA was stored at −20°C.

The first round of PCR was carried out in a 50 μL reaction mixture containing 1.5 μL of dNTP (2.5 mmol), 10 μL of cDNA, 0.5 μmol each of forward primer HA-F1 and reverse primer HA-R1 (Table 2), and 5 units of TaKaRa Ex Taq polymerase (TAKARA Bio Inc., Shiga, Japan). Amplification was carried out with denaturation at 95°C for 2 min, 40 cycles of denaturation-annealing extension at 94°C for 15 s, 50°C for 15 s and 72°C for 45 s, and a final extension at 72°C for 5 min. Thereafter, 1 μL of the products from the first round was used for 35 cycles of the second round PCR under the same conditions using the inner primers HA-F2 and HA-R2 (Table 2). The PCR products (10 μL) were analyzed by 8% polyacrylamide gel electrophoresis with a molecular marker, and then stained with ethidium bromide. The PCR products were then further electrophoresed on a 3% agarose gel. The HAV RNA was considered positive when the expected length of 239 base pairs (bp) was observed. All contamination prevention measures suggested by Kwok and Higuchi were strictly applied.[20]

Table 2 Nucleotide sequences of primers and TaqMan probe for nested reverse transcription–polymerase chain reaction (RT-PCR) and quantitative real-time PCR

Primers for nested RT-PCR	
HA-F1 (2799–2821)	5′-ATTCAGATTAGACTGCCTTGGTA-3′
HA-R1 (3273–3296)	5′-CCAAGAAACCTT CATTATTTCATG-3′
HA-F1 (2907–2929)	5′-GCAAATTACAATCATTCTGATGA-3′
HA-R2 (3162–3186)	5′-CTTCCTGAGCATACTTGAGTCTTTG-3′
Primers and TaqMan probe for quantitative real-time PCR	
HA-F3 (2749–2767)	5′-AAAACTGCYCTTGGAGCT-3′
HA-R3 (2873–2890)	5′-CAATCCRAATGTRGAATC-3′
TaqMan probe (2833–2855)	5′-FAM-ATGCHGTGTCTGGAGCACTGGATG-TAMRA-3′

Y = C, T. R = A, G. H = A, C, T.

Serum hepatitis A virus RNA quantification

Serum HAV RNA levels were quantitatively assessed by the two-step RT-PCR, which consisted of RT and amplification of cDNA by real-time PCR based on TaqMan chemistry. The extraction and synthesis of cDNA were carried out under the same conditions as those for the detection of HAV RNA, and the amplification of cDNA was carried out using a LightCycler instrument (Roche Diagnostics, Tokyo, Japan) according to the manufacturer's instructions. The primers set and probe consisted of forward primer HA-F3, reverse primer HA-R3 and a TaqMan probe (Table 2) derived from the VP1 region. Twenty microliters of the reaction mixture was added to the PCR tubes containing 1 μL of cDNA, 4.0 mmol $MgCl_2$, 0.5 μmol forward primer, 0.5 μmol reverse primer and 0.1 μmol TaqMan probe. Amplification was carried out by denaturation at 95°C for 10 s, 50 cycles of denaturation-annealing extension at 95°C for 10 s and 55°C for 21 s. The PCR product of 141 bp was purified using a High Pure PCR Product Purification kit (Roche Diagnostics) and applied to standard dilutions to construct the standard quantification curve. In this method, the quantitative linear range was 10^2–10^7 copies per ml of serum from the result of 10-fold serial dilution curves.

Genotyping of the hepatitis A virus strains

Direct sequencing was performed using the ALF Express DNA Sequencer (Amersham Biosciences, Tokyo, Japan). Sequencing analysis was carried out using GENETYX-WIN version 4 (Genetyx Co., Tokyo, Japan). The HAV genotype is defined as a group of viruses that differ from other strains by 15% or more in the VP1/2A junctional sequence (168 nucleotides) on the basis of the international criteria. Each genotype is divided into subgenotypes differing from each other at 7.5% in the VP1/2A junctional sequence.[21]

Statistical analysis

All data were expressed as mean ± SD. All of the HAV RNA determinations were analyzed after $\log_{10}$ transformation. Chi-squared analysis, Fisher's exact test and Student's *t*-test were used where appropriate in the present study. Pearson linear correlation was used to test the bivariate associations between the serum HAV RNA level and the various laboratory variables. To further investigate the association between the various variables and disease severity, multiple linear regression analysis was used for PT on the basis of univariable analysis of dependent variables. All *P*-values were two-tailed and $P < 0.05$ was considered significant. Statistical analysis was performed using STATVIEW 5.0 for Macintosh (SAS Institute Inc., NC, USA).

RESULTS

Detection of the serum hepatitis A virus RNA

Of the 58 patients with AH-A, all serum samples obtained on admission were positive for HAV RNA as determined by nested RT-PCR (Table 3). The phylogenetic analysis of the VP1/2A sequence revealed that 56 patients were of genotype IA (96.6%), which is the most predominant genotype in Japan, one was of IB (1.7%) and one was of IIIA (1.7%). The serum HAV RNA level was quantified in 53 patients (91.4%) on admission and 54 patients (93.1%) during the disease course. The mean viral load was 4.03 ± 1.62 log copies/mL on admission and 4.11 ± 1.52 log copies/mL at the peak.

Serial changes in serum hepatitis A virus RNA level according to severity of disease

To assess whether the immune response to the HAV influences the viral load decrease, we analyzed serial changes in the serum HAV RNA level in both the m-AH and s-AH groups. The mean period from the clinical onset to the first time point of assay at admission did not differ between the groups (7.9 ± 5.0 days in the m-AH group versus 10.8 ± 7.3 days in the s-AH group; $P = 0.1066$). The mean period from the clinical onset to the time point of the peak viral load was 8.5 ± 5.5 days in the m-AH group compared with 11.7 ± 7.6 days in the s-AH group ($P = 0.1978$). The serum HAV RNA levels after admission had reached their peak within 25 days in the m-AH group and 27 days in the s-AH group from the clinical onset. Although two of the 46 (4.3%) patients with m-AH had a peak viral load on admission when compared with two of the 12 (16.7%) patients with s-AH, there were no significant differences between the two groups (Fig. 1). Furthermore, the HAV RNA level in both groups decreased gradually in the same gradient after reaching the peak.

Clinical differences between mild and severe acute hepatitis A

The comparison of demographic and laboratory characteristics between the m-AH and s-AH groups is shown in Table 3. Regarding the background features of patients, the proportion of patients more than 40-years-old was significantly higher in the s-AH group than in the m-AH group (0/12 vs 17/29; $P = 0.0019$). Similarly, the proportion of patients with a history of heavy alcohol intake of more than 50 g of ethanol daily was significantly higher in the s-AH group than in the m-AH group (5/12 vs 2/45; $P = 0.0029$).

Regarding the peak laboratory data of the s-AH group compared with the m-AH group, there were significant increases in the white blood cell count, and the levels of FDP, bilirubin, AST, ALT, BUN, creatinine, IgM and C-reactive protein. Furthermore, there were

Table 3 Demographic and laboratory characteristics of mild and severe acute hepatitis A

Characteristics	Milder course ($n = 46$)	Severe course ($n = 12$)	P
Profile			
Age (years)			
<40	17	0	0.0019
>41	29	12	
Gender			
Male/Female	17/29	7/5	0.2052
Alcohol intake more than 50 g of ethanol daily	2 (4.3%)	5 (41.7%)	0.0029
Peak laboratory findings†			
White blood cells (per mm^3)	7479 ± 3262	10911 ± 4329	0.0037
Red blood cells ($\times 10^4$ per mm^3)	387 ± 42.9	329 ± 76.8	0.0011
Hemoglobin (g/dL)	11.5 ± 1.6	9.9 ± 2.5	0.0098
Hematocrit (%)	35.5 ± 4.3	30.3 ± 7.0	0.0019
Platelet ($\times 10^3$ per mm^3)	182 ± 75	110 ± 58	0.0034
Prothrombin time (%)	70.8 ± 21.3	27.2 ± 9.2	<0.0001
Fibrinogen (mg/dL)	216.2 ± 59.1	207.7 ± 83.4	0.6880
Anti-thrombin III (%)	75.0 ± 19.2	46.7 ± 16.4	<0.0001
Fibrin degradation product (μg/dL)	12.2 ± 21.1	29.8 ± 23.5	0.0181
Bilirubin (mg/dL)	7.7 ± 4.7	12.0 ± 4.1	0.0053
AST (IU/L)	2007 ± 1908	6585 ± 5993	<0.0001
ALT (IU/L)	2661 ± 1804	6156 ± 807	<0.0001
BUN (mg/dL)	14.1 ± 4.8	27.9 ± 19.7	<0.0001
Creatinine (mg/dL)	0.8 ± 0.2	2.7 ± 3.0	<0.0001
IgM (mg/dL)	529 ± 210	775 ± 402	0.0051
C-reactive protein (mg/dL)	1.7 ± 1.6	4.2 ± 3.4	0.0004
IgM-HA (cut-off index)	5.9 ± 1.7	6.4 ± 1.7	0.3666
Virological findings			
HAV RNA-positive no.	46 (100%)	12 (100%)	>0.9999
HAV genotype			
IA	44	12	0.7721
IB	1	0	
IIIA	1	0	
Peak viral load (log copies/mL)			0.5149
<4.00	17	3	
≥4.00	29	9	

†Values for the milder and severe courses are the means ± SD. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN; blood urea nitrogen; HAV, hepatitis A virus; IgM, immunoglobulin M.

significant decreases in the red blood cell count, hemoglobin level, hematocrit, platelet count, PT and AT-III level. However, there were no significant differences in the proportion of genotype and peak viral load between the two groups.

To further investigate the association between PT level and other variables, except for coagulation variables confounded with PT, we performed multiple linear regression analysis. In this analysis, the independent and significant variables associated with PT were ALT levels ($P = 0.0097$) and IgM levels ($P = 0.0098$). However, we found no significant correlation of PT with age, alcohol intake, hematological variables and levels of bilirubin, AST, BUN, creatinine and C-reactive protein.

Variables correlated with the serum hepatitis A virus RNA level

The relationship between the peak viral load and the peak values of variables was investigated in all of the patients (Table 4). As expected, a significant positive correlation of the peak viral load with the maximal ALT level was observed ($r = 0.363$; $P = 0.0048$) and a significant negative correlation with the minimal PT was also observed ($r = -0.330$; $P = 0.0110$). Interestingly, as the extrahepatic manifestation of HAV, a weak but significant positive correlation with the maximal C-reactive protein level was observed ($r = 0.270$; $P = 0.0400$) and a significant negative correlation with the minimal platelet counts was also observed ($r = -0.313$; $P = 0.0015$).

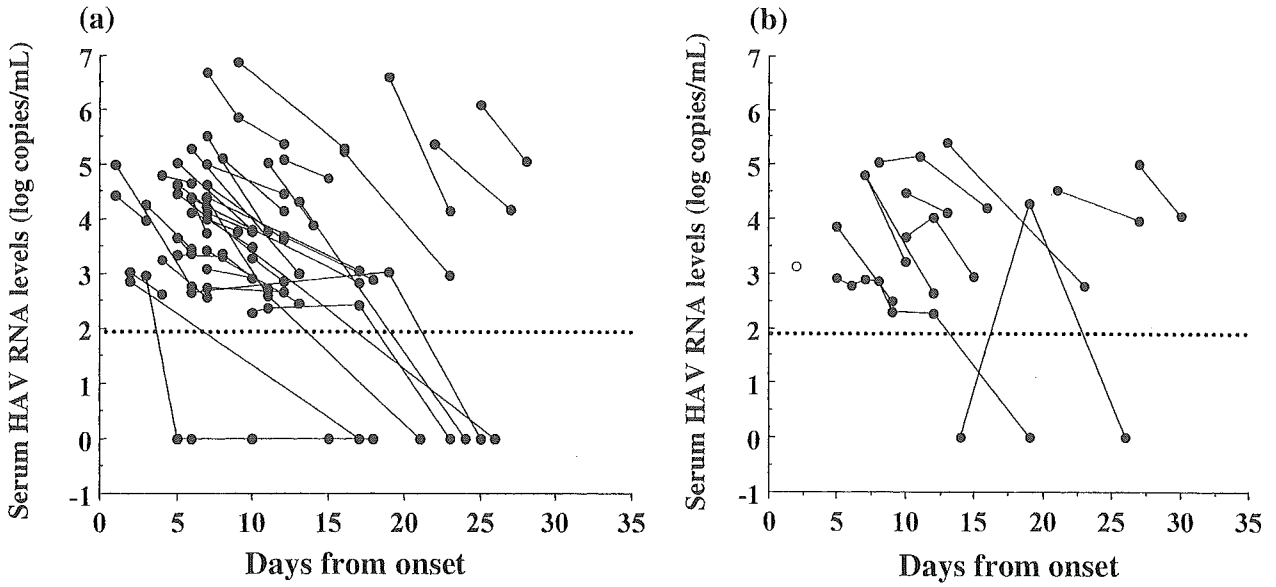

Figure 1 Serial changes in the serum hepatitis A virus (HAV) RNA level after the clinical onset in (a) mild ($n = 46$) and (b) severe ($n = 12$) AH. (····), quantification limit of the serum HAV RNA level; (○), fluminant case; (●), non-fluminant case.

Table 4 Correlation between the serum HAV RNA level and laboratory findings

Variables	$r^{\dagger}$	95% inferior	95% superior	P
White blood cells (per mm^3)	0.244	−0.015	0.473	0.0643
Red blood cells (×10^4 per mm^3)	−0.085	−0.336	0.177	0.5263
Hemoglobin (g/dL)	−0.064	−0.317	0.198	0.6362
Hematocrit (%)	−0.061	−0.315	0.200	0.6491
Platelet (×10^3 per mm^3)	−0.313	−0.529	−0.053	0.0015
Prothrombin time (%)	−0.330	−0.542	−0.060	0.0110
Fibrinogen (mg/dL)	0.086	−0.178	0.339	0.5246
Anti-thrombin III (%)	−0.209	−0.445	0.057	0.1227
Fibrin degradation product (μg/dL)	0.263	0.001	0.492	0.0495
Bilirubin (mg/dL)	−0.007	−0.265	0.252	0.9603
AST (IU/L)	0.210	−0.051	0.444	0.1139
ALT (IU/L)	0.363	0.115	0.568	0.0048
BUN (mg/dL)	0.074	−0.188	0.326	0.5823
Creatinine (mg/dL)	0.063	−0.199	0.316	0.6400
IgM (mg/dL)	0.013	−0.249	0.272	0.9261
C-reactive protein (mg/dL)	0.270	0.013	0.494	0.0400
IgM anti-HAV (cut-off index)	−0.231	−0.462	0.029	0.0808

†Pearson correlation coefficient. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN; blood urea nitrogen; HAV, hepatitis A virus; IgM, immunoglobulin M.

In addition, a weak but significant positive correlation with the FDP level was also observed ($r = 0.236$; $P = 0.0495$).

In a detailed investigation of disease severity, there was a significant negative correlation of viral load with the minimal PT level in the m-AH group ($r = -0.400$; $P = 0.0059$), but not in the s-AH group (Fig. 2a). Similarly, there was a significant positive correlation of viral load with the maximal ALT levels in the m-AH group ($r = 0.462$; $P = 0.0012$), but not in the s-AH group (Fig. 2b). These results indicate that serum HAV plays an important role in hepatic injury, but other factors in addition to viral load might be associated with the development of severe hepatitis.

Comparison of peak laboratory findings according to peak viral load

To assess the influence of serum HAV on the laboratory variables, we compared the laboratory variables that