

Figure 6 Inter-reagent variation in prothrombin time (PT) activity percent in the patients with liver disease. The horizontal line indicates the mean PT activity percent values measured using four different reagents in each samples. (a) The vertical lines indicate the maximum difference among the four values in each sample and (b) the ratio of the maximum difference to the mean value in each sample.

(Fig. 6) and the concerned range of the PT activity in clinical practice is below 80%, the inter-reagent variability and calibrant-dependent variability were examined regarding PT activity percent values lower than 80% (Table 3).

When the individually prepared calibrant was used, the maximum differences in the mean PT activity percent among the four thromboplastins was as high as 7.30%. The variability was relatively adjusted using common calibrants for RecombiPlastin and Thromborel S to 3.83 and 4.86, respectively.

On the other hand, the difference in the mean PT activity percent values among the reagents was very small (0.9) among the three thromboplastins except RecombiPlastin, even when the values were calculated using individually prepared calibrants.

DISCUSSION

ALTHOUGH THE PT is known to be a reliable marker of liver function, it is still controversial whether the activity percent and INR are appropriate for

reporting the PT in patients with liver disease. In particular, in clinical practice for patients with ALF, the PT is indispensable for the diagnosis, prediction of prognosis and indications for liver transplantation. In Japan, ALF has been designated as an intractable disease on the national list, and the Japanese government has conducted many studies on ALF, including national surveys,¹³⁻¹⁵ to establish and revise the diagnostic criteria^{8,16} or the indications for liver transplantation,¹⁷ and to develop potentially effective treatments for the disease. The diagnostic criteria have recently been revised to include the INR reporting style,^{8,16} in addition to the activity percent used in the previous version of criteria, although the criteria did not advocate the adoption of any of these reporting styles as being appropriate in clinical practice for ALF patients.

To compare the usefulness of these different PT reporting styles, we examined whether the INR or PT activity percent is more closely associated with the hepatic synthetic function, and which values, INR_w, INR_{LD} or PT activity percent, demonstrate low inter-reagent variation.

Table 3 Comparison of the mean values of the PT activity percent in patients with liver disease among the four thromboplastin reagents in patients with liver disease

	Individual calibrant		Common calibrant for RecombiPlastin		Common calibrant for Thromborel S	
	Mean	SD	Mean	SD	Mean	SD
RecombiPlastin	48.1	18.2	48.1	18.1	45.8	17.1
Thrombocheck PT	40.8	17.9	45.5	20.6	42.4	17.6
Thromborel S	41.0	15.7	44.5	17.6	41.0	15.8
Coagpia PT-N	41.7	19.3	44.3	19.3	43.5	19.5
Maximum difference	7.30		3.83		4.86	

PT, prothrombin time; SD, standard deviation.

As the gold standard marker for the hepatic synthetic function of clotting factors, we selected the plasma factor X level because only the factor X level, and not the level of factor VII and II, exhibited significant differences between the patients with and without hepatic encephalopathy. These findings indicate that the plasma factor X level mainly reflects the protein synthetic function of the liver through the PT value in relation to the severity of liver damage. In addition, this is supported by the fact that PT values correlated with the level of factor X more strongly than the levels of factors VII and II (prothrombin). Both INR_w and INR_{LD} exhibited closer correlations with the factor X level than PT activity percent. This finding suggests that the INR reporting system more closely reflects the liver synthetic function than PT activity percent and thus is more useful as a marker of the liver function.

The PT depends largely on plasma concentration and activity of clotting factors, including prothrombin, X, V, VII, IX and fibrinogen, all of which are synthesized uniquely by hepatocytes. The plasma levels of these factors, therefore, almost evenly decrease in the patients with liver disease according to the severity of liver dysfunction. In contrast, only vitamin K-dependent factors, namely, prothrombin and factors VII and X, decrease in patients receiving warfarin treatment. Furthermore, only the functional levels, not the antigen levels, of these factors decrease in warfarin-treated patients because warfarin does not affect the synthesis of clotting factors in hepatocytes, although it does disturb the maturation of these factors. Therefore, the prolongation of clotting time observed in PT measurement among patients with liver disease may be the result of uniform decreases of all clotting factors, whereas that observed in warfarin-treated patients may result from functional decreases in prothrombin and factors VII and X. The differences in the profiles of clotting factors may be reflected in the differences in ISI between liver disease patients and warfarin-treated patients.^{18,19} Therefore, liver disease-specific ISI instead of conventional ISI (ISI_w) is needed to be defined for evaluating liver function with PT, as demonstrated in the present study.

The system used to calibrate the activity percent employs serial titration of normal plasma, which is considered to simulate uniform decreases in the levels of multiple clotting factors in patients with liver disease. However, it has been reported that the PT clotting time is not equally influenced by the plasma activity of each clotting factor, rather it is largely influenced by the activity of factors VII and X but not that of other factors.²⁰ We believe that the specific effects of these vitamin

K-dependent factors on the INR value explains why the INR is more closely correlated to the plasma factor X level than the PT activity percent.

With regard to the inter-reagent variation in the PT activity percent reporting system, the difference in the mean PT activity percent values between the reagents was very small (0.9) among the three thromboplastins except for RecombiPlastin. There were few cases with a PT activity percent in the range below 20%, as measured by RecombiPlastin, although many cases were distributed in this range when measured by the other three thromboplastins (data not shown). This finding suggests that the PT activity percent measured by RecombiPlastin does not accurately evaluate the area below 20%, although RecombiPlastin is thought to be a preferential thromboplastin for INR measurement provided by the WHO, judging from the results of local ISI determination.

Regarding the inter-reagent variation in the INR reporting system, the INR_{LD} exhibited little inter-reagent variation, in contrast to the INR_w , when the PT-INR was measured in the liver disease patients. This finding suggests that INR_{LD} but not INR_w should be used to evaluate the liver function.

In conclusion, INR_{LD} is the most appropriate PT reporting method for use in patients with liver disease, although standardization of ISI_{LD} determination is needed.

ACKNOWLEDGMENTS

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Case Report

Elevation of serum cytokines preceding elevation of liver enzymes in a case of drug-induced liver injury

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A 50-year-old man who was being treated for both pneumonia and type 2 diabetes mellitus complained of abdominal distention on the 16th hospital day. Liver enzyme elevation without symptoms was detected on the 17th hospital day. Based on a Roussel Uclaf Causality Assessment Method score of 10 and a Japan Digestive Disease Week score of 9, we diagnosed the patient as having drug-induced liver injury (DILI). Simultaneous assays of the levels of cytokines revealed that the elevation of the levels of interleukin (IL)-1 β , IL-10, IL-12, IL-13 and

tumor necrosis factor- α preceded the elevation of the serum liver enzymes. This case suggests that some cytokines or related molecules are potentially useful as early-phase biomarkers for DILI.

Key words: Bio-Plex, interleukin-1 β , monocytic chemotactic protein-1, tumor necrosis factor- α

INTRODUCTION

DRUG-INDUCED LIVER INJURY (DILI) is the most common cause of death from acute liver failure in the USA¹ and has become a serious health problem. In order to predict and treat DILI, the detailed mechanisms underlying its development must be clarified. However, the pathogenesis of DILI remains unclear because the diagnosis is usually retrospective.

A subset of patients with DILI present with clinical findings associated with allergic reactions, such as rashes or eosinophilia.² These reactions in patients with DILI are associated with several cytokines.^{3,4} Therefore, cytokine interactions may play an important role in the pathogenesis of DILI.

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CASE REPORT

A 50-YEAR-OLD MAN who was being treated for type 2 diabetes mellitus and alcoholic liver injury with insulin by a general physician visited our department complaining of dyspnea and pyrexia. Moist rales were detected in the left lower lung. Cardiac and abdominal examinations were unremarkable. The laboratory data revealed leukocytosis, liver injury and hyperbilirubinemia: white blood cell (WBC) count, 14700/mL; alanine aminotransferase (ALT), 225 IU/L; and γ -glutamyl transpeptidase (γ -GTP), 1090 IU/L. Chest radiography revealed an infiltrative shadow accompanied by an air bronchogram in the right upper lobe. The patient was diagnosed with alcoholic liver injury and pneumonia. The pneumonia was treated with several antibiotics: tazobactam/piperacillin (TAZ/PIPC, 9 g/day) from the first hospital day to the seventh hospital day, micafungin (MCFG, 75 mg/day) from the eighth hospital day to the 17th hospital day and levofloxacin (LVFX, 500 mg/day) from the eighth hospital day to the 17th hospital day. On the 15th hospital day, the pneumonia improved and the liver enzyme level returned to normal. However, the patient complained of right upper abdominal distention on the 16th hospital day. Although this symptom rapidly disappeared after 4 h,

asymptomatic liver injury was detected on the 17th hospital day: ALT, 666 IU/L; γ -GTP, 621 IU/L; and alkaline phosphatase, 2113 IU/L (Fig. 1 and Table 1). No causes of acute liver injury, such as cholelithiasis, viral infection or autoimmune disease, were detected (Supporting Information Fig. S1). Therefore, a diagnosis of DILI due to antibiotics was suspected, and all medications were discontinued, except for insulin. The liver enzyme elevation improved by the 22nd hospital day without specific therapy, and the patient was discharged on the 26th hospital day. Although drug-induced lymphocyte stimulation test (DLST) was performed for TAZ/PIPC, MCFG and LVFX, DLST for all these medicines was negative.

The Roussel Uclaf Causality Assessment Method score in this case was 10 and the Japan Digestive Disease Week score was 9 (Table 2). According to the patient's clinical course, the antibiotics were considered to be the causal drugs (Fig. 1). Serum samples were collected on the 15th hospital day, when the serum liver enzyme levels were within the normal limits, and it was 2 days before marked elevation in the liver enzymes levels was observed. Serial changes in the cytokine levels were simultaneously evaluated with the Bio-Plex 200 (Bio-Rad, Tokyo, Japan), and the values were calculated using

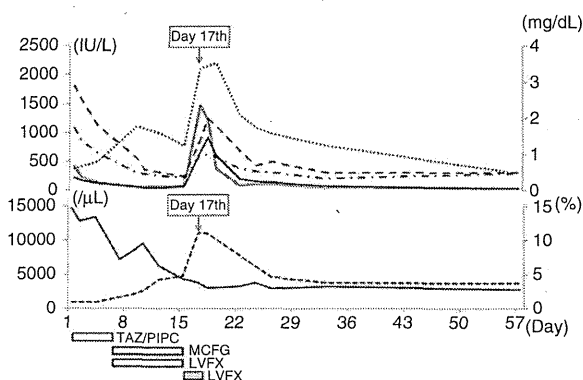


Figure 1 Time course of the laboratory data of the present patient with drug-induced liver injury. The upper line chart presents several biochemical parameters, including the levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ -glutamyl transpeptidase (γ -GTP) and total bilirubin (T-Bil). The lower line chart presents the white blood cell (WBC) count and the proportion of cells exhibiting eosinophilia relative to the total number of WBC. The bar chart indicates the duration of each antibiotic. —, AST; —, ALT; ·····, ALP; -·-·, γ -GTP; - - -, T-Bil; —, WBC; ---, eosinophils; □, drip infusion; □, oral medicine. MCFG, micafungin; LVFX, levofloxacin; PIPC, piperacillin; TAZ, tazobactam.

the Bio-Plex manager software program, version 5.0 (Bio-Rad). The levels of interleukin (IL)-1 β , IL-10, IL-12, IL-13 and tumor necrosis factor (TNF)- α were elevated before the liver enzyme elevation (Fig. 2). The levels of IL-4, IL-5, IL-6, IL-8, IL-17, monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1 β (MIP-1 β) immediately became elevated after the liver enzyme elevation and then dramatically decreased 2–3 days after peaking (Fig. 2).

DISCUSSION

DRUG-INDUCED LIVER INJURY is classified into two types: the intrinsic type and the idiosyncratic type.^{2,5,6} Most cases of DILI are idiosyncratic, accounting for 13% of cases of acute liver failure in the USA.¹ In order to prevent and treat idiosyncratic DILI, the pathogenesis of the condition must be understood. However, because DILI is usually diagnosed retrospectively, the detailed mechanisms underlying the development of DILI remain unclear. Because a subset of idiosyncratic DILI patients present with rashes, fever or eosinophilia, the disease is considered to be associated with the immune response.² Therefore, cytokine interactions may play an important role in the pathogenesis of DILI.

We first reported the simultaneous evaluation of serial changes in the levels of several cytokines before the initiation of liver injury in humans and found that the elevation of the IL-1 β , IL-10, IL-12, IL-13 and TNF- α levels preceded the liver enzyme elevation. These findings suggest that these cytokines play important roles in the initial stage of DILI. Because alcoholic liver injury and pneumonia were present as pre-existing diseases in this case, the levels of several cytokines were not within the normal ranges on the 15th hospital day, although the patient was asymptomatic. Because we did not store any samples before the 15th hospital day in this case, we were not able to ascertain what cytokine was the initiation cytokine in onset of DILI. Sustained high levels of several cytokines in patients with pneumonia or alcoholic liver injury after treatment have been previously reported.^{7–10} Therefore, the influence of alcoholic liver injury and pneumonia on the cytokine levels cannot be completely excluded in this case. In fact, the levels of most cytokines in this case were high to normal even within 52 days after the administration of treatment.¹¹ However, the levels of several cytokines that were high before onset immediately decreased after the administration of antibiotics was discontinued as shown in the profile of IL-1 β . Therefore, these cytokines acted as pre-conditioning cytokines in this case.

Table 1 Laboratory data of the present patient with drug-induced liver injury on the 1st and 17th hospital days

Administration			17th hospital day					
Hematology			Virus markers			Hematology		
WBC	14.7	10 ³ /mL	HBsAg	(-)		WBC	3.1	10 ³ /mL
Neutrophils	81.2	%	HCVAb	(-)		Neutrophils	44.0	%
Lymphocytes	16.6	%	IgM HA	(-)		Lymphocytes	33.1	%
Monocytes	1.9	%	HSV IgM	(-)		Monocytes	8.0	%
Eosinophils	0.1	%	HSV IgG	(+)		Eosinophils	11.1	%
Basophils	0.1	%	CMV IgM	(-)		Basophils	3.8	%
RBC	412	10 ⁶ /mL	CMV IgG	(+)		RBC	447	10 ⁶ /mL
Hb	13.9	g/dL	EBVCA IgG	(-)		Hb	14.4	g/dL
Plt	99	10 ³ /mL	EBVCA IgM	(-)		Plt	369	10 ³ /mL
			EBNA Ab	(-)				
Blood chemistry			Autoantibodies			Blood chemistry		
TP	6.6	g/dL	ANA	<x40		TP	6.8	g/dL
Albumin	3.3	g/dL	AMA	(-)		Albumin	3.4	g/dL
T-Bil	2.9	mg/dL				T-Bil	1.2	mg/dL
AST	424	IU/L	Tumor markers			AST	1484	IU/L
ALT	225	IU/L	CEA	2.1	ng/mL	ALT	666	IU/L
ALP	385	IU/L	CA19-9	17.3	U/mL	ALP	2113	IU/L
γ-GTP	1090	IU/L	AFP	2.3	ng/mL	γ-GTP	621	IU/L
ChE	138	IU/L	PIVKA-2	21	mAU/mL	ALP	2113	IU/L
BUN	22.6	mg/dL				BUN	10.8	mg/dL
Cre	1.23	mg/dL				Cre	1.03	mg/dL
AMY	33	IU/L				AMY	97	IU/L
NH3	57	mg/dL				NH3	36	mg/dL
CRP	36.13	mg/dL				CRP	0.26	mg/dL
Blood coagulation			Blood coagulation			Blood coagulation		
PT	65	%				PT	72	%
HPT	54	%				HPT	64	%
Fib	643	mg/dL				Fib	406	mg/dL
FDP	11.8	mg/mL				FDP	2.1	mg/mL

Ab, antibody; AFP, α -fetoprotein; Ag, antigen; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AMA, antimitochondrial antibody; AMY, amylase; ANA, antinuclear antibody; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; ChE, choline esterase; CMV, cytomegalovirus; Cre, creatinine; CRP, C-reactive protein; EB, Epstein-Barr virus; EBNA, Epstein-Barr virus nuclear antigen; EBVCA, Epstein-Barr virus capsid antigen; FDP, fibrin degradation products; Fib, fibrinogen; HA, hepatitis A virus; Hb, hemoglobin; HB, hepatitis B virus; HCV, hepatitis C virus; HPT, hepaplastine test; HSV, Herpes simplex virus; Ig, immunoglobulin; NH3, ammonia; PIVKA-II, protein induced by vitamin K absence/antagonist-II; Plt, platelets; PT, prothrombin time; RBC, red blood cells; T-BIL, total bilirubin; TP, total protein; WBC, white blood cells; γ -GTP, γ -glutamyl transpeptidase.

We hypothesized the following mechanism of liver injury in the present case. Because IL-1 β and TNF- α , which are pro-inflammatory cytokines, were at a high level before the elevation of liver enzymes, these cytokines functioned as preconditioning cytokines. IL-10, an anti-inflammatory cytokine, was also at a high level at that time. The IL-10 level may be elevated as a reaction to high levels of pro-inflammatory cytokines. The IL-1 β , TNF- α and IL-10 levels were decreased after the administration of antibiotics was discontinued. In contrast, the elevation of IL-12, which is secreted from

activated hepatocytes, and IL-13, which is secreted from T-helper (Th)2 cells, was sustained at a high level for several days after the elevation of the liver enzymes. However, the role of sustained high levels of IL-12 and IL-13 remains unclear. Several cytokines originating from Th2 cells, such as IL-4, IL-5 and IL-6, in addition to IL-17 from Th17 cells, and several chemokines, such as IL-8, MCP-1 and MIP-1 β , were increased following the elevation of liver enzymes and rapidly decreased after several days of elevation of liver enzymes. Therefore, these cytokines and chemokines may have been elevated

Table 2 Assessments used to diagnose drug-induced liver injury

RUCAM score			J-DDW score			
Type of liver injury	Cholestatic/mixed		Type of liver injury	Cholestatic/mixed		
Time of onset of the event	First exposure		Time of onset of the event	Initial treatment		
Time from drug intake until reaction onset	5–90 days	2	Time to onset	After cessation of the drug from the beginning of the drug	5–90 days	2
Alcohol or pregnancy risk factor	Present	1	Risk factors	Presence of ethanol or pregnancy	Alcohol	1
Age risk factor	≥55 years	1				
Course of the reaction	≥50% improvement 180 days	2	After cessation of the drug	Difference between the peak of ALP and upper limit of normal value	Decrease >50% within 180 days	2
Exclusion of non drug-related causes	Ruled out	2	Search for non drug causes	All causes – groups I and II – reasonably ruled out	Ruled out	2
Previous information on hepatotoxicity	Reaction labeled in the product's characteristics	2	Previous information on hepatotoxicity	Reaction labeled in the product characteristics	+	1
			Eosinophilia (>6%)	With eosinophilia	+	1
			DLST	Negative or unavailable	Negative	0
	Total	10			Total	9

DLST, drug-induced lymphocyte stimulation test; J-DDW, Japan Digestive Disease Week; RUCAM, The Roussel Uclaf Causality Assessment Method.

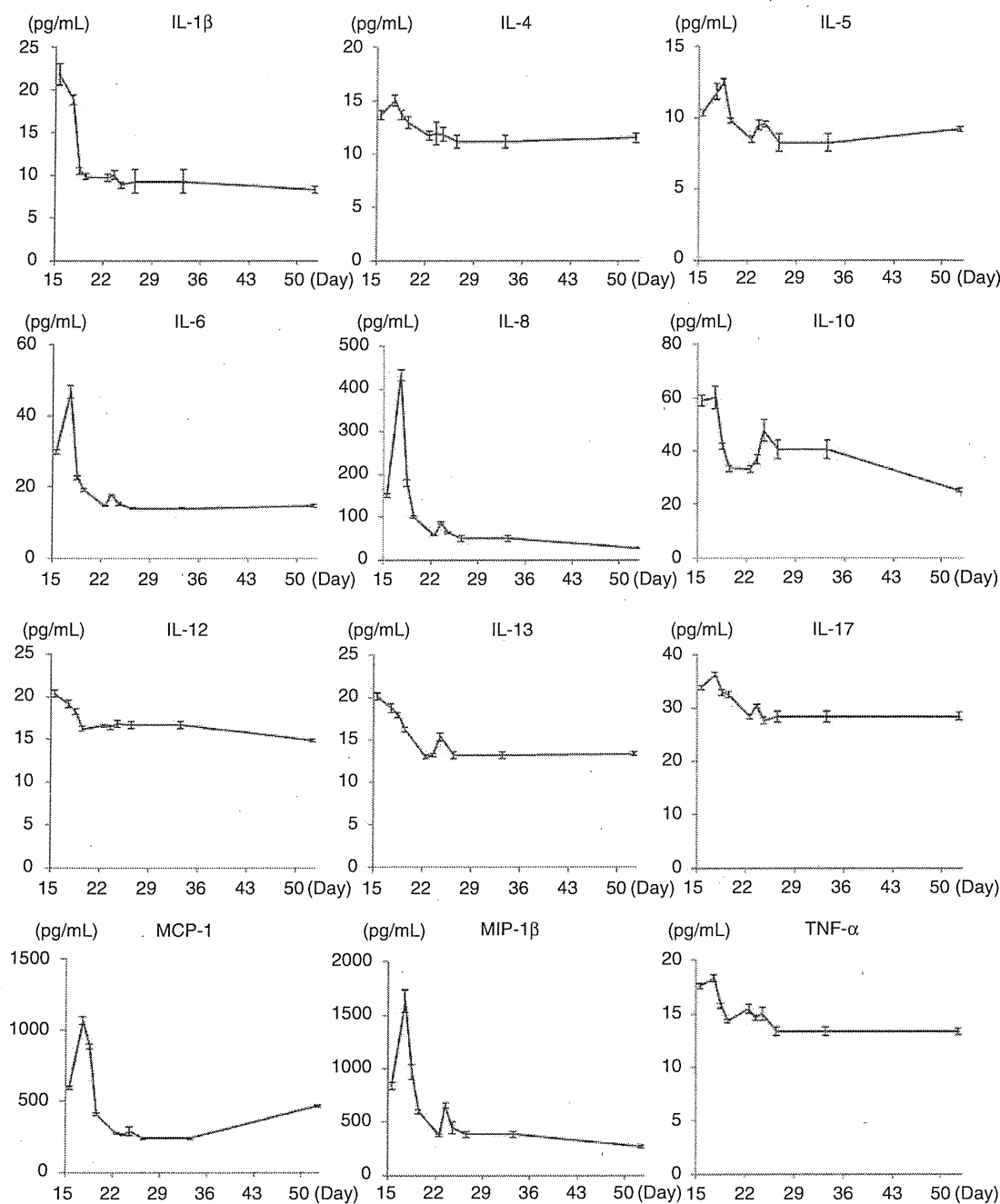


Figure 2 Simultaneous evaluation of serial changes in the levels of several cytokines before the onset of drug-induced liver injury. The line charts indicate the serial changes in the levels of cytokines evaluated using BioPlex, including interleukin (IL)-1 β , IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IL-17, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 β (MIP-1 β) and tumor necrosis factor- α (TNF- α). We collected serum samples on the 15th, 17th, 18th, 19th, 22nd, 23rd, 24th, 26th, 33rd and 52nd days. The normal ranges of several cytokines have been previously reported by us. The normal range of IL-1 β , IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IL-17 and TNF- α is up to 2.0 pg/mL. The normal range of MCP-1 or MIP-1 β is up to 174.8 pg/mL or 159.3 pg/mL, respectively.

as enhanced or inhibited factors due to the influence of IL-1 β , TNF- α and IL-10. Because IL-1 β , TNF- α and IL-10 were secreted from macrophage or antigen-presenting cells, these cells played an initial important role in the development of liver enzyme elevation in this case.

Intriguingly, the inhibition of IL-1 β was found to attenuate liver damage in an animal model of DILI.¹² IL-10 and TNF- α polymorphisms are associated with DILI.⁴ The identification of early-phase biomarkers for DILI is urgently needed because the prognosis of patients with overt idiosyncratic DILI remains very poor. The present case report therefore suggests that early-phase cytokines or some related molecules may be potentially useful as early-phase biomarkers for DILI. Although interaction between preceding inflammatory diseases and the cytokines at the onset of DILI and the mechanisms through which cytokines interact in patients with DILI remain unclear, this case report may provide new insight into the initial stages of DILI.

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SUPPORTING INFORMATION

ADDITIONAL SUPPORTING INFORMATION may be found in the online version of this article at the publisher's website:

Figure S1 Imaging findings of the present patient with drug-induced liver injury on the 17th hospital day. (a) Abdominal computed tomography showed mild splenomegaly and without dilatation of the intrahepatic biliary ducts. (b) Magnetic resonance cholangiopancreatography showed no evidence of obstructive jaundice or cholelithiasis.

Proliferation of mouse liver stem/progenitor cells induced by plasma from patients with acute liver failure is modulated by P2Y₂ receptor-mediated JNK activation

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Abstract

Background We recently reported that acute liver failure plasma (ALF-P) promotes the proliferation of mouse liver oval cells (OCs) through c-jun N-terminal kinase (JNK) activation. The aim of this study was to investigate the mechanism by which ALF-P induces JNK activation and OC proliferation.

Methods OCs and primary hepatocytes were exposed to ALF-P or normal control plasma (NC-P). Cell proliferation and activation of JNK and other JNK signaling molecules were detected subsequently. Next, we determined the effects of extracellular adenosine triphosphate (ATP) and ATP receptors on ALF-P-stimulated cell growth. Finally, the relationship between the tumor necrosis factor alpha (TNF α) and ATP receptor pathways was investigated.

Results Cell proliferation accompanied by JNK activation was only observed in ALF-P-stimulated OCs. ALF-P stimulated the activation of SEK1/MKK4 and ATF2, but not c-Jun. Both PPADS (pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid) treatment and P2Y₂ (G-protein-coupled) small interfering RNA (siRNA) transfection blocked the effects of ALF-P on cell proliferation and JNK activation. However, ATP levels in ALF-P were significantly lower than that in NC-P, and ATP did not stimulate the

proliferation of OCs. On the other hand, TNF α stimulated JNK activation and proliferation of OCs. TNF α receptor antagonist partly inhibited the ALF-P-stimulated proliferation of OCs. Moreover, PPADS significantly inhibited TNF α -stimulated cell proliferation, induced apoptosis, and inhibited the activation of JNK. However, our data showed no significant difference in plasma TNF α levels between the NC-P and ALF-P samples.

Conclusions JNK activation induced by P2Y₂ receptor crosstalk with the TNF α signaling pathway is important in mediating the effects of ALF-P on the proliferation and survival of OCs.

Keywords ALF-P · JNK · OCs · P2Y · TNF α

Abbreviations

ALF	Acute liver failure
ALF-P	ALF plasma
NC-P	Normal control plasma
HPCs	Hepatic progenitor cells
OC	Oval cell
JNK	c-jun N-terminal kinase
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
AFP	Alpha-fetoprotein
Alb	Albumin
CK19	Cytokeratin 19
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
TAT	Tyrosine aminotransferase
G6Pase	Glucose-6-phosphatase
IL	Interleukin
IRB	Institutional review board
HGF	Hepatocyte growth factor
TUDC	Tauroursodeoxycholate
EGF	Epidermal growth factor

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IFN	Interferon
TGF β	Transforming growth factor beta
TNF	Tumor necrosis factor
TNFR	TNF receptor
AP-1	Activating protein 1
ATP	Adenosine triphosphate
PPADS	Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid
DAPI	4',6-diamidino-2-phenylindole dihydrochloride
PI	Propidium iodide
ATF2	Activating transcriptional factor 2
RT-PCR	Reverse transcriptase-polymerase chain reaction
siRNA	Small interfering RNA
TPE	Total plasma exchange
RLUs	Relative light units

Introduction

In spite of its vigorous capacity for regeneration in response to resection of more than 50 % of its mass, the liver becomes disabled in the case of acute liver failure (ALF). ALF is a fatal clinical syndrome characterized by the sudden initiation of irreversible hepatocyte death, which leads to hepatic encephalopathy and finally to multi-organ failure [1, 2]. Although liver transplantation is currently the best option for improving the survival rate, the rapid progression and variable course of ALF limit its use [2]. As an alternative to transplantation, developing effective methods to improve the regenerative capacity of the failing liver is regarded as an ideal goal that could save patients' lives.

Hepatic progenitor cells (HPCs), also named oval cells (OCs) in rodents, have attracted a great deal of attention because they appear only when the regenerative capacity of the liver is blocked [3, 4]. Because they generate hepatocytes and biliary epithelial cells [5, 6], HPCs are generally recognized as a major resource for pathological liver repair. However, clinical studies have provided evidence that extensive HPC activation is negatively correlated with the survival rate of ALF patients and is a sign of disease severity [7]. The findings indicate that in ALF, rather than differentiating into functional hepatocytes, HPCs continue proliferating, which might impair liver regeneration. Investigating the mechanism underlying the proliferation of HPCs in ALF is therefore of clinical significance.

Plasma or serum is usually used to evaluate the *in vivo* microenvironment. The cytotoxicity of plasma or serum from patients with ALF has been demonstrated. The cytotoxic effects include inhibiting the proliferation of

hepatocytes, interfering with protein synthesis, and causing cell death [8–11]. Conversely, ALF plasma (ALF-P) was not toxic to the function of primary rat hepatocytes in three-dimensional culture [12]. Our previous study showed that ALF-P stimulated the proliferation of OCs. This effect was blocked by a specific c-Jun N-terminal kinase (JNK) inhibitor (SP600125), which indicates that JNK activation is required for the ALF-P-stimulated proliferation of OCs [13]. Although the relationship between JNK and the proliferation and death of hepatocytes is well-described [14, 15], the upstream molecular mechanism that leads to the activation of JNK signaling in OCs stimulated by ALF-P remains unknown.

Extracellular adenosine triphosphate (ATP), an important signaling molecule in the inflammatory and cellular stress responses, was recently reported to affect liver regeneration by activating JNK signaling [16]. ATP exerts its function through P2 receptors. Mammalian P2 receptors consist of ion channels (P2X) and G-protein-coupled (P2Y) subtypes. P2Y receptors control glycogen metabolism and proliferation-associated responses, such as increased $[Ca^{2+}]$ and mitogen-activated protein kinase cascades, in primary human hepatocytes [17]. Among the members of the P2Y receptor family, the P2Y₂ receptor mediates the ATP-activated JNK pathway and consequently contributes to hepatocyte proliferation *in vitro* and cell cycle progression in rat liver after partial hepatectomy [16]. However, ATP and the P2Y₂ receptor promote cell death in mice with acute liver injury [18, 19]. These findings appear to reflect dual roles of ATP receptors in the regulation of cell growth under pathological conditions.

Tumor necrosis factor (TNF) signaling via TNF receptor (TNFR)-1 is one of best-studied pathways leading to JNK activation. The initial TNFR-1-mediated JNK activation is transient and associated with cell survival and proliferation through activating protein 1 (AP-1), whereas sustained JNK activation is closely related to TNF α -induced programmed cell death in the liver [14, 15, 20–22]. Moreover, TNF α takes a central role in the pathogenesis of ALF. The levels of circulating TNF α are increased in ALF patients and are associated with a poor prognosis [23–25].

Notably, a close relationship exists between extracellular ATP and TNF α in the development of ALF. TNF α mediates hepatic apoptosis during ALF, and the apoptosis itself is a highly ATP-dependent process. ATP depletion upon massive cell injury induces the expression of inflammatory cytokines including TNF α . However, little is known about the roles of ATP, TNF α , and their pathways in the regulation of OCs in ALF.

Here, we demonstrate that one of the P2Y receptors, P2Y₂ subtype, activates JNK and plays an important role in mediating the proliferative and anti-apoptotic effects of

ALF-P in OCs. The present study also provides evidence that P2Y₂ receptor signaling in ALF-P is ATP-independent and closely linked with the TNF α receptor signaling pathway.

Materials and methods

Patients

Three patients with ALF in maximum coma grades 2, 3, and 5 were studied. The etiology of the three patients were B hepatitis in two cases and non-A and non-B hepatitis in one. The patients consisted of two males and one female with an age range of 53–68 years. None of the patients survived. The laboratory parameters were shown as total bilirubin of 11.4 (range 4.6–14.9) mg/dL, aspartate aminotransferase (AST) 2534 (626–5496) IU/L, alanine aminotransferase (ALT) 1307 (1173–1480) IU/L, total bile acid 217.5 (177.0–318.0) μ M/L, prothrombin time 27.7 (22.4–32.4) %, alpha-fetoprotein (AFP) 738.4 (2.0–2149.0) ng/mL, and hepatocyte growth factor (HGF) 2.06 (0.58–3.70) ng/mL. Control samples were taken from three normal subjects, one male and two females with an age range of 35–45 years. Blood in the acute phase of the disease was taken on ice into endotoxin-free heparinized vacutainers. The blood was centrifuged at 1,500g for 10 min. The resulting plasma was stored in aliquots at –80 °C. Approval for the study was obtained from the institutional review board (IRB, H19-87) of Iwate Medical University, Morioka, Japan, and informed consent was obtained from the patients' relatives.

Oval cells

A cell line of mouse liver stem/progenitor cells was established and provided by Professor Atsushi Miyajima and Dr. Minoru Tanaka of Tokyo University [26].

Isolation and primary culture of mouse hepatocytes

Mouse hepatocytes were isolated by the two-step collagenase perfusion method using 0.025 % collagenase [27]. The hepatocytes were plated in collagen 1-coated dishes. After the cells were attached, the medium was changed to serum-free medium for the proliferation assay.

Proliferation assay

Serum-starved oval cells or primary hepatocytes (3×10^4 cells/mL) were treated with 30 % ALF-P, 30 % normal control plasma (NC-P), recombinant murine tumor necrosis factor α (rmTNF α : R&D systems, USA), or the

adenosine triphosphate receptor agonists (ATP: R&D systems, USA; ATP γ S and 2MeSATP: Sigma-Aldrich, Germany) with and without the relative inhibitors (R-7050: Calbiochem, USA; pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADs), NF023, and Apyrase: Sigma-Aldrich, Germany; AG1478 and SB431542: Santa Cruz, USA) for the indicated period. The number of viable cells were evaluated by adding the cell count reagent SF (Nacalai Tesque Inc., Japan) directly to the cells [28]. Absorbance was measured at 450 nm by a microplate photometer (Immuno Mini NJ-2300: InterMed, Japan).

Evaluation of apoptosis and live/dead cell staining

Oval cells were seeded at a density of 5×10^4 cells/mL into sterile culture plate and left overnight for adherence. After serum starvation, cells were treated with 30 % ALF-P, 30 % NC-P, or TNF α with and without PPADs and NF023 for 72 h. Apoptotic cells were stained by the 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Dojindo, Japan). In addition, Live-dyeTM (MBL, USA), a cell-permeable green fluorescent dye and a non-cell-permeable red fluorescent dye propidium iodide (PI, MBL, USA) were used for the differential staining of live and dead cells, according to manufacturer's instructions. The cells were scored under inverted fluorescence microscopy (ECLIPSE TE300: Nikon, Japan) at 20 \times magnification, and were photographed using a digital camera (DXC-S500/OL; Olympus, Tokyo, Japan).

Western blot analysis

Total protein was isolated from the OCs using a total protein extraction kit from BioChain Institute Inc. (Hayward, CA, USA). A total of 20 μ g of protein from each sample was separated using 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrotransferred onto a polyvinylidene difluoride membrane. Immunoblotting was performed using specific antibodies against p-JNK, p-ATF2, p-MKK4, p-c-Jun (Cell Signaling Technology, USA), and β -actin (Santa Cruz Biotechnology, USA). The immunoreactive bands were visualized with an enhanced chemiluminescence reagent (Amersham Biosciences, UK).

ATP determination assay

The plasma ATP concentration was measured using an ATP assay kit (Toyo-ink, Japan), according to the manufacturer's instructions. In brief, plasma ATP was extracted using the ATP extraction reagent. Luminescence reagents were added to the samples and the relative light units (RLUs) were measured immediately with the Lumitester

C-110 (LuminUltra Technologies Ltd., Canada). The method of standard additions was used to convert RLU into plasma ATP concentration.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted using the RNAqueous-4PCR isolation kit (Ambion, USA). Reverse transcription was performed using the high-capacity cDNA reverse transcription kit (Applied Biosystems, USA). PCR was performed using the Platinum PCR SuperMix High Fidelity (Invitrogen, USA) system. The primer sets were prepared using published sequence data for 14 different P2 receptor subtypes, albumin (Alb), alpha-fetoprotein (AFP), cytokeratin 19 (CK19), tyrosine aminotransferase (TAT), glucose-6-phosphatase (G6Pase) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [26, 29]. The PCR products were separated by electrophoresis on a 1 % agarose gel, and visualized by ethidium bromide staining under ultraviolet light.

Small interfering RNA (siRNA) transfection

The siRNAs used for the knockdown of endogenous P2Y₁ and P2Y₂ proteins and the negative control siRNA were purchased from Santa Cruz Biotechnology, CA. These siRNAs were transfected into cells using Lipofectamine 2000 (Invitrogen, USA), according to manufacturer's instructions.

Measurement of cytokines in human plasma

Plasma cytokines were measured using Bio-Plex human cytokine multiplex kits (Bio-Plex human Cytokine 17-Plex Panel, Bio-Rad Laboratories, Hercules, CA, USA), according to manufacturer's instructions. The level of each cytokine is indicated by the fluorescence intensity (FI), according to the manufacturer's instructions.

Statistical analysis

The statistical analysis was carried out using the Student's *t* test. A *p*-value of less than 0.05 was considered to be significant. The results are presented as the mean \pm SD.

Results

Effects of ALF-P on cell proliferation and JNK activation in OCs and primary hepatocytes

We studied the effects of ALF-P on the proliferation of primary hepatocytes and OCs because the reports

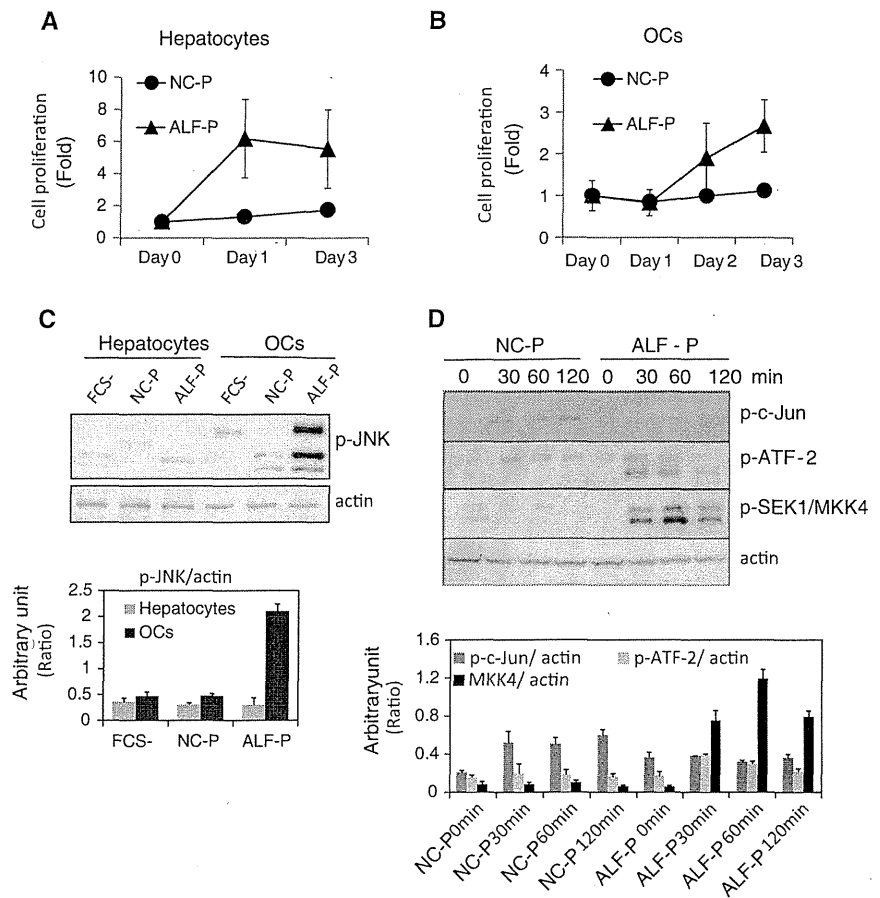
describing the effects of ALF-P on the proliferation of hepatocytes present paradoxical results [8–12]. ALF-P stimulated the proliferation of normal hepatocytes about 6-fold at 24 h and 4-fold at 72 h, relative to the starting point at day 0 (Fig. 1a). In OCs, although ALF-P increased the number of cells slowly after 24 h, the increased proliferative capacity of the cells was sustained for 72 h (Fig. 1b). In addition, gene expression of Alb, AFP, and CK19, which are phenotypic markers of OC presence [26], was not affected by ALF-P treatment for 72 h. Under the same experimental condition, TAT and G6Pase, differential markers of hepatocytes, were not detectable (Supplemental data, Fig. 1). The data indicate that under the experimental conditions, ALF-P treatment stimulated the proliferation of OCs without initiating the differentiation.

ALF-P markedly stimulated the activation of JNK in OCs, which is consistent with our previous data. However, it did not induce JNK phosphorylation in primary hepatocytes. To confirm the effects of ALF-P on the activation of JNK signaling in OCs, we assessed the phosphorylation of SEK-1/MKK4 and c-Jun, which are located immediately upstream and downstream, respectively, of JNK [30–32]. Elevated SEK-1/MKK4 phosphorylation was observed after a short (30–120 min) cell culture period. However, the activation of c-Jun was not detected in the ALF-P-stimulated cells during this period. Instead, activating transcriptional factor 2 (ATF2), another target of JNK signaling in response to cellular stress [33], was activated (Fig. 1d). On the other hand, normal control plasma (NC-P) did not stimulate cell proliferation or JNK activation in either of the two types of cells (Fig. 1a–c). However, increased activation of c-Jun was observed at each indicated time point in OCs (Fig. 1d).

P2Y₂ receptor mediates the effects of ALF-P on the growth of OCs

Considering the importance of ATP signaling in the development of ALF and in the regulation of JNK, we sought to determine if P2 receptors contribute to the effects of ALF-P on the growth of OCs. The mRNA expression of the 14 different P2 receptor subunits, except for P2Y₁₁, which is absent in mice and other rodents [34], was analyzed in OCs. Our data showed that all P2Y subtypes, including P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₂, P2Y₁₃, and P2Y₁₄, were expressed in OCs. However, except for P2X₂, P2X receptors were not detected by RT-PCR (Fig. 2a). To dissect the role of specific P2 receptors, we firstly added P2 antagonists and ALF-P to OCs. PPADS (pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid), a non-selective antagonist of P2 receptors, blocked the stimulatory effects of ALF-P on the proliferation of OCs. However, NF023, a selective and competitive antagonist of P2X receptors, did

Fig. 1 Effects of ALF-P on cell proliferation and JNK phosphorylation. Mouse primary hepatocytes were prepared as described in “Materials and methods”. The hepatocytes (a) and oval cells (OCs) (b) were stimulated with 30 % ALF-P or 30 % NC-P. The proliferation of the cells was measured as described in “Materials and methods”. c OCs and primary hepatocytes received the same treatments as described above and were collected 72 h later. The phosphorylation of JNK was detected by western blot analysis. d OCs were treated with 30 % ALF-P and 30 % NC-P for the indicated times. Western blot analysis was performed using cell lysates and antibodies specific for p-c-Jun, p-ATF2, and p-SEK1/MKK4, as indicated. Actin was used as the internal control. Data are expressed as the mean \pm SD ($n = 3$)



not block the proliferative effects of ALF-P in OCs (Fig. 2b). On the other hand, the live and dead/apoptotic cell staining was performed to investigate changes in proliferation rates in relation to the induction of apoptosis. Compared to the NC-P-treated cells, most of the cells treated with ALF-P or ALF-P and co-treated with NF023 were strongly stained by Live-dye, and quite a few of the cells were stained with DAPI or PI, indicating that either apoptosis or cell death rarely occur in the above conditions. However, co-treatment of PPADS and ALF-P increased the amounts of apoptotic or dead cells, which indicates that the reduction of proliferation in the described condition was attributable to the apoptosis-induced cell death. These results thus suggest that P2Y, but not P2X receptor, contributes to the effects of ALF-P. We next determined which subtype of P2Y receptors mediates the effects of ALF-P. Since both P2Y₁ and P2Y₂ are related to the action of extracellular ATP in regulating liver function [17], we blocked the gene expression of these two receptors using their specific siRNAs (Fig. 2d). P2Y₂ gene silencing reversed the effects of ALF-P on the phosphorylation of JNK and the proliferation of OCs. By contrast, P2Y₁ gene silencing did not alter the effects of ALF-P (Fig. 2e, f).

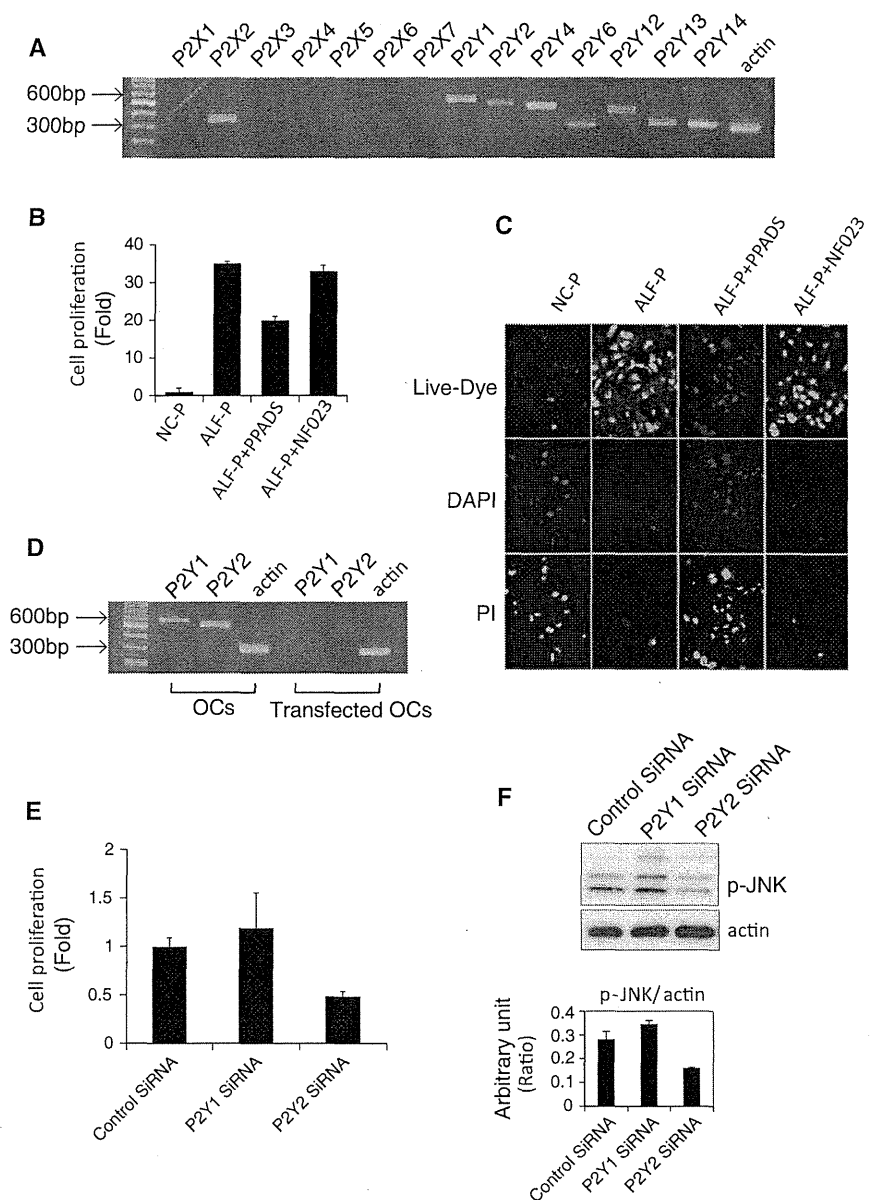
Extracellular ATP does not contribute to the ALF-P-induced proliferation of OCs

We next sought to identify whether the effects of ALF-P are due to the main ligand of P2 receptors, extracellular ATP, which increases inflammatory processes in the liver. Unexpectedly, we found that ATP levels in ALF-P were significantly lower than those in NC-P (Fig. 3a). Although ATP stimulated the activation of JNK in OCs (Fig. 3b), it did not promote cellular proliferation, unlike two other P2 receptor agonists, ATP γ S and 2MeSATP (Fig. 3c). Moreover, when cells were treated with apyrase, an ATP di-phosphohydrolase that catalyzes the hydrolysis of ATP to yield AMP and inorganic phosphate, the ALF-P-stimulated activation of JNK was not inhibited, whereas it was inhibited when cells were treated with PPADS (Fig. 3d).

TNF receptor signaling is involved in the ALF-P-stimulated proliferation of OCs in a P2Y-dependent manner

We therefore focused on other molecules that could mediate the proliferatory effects of ALF-P, such as

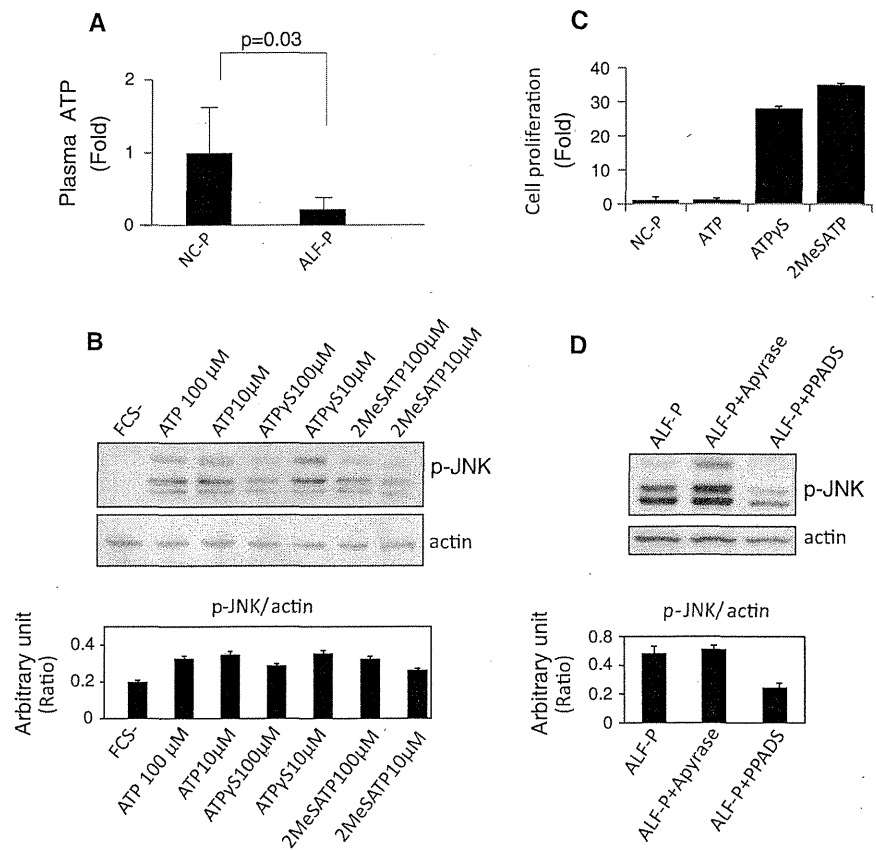
Fig. 2 ALF-P regulates the growth of OCs through P2Y₂ receptor. **a** The expression of P2 receptors in OCs was detected using RT-PCR analysis as described in “Materials and methods”. **b** OCs were incubated with 30 % NC-P or 30 % ALF-P with or without 250 μM PPADS or 200 μM NF023. The proliferation of the cells was evaluated after 72 h. Data are expressed as the mean ± SD (*n* = 3). **c** OCs were treated as described in **b**. Apoptotic and dead cells were evaluated as described in “Materials and methods”. **d** P2Y₁ siRNA, P2Y₂ siRNA, or control siRNA was transfected into OCs as described in “Materials and methods”. Blockage of receptor mRNA expression was confirmed by RT-PCR analysis. **e, f** The control cells and the cells with knockdown of endogenous P2Y₁ or P2Y₂ were treated with ALF-P for 3 days. The proliferation of the cells (**e**) and the phosphorylation of JNK (**f**) were examined subsequently. Data are expressed as the mean ± SD (*n* = 3)



hepatocyte growth factor (HGF), tauroursodeoxycholate (TUDC), insulin, epidermal growth factor (EGF), interferon (IFN) γ , transforming growth factor beta (TGF β), and tumor necrosis factor (TNF) α . Similar to ALF-P, EGF, TGF β 1/2, TGF β 3, and TNF α stimulated the phosphorylation of JNK, whereas the other reagents, including HGF, TUDC, insulin, and IFN γ , did not have an effect (Fig. 4a). Furthermore, when we used SB-431542, AG1478, and R-7050, specific inhibitors of EGF, TGF β , and TNF α , respectively, we found that SB-431542 and AG147 had no significant effect on ALF-P-stimulated cell proliferation or JNK activation, whereas

R-7050 markedly suppressed the effects of ALF-P. PPADS inhibited the effects of ALF-P to a greater degree than R-7050 (Fig. 4b, c). Treatment with TNF α for 72 h stimulated the proliferation of OCs and protected the cells from apoptosis, and this effect was abolished by co-treatment with PPADS, but not NF023 (Figs. 4d, e). However, our data showed no significant difference in plasma TNF α levels between the NC-P and the ALF-P samples (Fig. 4f). By contrast, Interleukin 8 (IL-8) levels increased, whereas IL-5 and IL-17 levels decreased in the ALF-P compared with those in NC-P (Supplemental data, Table 1).

Fig. 3 Extracellular ATP does not contribute to the effects of ALF-P on the growth of OCs. **a** Plasma ATP was measured using samples from patients with ALF and normal control subjects. Data are expressed as the mean \pm SD ($n = 3$). **b** OCs were treated with ATP or two other P2 receptor agonists at the indicated concentrations. The proliferation of the cells was evaluated after 72 h. **c** The cells were treated as described in **b**. The phosphorylation of JNK was determined by western blot analysis. **d** OCs were treated with ALF-P with or without the indicated inhibitors for 72 h. The phosphorylation of JNK was determined by western blot analysis. Actin was used as the internal control. Data in **b–d** are expressed as the mean \pm SD ($n = 3$)



Discussion

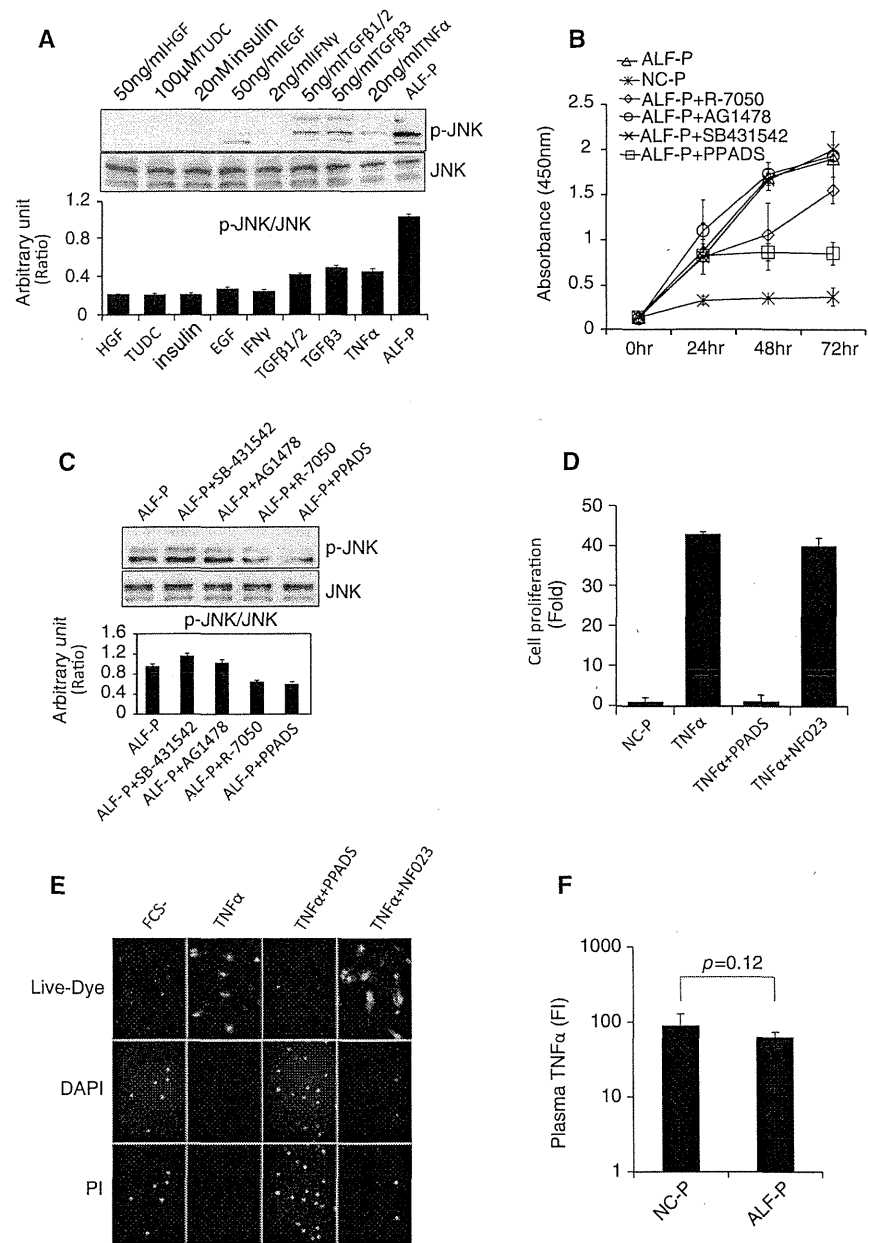
Total plasma exchange (TPE) was significantly effective for correcting coagulopathy and improving liver tests in the treatment of ALF [35], indicating that some hepatotoxins/cytokines in ALF plasma can pathologically affect the growth of hepatocytes and OCs. The results of the present study showed that ALF-P promoted the proliferation of primary hepatocytes and OCs. However, in contrast to the sustained increase in cell proliferation and JNK activation in OCs, the proliferation of primary hepatocytes peaked during the first 24 h of stimulation and declined thereafter. Moreover, no activation of JNK was observed in these cells. The data suggest that although ALF plasma stimulates the proliferation of primary hepatocytes, it exerts its effect early and transiently through a JNK-independent signaling pathway, which is considerably different from the action of ALF-P in OCs. Clinically, liver progenitor cells may therefore proliferate much more than normal hepatocytes in response to sustained stimulation with ALF-P. Normal control plasma did not stimulate JNK activation in OCs or primary hepatocytes. JNK activation was observed in ALF-P-stimulated OCs, but not in primary hepatocytes. The findings demonstrate the pathologic roles of the JNK

pathway in regulating the proliferation of OCs, which may be related to the impairment of liver regeneration in ALF.

The transcription factor c-Jun is required for hepatocyte survival and proliferation [36–39]. c-Jun phosphorylation is usually activated by JNK but sometimes is JNK-independent [40]. The present study showed that NC-P stimulated the phosphorylation of c-Jun in OCs in a JNK-independent manner. In addition, NC-P negatively regulated the proliferation of OCs by inducing apoptosis. Since Harrington has demonstrated the negative effect of serum on the proliferation of primary hepatocytes [41], which was also shown in the present study using NC-P, our results demonstrate the physiological significance of c-Jun in the growth of OCs. Contrary to NC-P, ALF-P activated ATF2 and JNK but not c-Jun. To date, although the functional properties of ATF2 remain poorly understood, significant in vitro and in situ experiments demonstrate mainly a proliferative role for ATF2 in several types of cancer, including hepatic cancer [42]. Taken together, the data suggest that the activation of different JNK downstream target genes may result in different outcomes for the growth of OCs.

The present study addressed the role of P2 receptors in the ALF-P-stimulated proliferation of OCs. It has been

Fig. 4 TNF α receptor signaling mediates the function of ALF-P in a P2 receptor-dependent manner. **a** Cells were incubated with 30 % ALF-P and the other indicated reagents. The phosphorylation of JNK was detected after 3 days of stimulation. JNK was also detected as an internal control. Data are expressed as the mean \pm SD ($n = 3$). **b** Cells were incubated with 30 % NC-P or 30 % ALF-P with or without the indicated inhibitors. The proliferation of the cells was measured daily by using cell count reagent SF. In addition, the phosphorylation of JNK (c) was detected after 72 h of stimulation. Data are expressed as the mean \pm SD ($n = 3$). **d** OCs were treated with 10 μ g/mL TNF α with or without 250 μ M PPADS or 200 μ M NF023. Cells proliferation was determined after 3 days of stimulation. Data are expressed as the mean \pm SD ($n = 3$). **e** OCs were treated as described in d. Apoptotic and dead cells were evaluated as described in “Materials and methods”. **f** Plasma TNF α levels were measured as described in “Materials and methods” using samples from patients with ALF and normal control-subjects. Data are expressed as the mean \pm SD ($n = 3$). * $p < 0.05$ versus control



reported that P2X₄ and P2X₇ receptors are expressed predominantly in hepatocytes, and the former contributes to ATP-dependent calcium signaling and glucose release [43]. However, our results showed that P2X receptor transcripts other than P2X₂ were not detectable. Moreover, a P2X-specific inhibitor did not block the effects of ALF-P on the proliferation of OCs, suggesting that P2X receptors are not involved in the function of ALF-P. On the other hand, pharmacological and RNA interference (RNAi) approaches provided evidence that P2Y₂ rather than P2Y₁ participates in the regulation of OCs by ALF-P. Notably, although the expression of P2Y₂ mRNA was blocked by siRNA

transfection, neither the proliferation of OCs nor JNK activation was completely abolished in response to stimulation with ALF-P, as shown in Fig. 2. Since in addition to P2Y₂, P2Y₄, and P2Y₆ have roles in the proliferation of cell lines other than hepatocytes [44, 45], it is reasonable to assume, in combination with our findings, that P2Y receptors other than P2Y₂ may participate in the effects of ALF-P.

Interestingly, despite the importance of P2Y receptors demonstrated in the present study, our findings showed that extracellular ATP does not participate in the function of ALF-P, suggesting that P2 agonists other than ATP may be

involved. Although all P2Y receptors are activated by ATP, at P2Y₁, ADP is reported to be equipotent or more potent than ATP, and at P2Y₂, UTP and ATP are equipotent [46]. In addition, UTP levels are critical for efficient hepatitis C virus replication, and UTP depletion contributes to the development of liver injury [47, 48]. Whether ADP or UTP contributes to the effects of ALF-P on the growth of OCs is now under investigation.

Given the evidence on the importance of TNF α in the development of ALF and the negative correlation between ALF prognosis and TNF α levels, the present study suggests that a TNF α receptor signal is involved in the effects of ALF-P. Moreover, P2 receptor inhibition completely abolished the effects of TNF α on the proliferation and apoptosis of OCs, indicating that the TNF α pathway is P2 receptor-dependent. Our data showed no significant difference in plasma TNF α levels between the NC-P and the ALF-P samples. Although the plasma levels of other cytokines, including IL-5, IL-8, and IL-17, were significantly changed in the ALF-P compared with those in NC-P, because these interleukins do not interact with ATP or TNF α receptors, we can therefore predict that all the above cytokines do not contribute to the ALF-P-induced action of the TNF α receptors. On the other hand, several members of the TNF family besides TNF α serve as ligands of the TNF receptors [49]. There is another possibility that some unknown TNF ligands may contribute to the effects of ALF-P through TNF receptor signaling. These factors should thus be clarified in future investigations.

The components of normal human plasma have been reported [50, 51]. However, the exact nature of toxic molecules in the plasma during liver failure is unknown and the toxicity effects may vary among different organ systems [52]. In the present study, we focused on the effects of ATP, TNF α , and their related signals on the proliferation of oval cells based on the fact that both ATP and TNF α play essential roles in the development of fulminant hepatitis and in regulating the proliferation of hepatocytes. Although our data did not support that either ATP or TNF α should be the target molecule in the ALF plasma, the importance of P2Y₂ receptor crosstalk with the TNF α signaling pathway has been clearly addressed and subsequently will be valuable for our later investigations.

In conclusion, the present study demonstrated the specific involvement of JNK activation, the important roles of ATP receptor P2Y₂, and the crosstalk of P2Y₂ with TNF α receptor signaling in mediating the effects ALF-P on the regulation of OC growth. The data also suggested that targeting the JNK pathway could selectively inhibit the abnormal proliferation of OCs in ALF without affecting the growth of normal hepatocytes, which may be of clinical significance in the treatment of ALF.

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

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急性肝不全の予後

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1 はじめに

急性肝不全 (Acute liver failure : ALF) とは急激かつ高度の肝細胞機能障害に基づいて肝性脳症をはじめとする肝不全症状をきたす予後不良の疾患群であり、多くの場合、病理学的には広汎あるいは亜広汎肝細胞死の像を呈する。肝細胞死(急性肝障害)の原因はウイルスなどによる炎症の場合と薬物中毒や循環障害などによる非炎症の場合に大別される。

わが国では急性肝障害の原因の大多数がウイルス性と考えられてきたことから、肝炎(ウイルス性肝炎，薬剤アレルギー性肝炎，急性発症自己免疫性肝炎)による急性肝不全を「劇症肝炎」とし、急性肝不全の代表として扱ってきた経緯がある。したがって、米国や英国で急性肝不全の成因の第1位を占めるアセトアミノフェン中毒や循環障害などはわが国の劇症肝炎からは除外されてきた。一方で、劇症肝炎の成因のひとつとして、その多くを占める「成因不明(あるいは非A非B型肝炎)」例が本当に「肝炎」であるのかは明確ではなかった。

これらの問題点を解消し、欧米の概念との整合性を得る目的から、わが国でも急性肝不全の概念の導入が検討され、2011年厚生労働省科学研究費補助金(難治性疾患克服事業)「難治性の肝・胆道疾患に関する調査研究」班において定義が定められた(表1)²⁾。この定義では、従来劇症肝炎は、成因不明を含む肝炎による昏睡型急性肝不全に相当する。

2 わが国における劇症肝炎の予後の変遷

前述の経緯から、わが国では急性肝不全としての全国統計はなく、劇症肝炎の全国統計が1983年以来行われてきた。これを基に、わが国の急性肝不全の予後の変遷を述べる。

1. 臨床病型と予後

昏睡型の急性肝不全の予後は発症あるいは黄疸の発現から肝性昏睡の発現までの期間により異なることが知られており、この期間によっていくつかの臨床病型に分けられている。わが国では劇症肝炎の全国集計を基に、初発症状から昏睡までの期間が10日以内の

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