(450G/A) were observed. In patient no. 16, only 7.94% nucleotide substitution (242C/T) was observed. These results showed no specific mutations in HAV IRES associated with severe form existing in this outbreak.

UDPS of HAV IRES of acute hepatitis, severe form, in sporadic case

We performed UDPS in the one HAV IRES derived from one patient with acute hepatitis, severe form, who was not involved in this outbreak. In patient no. 17, 8 nucleotides at 8 positions were different from the reference sequence (Table 2), and 64.72% (578T/C), 98.14% (208C/T), 99.71% (527G/T), 99.83% (466T/C), 100% (204A/G), 100% (275A/G), 100% (276T/C) and 100% (378G/A) nucleotide substitutions were observed. These results showed that UDPS of the sporadic case was obviously different from those of the outbreak cases.

Comparison of UDPSs among HAV with different severities and HAV from different sources

The substitution rates were analyzed in comparison to the reference sequence [19]. We also counted insertions as substitutions. In HAV IRES (nt. 90-620), total nucleotide substitutions of acute hepatitis, non-severe and severe forms from the outbreak, and sporadic acute hepatitis, severe form, were 0.020, 0.014, and 1.43%, respectively. In HAV IRES domain III (nt. 90-300), nucleotide substitutions of acute hepatitis, non-severe and severe forms from the outbreak, and sporadic acute hepatitis, severe form, were 0.047, 0.013, and 1.88%, respectively. In HAV IRES domain IV (nt. 301-580), nucleotide substitutions of acute hepatitis, non-severe and severe forms from the outbreak, and sporadic acute hepatitis, severe form, were 0.0034, 0.018, and 13.0%, respectively. In HAV IRES domain V (nt. 581-620), nucleotide substitutions of acute hepatitis, non-severe and severe forms from the outbreak, and sporadic acute hepatitis, severe form, were 0.0020, 0, and 0%, respectively. All nucleotide substitutions from acute hepatitis from the outbreak were transition mutations. Three nucleotide insertions were observed in only acute hepatitis, severe form, from the outbreak (Table 3).

Table 3. Nucleotide substitutions of hepatitis A virus (HAV) internal ribosomal entry site (IRES).

Type of Mutations	Insertion	Transition				Transversion	Total Numbers
		A/G	G/A	C/T	T/C	G/T	
Total Numbers	3	10	5	4	12	1	35
Numbers in Outbreak Cases	3	8	4	3	9	0	27
AH of Outbreak Cases	3	8	2	2	8	0	23
AH-S of Outbreak Cases	0	0	2	1	1	0	4
Numbers in Patient no. 17	0	2	1	1	3	1	8

"Numbers" equivalent to "nucleotide substitutions".

DISCUSSION

In the present study, we analyzed the UDPSs of HAV associated with the outbreak and tried to detect specific mutations in HAV 5′UTR associated with hepatitis A, severe form. Our result showed that no specific mutations in HAV 5′UTR associated with severe form existed in this outbreak. UDPS analysis of HAV 5′UTR revealed no association between the disease severity of hepatitis A and HAV 5′UTR substitutions. It might be more interesting to perform ultra-deep sequencing of the full-length HAV genome in order to uncover possible unknown genomic determinants associated with disease severity. Further studies will be needed regarding this point. We also found minor nucleotide sequence variations, which seemed undetectable by the Sanger method. We do not yet know what these variations mean.

The sensitivity of UDPS is higher than that of Sanger sequencing. Next-generation sequencing technologies are increasingly being used to identify low-abundance minority genetic variants within a heterogeneous pool of amplified DNA molecules, such as those within a virus population, which are especially valuable for the detection of drug resistance mutations [20]. We characterized HAV minority variants in the HAV IRES region.

In general, HAV infection risk is inversely correlated to sanitation and other socio-economic indicators [21]. Although Japan is one of the developed countries in Asia, a universal vaccination program against HAV and HBV has not yet been initiated [17]. Although the present study did not include acute liver failure with hepatic encephalopathy such as fulminant hepatitis A, our study is important because UDPS analysis of these HAV strains reconfirmed that a single source might have caused this outbreak as previously reported [16], suggesting that UDPS analysis might be a new analytical tool for the source of hepatitis A outbreaks.

The effect of mutations in 5′UTR of HAV on the severity of the disease is a long story that has never been clearly proven. Fujiwara et al. reported an association between the severity of hepatitis A and nucleotide substitutions in 5′UTR of HAV RNA [8-10]. However, there have been several contrary observations [7,11-13]. As the definition of acute liver failure is also different among different countries [11,14], it seemed difficult to compare them using different criteria. Then we compared the different HAV IRES sequences derived from a single-source outbreak, based on a single definition of liver failure. In this outbreak, the proportion of acute liver failure was very high (3/29, 10.3%), compared to the previous report [22], although the calculation was performed on the basis of patients admitted into two hospitals [15,16]. The reference clonal sequence from patient 12 had only one different nucleotide from the sequence of the severe hepatitis strain HAV PT (A10), which was reported by Fujiwara et al [10], suggesting that our present study might be conducted among specific HAV strains.

The technical approach used in the present study had the advantage of having great power in detecting mutations present at a very low frequency in the swarm of mutants. However, our results confirm the old paradigm that 5′UTR of picornaviruses and particularly of HAV is highly conserved. This reason might be associated with the function of HAV IRES, preventing the occurrence of variability.

In conclusion, there were no different HAV IRES sequences between severe and non-severe forms in this outbreak. To our surprise, HAV strains in this outbreak conserved HAV IRES sequence even if we performed analysis of UDPSs. Further analysis of HAV UDPSs could give us new information concerning the association between the disease severity of hepatitis A and HAV genome substitutions.

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COMPETING INTERESTS

The authors have declared that no competing interest exists.

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Hepatology Research

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Original Article

Usefulness and accuracy of the international normalized ratio and activity percent of prothrombin time in patients with liver disease

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Aim: In order to determine the most reliable reporting style for prothrombin time (PT) in patients with liver disease, we examined the correlations between the plasma antigen levels of clotting factors and the PT activity percent or two international normalized ratios (INR), and compared the inter-reagent variation among these PT reporting styles.

Methods: The PT was measured in 81 patients with liver diseases, including acute liver failure (ALF) ($n = 10$), acute liver injury ($n = 52$), chronic hepatitis ($n = 8$) and liver cirrhosis ($n = 11$), and in 75 warfarin-treated patients and 32 healthy volunteers. The PT of each plasma sample was determined with four commercial thromboplastins using an automated photo-optical coagulometer. For individual thromboplastin reagents, a locally determined international sensitivity index (local ISI) was derived using plasma obtained from healthy volunteers, warfarin-treated patients and liver disease patients. The INR_{W} and INR_{LD} were calculated using the corresponding local ISI. The PT activity percent was calibrated according to the Lineweaver–Burk equation. The PT values were compared with the plasma antigen levels of clotting factors X, II and VII measured using the enzyme-linked immunoassay method.

Results: The plasma factor X level was selected as the gold standard for measuring the synthetic liver function among the three clotting factors due to its significant relationship ($P = 0.007$) with the prognosis of ALF. The INR_{LD} exhibited the closest correlation to the factor X level ($r = 0.723–0.759$), with the smallest inter-reagent variation among these reporting styles.

Conclusion: The INR_{LD} is the most appropriate PT reporting style for use in patients with liver disease.

Key words: activity percent, international normalized ratio, liver failure, liver function test, prothrombin time

INTRODUCTION

THE PROTHROMBIN TIME (PT) is recognized to be a reliable marker of the protein synthetic function of the liver and thus a marker of the hepatic functional reserve. It is included in many authorized criteria for the diagnosis of liver failure and is used as an indication for liver transplantation worldwide.[1–8] The PT is directly measured as the clotting time (s) of citrated plasma mixed with a tissue thromboplastin reagent. Because the clotting time closely correlates with the concentrations of several clotting factors, including factors VII, X, V, II (prothrombin) and I (fibrinogen), it indicates the plasma levels of these clotting factors. These clotting factors are synthesized uniquely in hepatocytes and rapidly (several hours to a few days) disappear from the circulation after excretion from hepatocytes, in contrast to other liver-specific proteins such as albumin, which has a half-life of 2–3 weeks. In addition, the levels of clotting factors are hardly affected by the nutritional status of the patient, also in sharp contrast to albumin and prealbumin.[9] This is why the PT is considered to be an excellent and real-time marker of the protein synthetic function of the liver. However, the method used to report the results of PT measurement has not yet been standardized.

Although the international normalized ratio (INR) has been adopted in the model for end-stage liver disease (MELD) score and the diagnostic criteria of acute

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liver failure in the USA,[6–8] its validity is controversial because it has been established as a marker for evaluating the effects of an oral anti-coagulant (warfarin), an antagonist of vitamin K carboxylase.[10] In contrast to the US criteria, the Japanese[8] and French (Clity) criteria[2,3] include the PT activity percent in the diagnostic criteria for acute liver failure (ALF). Indeed, one paper reported that activity percent is more accurate for evaluating liver damage than INR.[11] The important issues in comparing the validity of PT reporting methods are which method exhibits low variability across different laboratory conditions (thromboplastin reagents and equipment) and which method closely expresses the hepatic synthetic function.

In order to determine the superior reporting method for evaluating the hepatic functional reserve using PT, we analyzed the variability among different thromboplastin reagents (inter-reagent variation) and the correlations between the plasma antigen levels of clotting factors and INR and PT activity percent.

METHODS

Patients and plasma samples

THE PT WAS measured in 81 patients with liver diseases including ALF ($n = 10$), acute liver injury (hepatitis A, acute hepatitis B, drug-induced liver injury and unknown etiology) ($n = 52$), chronic hepatitis ($n = 8$) and liver cirrhosis (hepatitis B, hepatitis C and alcoholic hepatic injury) ($n = 11$), and in 75 warfarin-treated patients and 32 healthy volunteers.

Blood plasma samples were collected from 81 adult patients with liver disease, 75 adult patients who underwent stable warfarin treatment and 32 healthy adult volunteers using phlebotomy into vacutainer tubes containing 3.2% sodium citrate. Platelet-poor plasma was prepared using centrifugation at 2500 g for 15 min at 4 °C, aliquoted and stored at −80 °C until assay.

Thromboplastins and ISI calibration

Four thromboplastins that are commercially popular in Japan were selected for the present study, as listed in Table 1. The international sensitivity index (ISI) of each thromboplastin provided by the respective manufacturers was compared with the local ISI, which was calibrated according to the World Health Organization (WHO) guidelines for thromboplastins and plasma used to control oral anticoagulant therapy,[12] with the following differences: the use of frozen instead of fresh plasma, and the use of plasmas obtained from patients with liver disease and warfarin-treated patients instead of plasma obtained from patients on stable oral anticoagulation.

To reference standard thromboplastin in order to determine the ISI, the 3rd International Standard for human, recombinant plain thromboplastin (rTF/95, ISI 0.94) was obtained from a WHO laboratory (Central Laboratory of the Netherlands Red Cross blood transfusion service, Amsterdam, the Netherlands).

The PT (s) of the 32 healthy volunteers and the 75 warfarin-treated patients or 81 patients with liver disease were plotted on a double logarithmic scale with rTF/09 on the vertical axis and each commercial thromboplastin on the horizontal axis. The slope of the regression line was used as the ISI.

PT measurement and definition of ISI

The PT (s) of each plasma was determined with the four commercial thromboplastins using an automated photo-optical coagulometer, ACL TOP (Mitsubishi Chemical Medience, Tokyo, Japan) according to the manufacturer's instruction manual.

Table 1 Characteristics of thromboplastin reagents and determined ISI

Name	RecombiPlastin	Thrombocheck PT	Thromborel S	Coagpia PT-N
Manufacturer	Intrumentation Laboratory, Bedford, MA, USA	Sysmex, Kobe, Japan	Dade Behring, Deerfield, IL, USA	Sekisui Medical, Tokyo, Japan
Source	Human recombinant	Rabbit brain	Human placenta	Rabbit brain
ISI	1.01	1.70	1.01	1.07
Local ISI†	0.96	1.85	1.09	1.04
ISI_W	0.95	1.74	1.09	1.11
ISI_{LD}	0.95	1.30	0.94	0.96
Control PT (s)	10.5	11.8	12.5	12.8

†Local ISI values were calibrated for a photo-optical coagulometer, ACL TOP, using AK calibrant, which consists of four INR-known plasma samples.
INR, international normalized ratio; ISI, international sensitivity index; PT, prothrombin time.

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For each individual thromboplastin, an ISI was calculated according to the WHO guidelines and defined as follows: ISI_W was defined as the locally determined ISI derived using plasma obtained from healthy volunteers and warfarin-treated patients, while ISI_{LD} was defined as the locally determined ISI derived using plasma obtained from healthy volunteers and patients with liver disease. Because the ISI provided by each manufacturer were calculated using an individually-fixed coagulometer but not always the ACL TOP, different ISI values might have been obtained when the thromboplastins were used in the ACL TOP. Therefore, we calculated the "local ISI" of each individual thromboplastin using the ACL TOP with AK calibrant (Technoclone, Vienna, Austria), which consists of four INR-known control plasmas (INR, 1.02, 2.13, 3.10 and 4.73). The local ISI of the individual thromboplastins were reverse calculated using simultaneous measurement of the PT of the AK calibrant with rTF/09 and each thromboplastin.

The INR was calculated using the formula: $INR = (\text{patient PT} / \text{control PT})^{ISI}$, where the median value of the 32 healthy volunteer was used as the "control PT". The INR calculated using ISI_W or ISI_{LD} was represented as INR_W or INR_{LD}, respectively.

Calibration of PT activity percent

The PT (s) of each plasma with the four commercial thromboplastins was calibrated into the activity percent using a calibration equation (Lineweaver–Burk equation), obtained based on the regression between the reciprocal of a known activity percent of standard plasma (calibrant) and its measured PT (s) for three points made using stepwise dilution of the calibrant with the provided diluent.

The calibration equation for each thromboplastin was formulated using the calibrant provided by each representative manufacturer. In addition, two other activity percent values were calculated for each plasma using two other calibrants as common calibrants: calibrant A for RecombiPlastin and calibrant C for Thromborel S.

Enzyme-linked immunosorbent assay (ELISA) of the plasma clotting factors

The plasma antigen levels of factor II (prothrombin), factor VII and factor X were determined using ELISA with Assay Max Human Prothrombin, Assay Max Human Factor VII and Assay Max Human Factor X, respectively (AssayPro, St Charles, MO, USA), according to each manufacturer's instruction.

Statistical analysis

The central tendency of variants that showed a normal distribution was presented as the mean ± standard deviation (SD), while that of variants that showed another distribution type was presented as the median (25th–75th percentile).

The Mann–Whitney *U*-test was used to compare the mean values between two independent groups with respect to variants that did not show a normal distribution.

To compare correlation coefficients between the INR or activity percent and the antigen levels of clotting factors, each variant was first converted to a standard normal distribution with a mean of 0 and an SD of 1, then the Pearson product moment correlation coefficient was calculated.

The variability in PT among the four thromboplastin reagents (inter-reagent variation) was compared between different PT reporting styles using the Bland and Altman plot method, that is, the mean of the four PT values was determined according to the four different reagents in each plasma, and the differences between the maximum and minimum values among the four values were plotted on the horizontal axis and the vertical axis, respectively. The vertical axis was expressed according to two methods: raw differences in each PT reporting style and the ratio of the difference to the corresponding mean value.

RESULTS

Local ISI, originally determined ISI using plasma obtained from warfarin-treated patients (ISI_W) and liver disease patients (ISI_{LD})

THE ISI_W AND ISI_{LD} of the individual thromboplastins were compared with the local ISI and the manufacturer-provided ISI, as shown in Table 1. The ISI_W exhibited the closest approximation to the corresponding local ISI for each thromboplastin reagent, whereas the ISI_{LD} exhibited substantial differences from the local ISI. Because RecombiPlastin exhibited similar values among the ISI, it was thought to be a preferential thromboplastin provided by the WHO.

Association between the plasma antigen levels of clotting factors and the severity of acute liver injury

The plasma antigen levels of prothrombin, factor VII and factor X were compared between the acute liver injury

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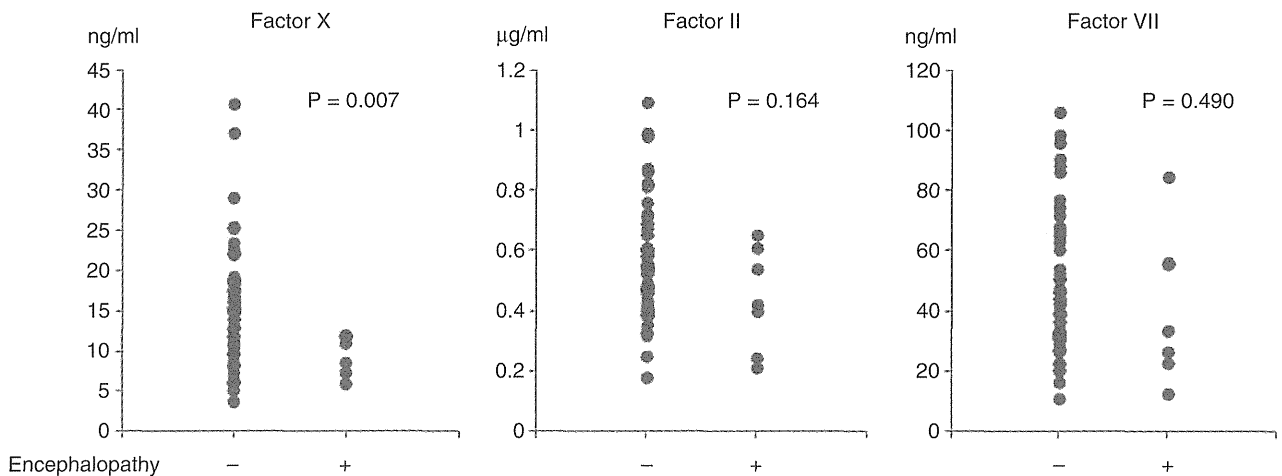

Figure 1 Differences in the plasma antigen levels of clotting factors X, II and VII between the patients with and without hepatic encephalopathy among those with acute liver injury. The *P*-values indicate the risk in Mann–Whitey *U*-test.

patients with and without hepatic encephalopathy (Fig. 1). Because only the factor X level significantly reflected the severity of liver damage among the three factors, we adopted the factor X level as the gold standard marker for the protein synthetic function of the liver.

Correlation between the INR or PT activity percent and the plasma antigen levels of clotting factors

Because the plasma antigen levels of factor X exhibited a logarithmic distribution (Fig. 2a), the values were converted to a normal distribution (mean = –2.609, SD = 0.446) (Fig. 2b) using the formula: x = LN (1 / factor X) and then standardized using the mean and SD. Similarly, the INR_W and INR_{LD} were converted to a normal distribution using the formula: x = LN (INR – 0.84), and then standardized using the individual mean and SD (Figs 3,4). Because the PT activity percent exhibited nearly normal or symmetrical distribution (data not shown), these values were directly standardized.

After the values were standardized, correlation coefficients between the plasma factor X levels and the INR_W, INR_{LD} and PT activity percent in the four different thromboplastins were calculated (Table 2). All five types of PT reporting styles were highly correlated with the plasma antigen levels of factor X. In particular, INR_{LD} exhibited largest correlation coefficient among the five types of PT reporting styles for every thromboplastin, while INR_W exhibited almost identical values to INR_{LD}. Therefore, the INR type reporting style of PT was always superior to the PT activity percent type reporting style for every thromboplastin reagent.

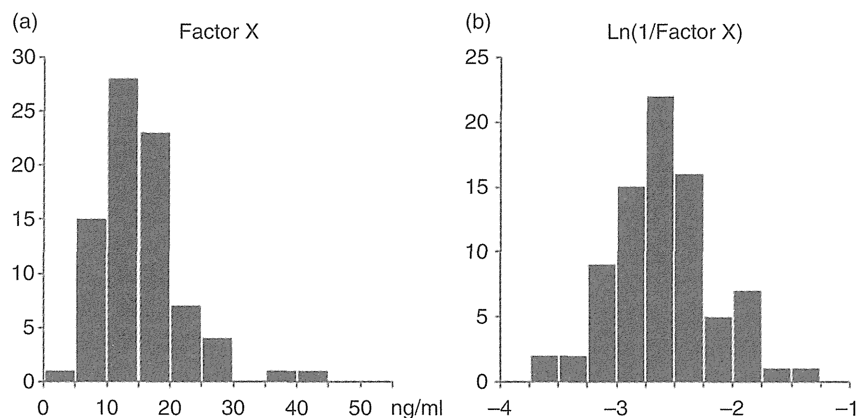

Figure 2 Distribution of the plasma antigen levels of factor X in the patients with liver disease. (a) Distribution of the crude values. (b) Distribution of the logarithmically converted values.

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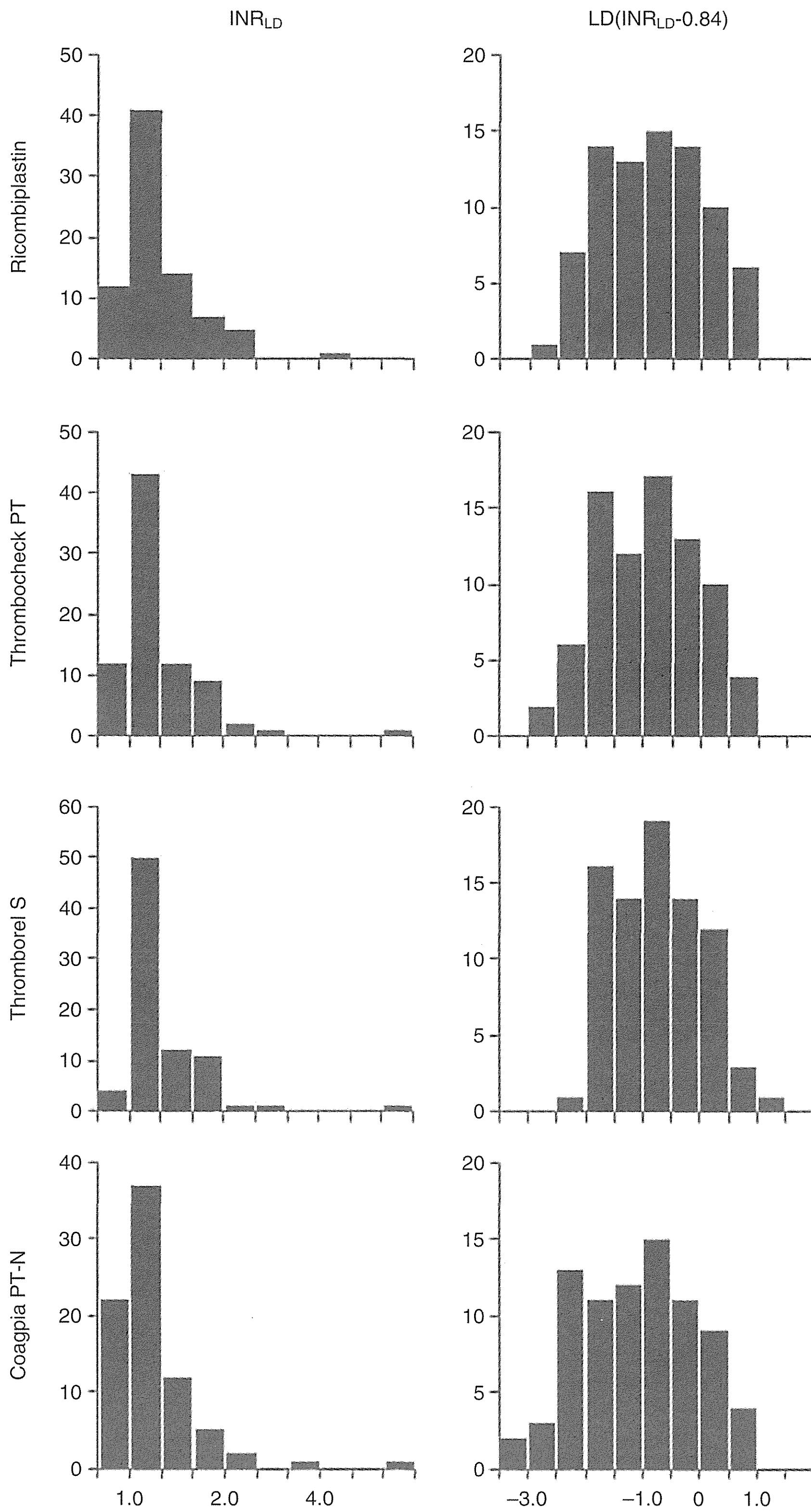

Figure 3 Distributions of the INR$_{LD}$ values in patients with liver disease measured using four different reagents. The left side panel for each reagent indicates the distribution of the crude INR$_{LD}$ values and the right side panel indicates the distribution of the logarithmically converted values.

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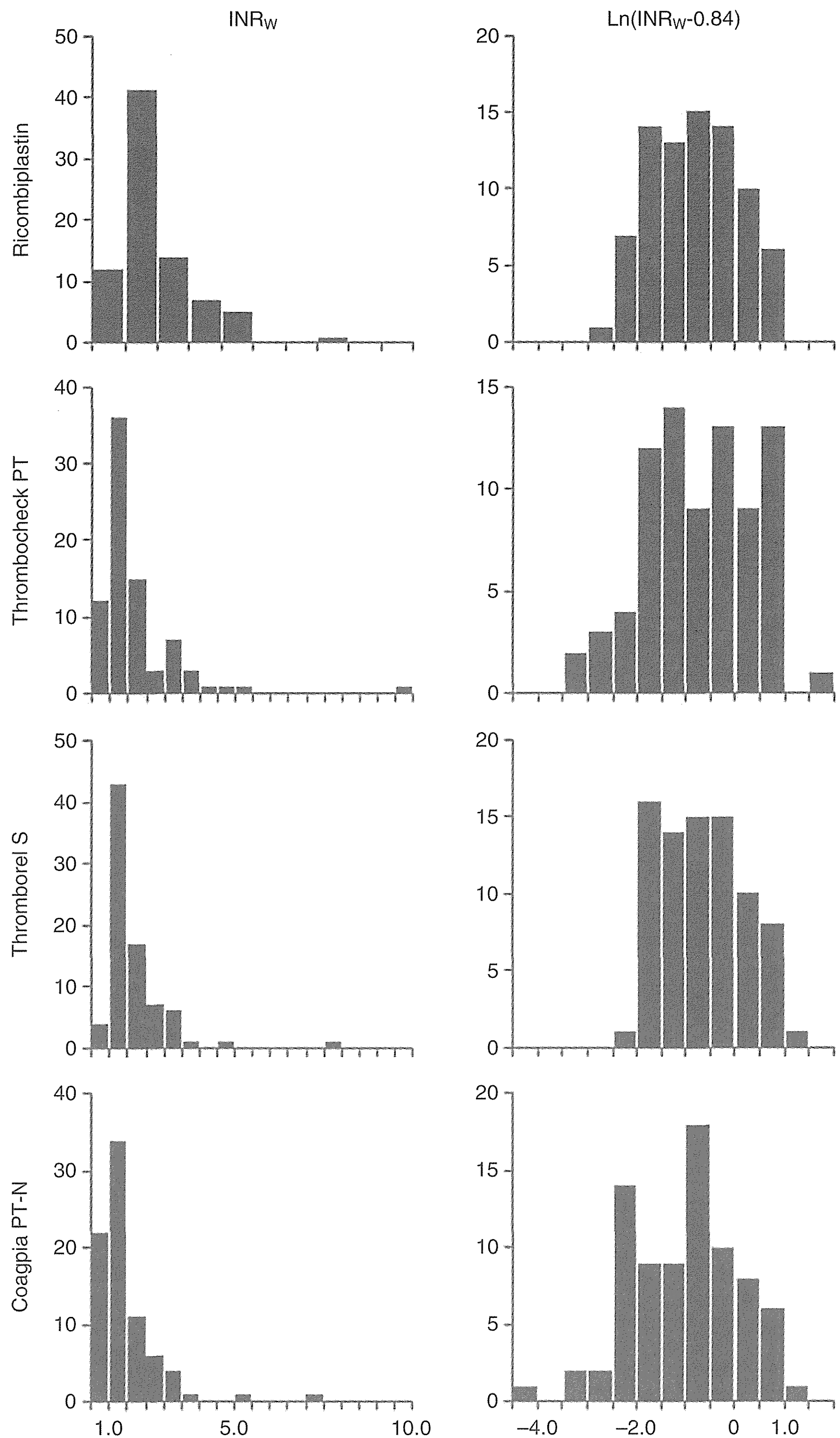

Figure 4 Distributions of the INR_W values in patients with liver disease measured using four different reagents. The left side panel for each reagent indicates the distribution of the crude INR_W values and the right side panel indicates the distribution of the logarithmically converted values.

For confirmation, we checked the correlation between each of these PT reporting styles and the level of prothrombin or factor VII. The factor VII level exhibited no significant correlation with any of the PT reporting styles. Although the prothrombin level showed a significant correlation with all three PT reporting styles, the correlation coefficients (0.44–0.54) were substantially lower than those of factor X.

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Table 2 Comparison of correlation coefficients between the plasma factor X level and PT activity percent, INR_{LD} and INR_W in patients with liver disease

Reagents	PT activity percent			INR	
	Individual calibrant	Common calibrant for RecombiPlastin	Common calibrant for Thromborel S	INR_{LD}	INR_W
RecombiPlastin	0.717	0.717	0.720	0.734	0.734
Thrombocheck PT	0.678	0.666	0.696	0.744	0.726
Thromborel S	0.733	0.730	0.733	0.759	0.758
Coagpia PT-N	0.683	0.695	0.688	0.723	0.704

INR, international normalized ratio; PT, prothrombin time.

Inter-reagent variation of INR

The variability of the INR values among the thromboplastin reagents was compared between INR_W and INR_{LD} (Fig. 5). When the INR in the patients with liver disease was calculated using ISI_W (Fig. 5a), the differences among the thromboplastins were very large, especially in patients with a prolonged PT. In contrast, when the INR was calculated using ISI_{LD} (Fig. 5b), the differences among the thromboplastin reagents were substantially adjusted. As a result, the regression lines converged to the line, y = x, in INR_{LD}.

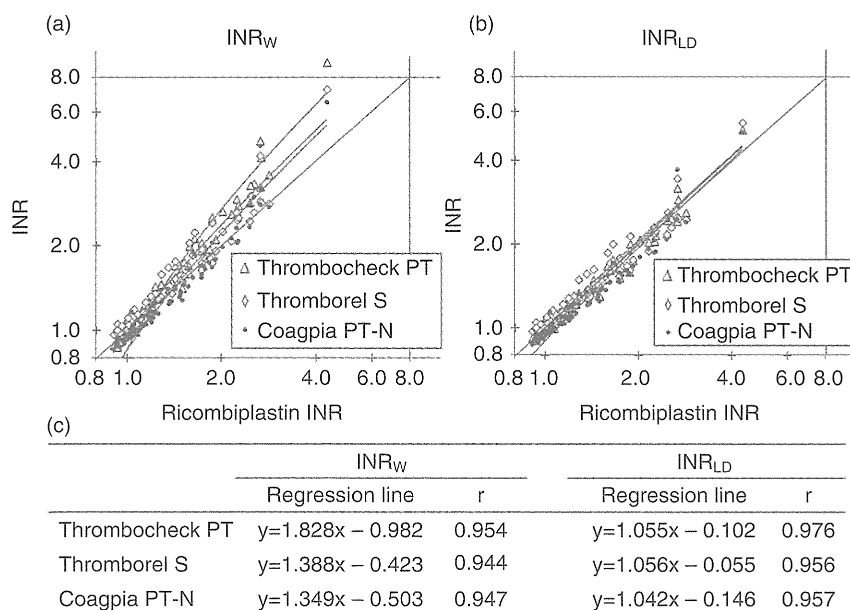
Inter-reagent variation of PT activity percent

Because the value of PT activity percent depends on both the sensitivity to the thromboplastin reagent and the accuracy of the calibrant titer, we used the same calibrant (a calibrant for RecombiPlastin) to examine the variability of PT activity percent depending on the difference in thromboplastin.

Although the variability among the thromboplastin reagents appeared to be smaller in lower PT activity percent samples (Fig. 6a), nearly 10% differences were seen in the area around the mean PT value of 20%. When the ratios of the maximum differences among the four reagents was plotted against mean PT values, the ratios were relatively high in both the normal PT and the highly prolonged PT areas (Fig. 6b).

Calibrant-dependent variation of PT activity percent

Because the inter-reagent variability of the raw PT activity percent was too high in the normal PT range (>80%)



	INR_W		INR_{LD}	
	Regression line	r	Regression line	r
Thrombocheck PT	y=1.828x – 0.982	0.954	y=1.055x – 0.102	0.976
Thromborel S	y=1.388x – 0.423	0.944	y=1.056x – 0.055	0.956
Coagpia PT-N	y=1.349x – 0.503	0.947	y=1.042x – 0.146	0.957

Figure 5 Correlation between the international normalized ratio (INR) values in patients with liver disease measured with Thrombocheck PT, Thromborel S and Coagpia PT-N and those measured with RecombiPlastin. (a) Correlation between the INR_W and (b) INR_{LD} values. The lines in the graph indicate the regression lines. (c) Formulas of the regression lines. The *r*-values indicate the correlation coefficients. △, Thrombocheck PT; ◊, Thromborel S; ◆, Coagpia PT-N.

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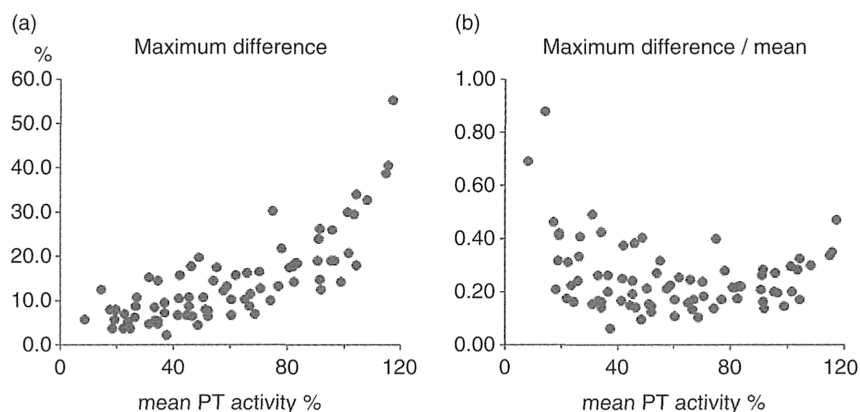

Figure 6 Inter-reagent variation in prothrombin time (PT) activity percent in the patients with liver disease. The horizontal line indicates the mean PT activity percent values measured using four different reagents in each samples. (a) The vertical lines indicate the maximum difference among the four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

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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.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Proliferation of mouse liver stem/progenitor cells induced by plasma from patients with acute liver failure is modulated by $P2Y_2$ 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 (TNFα) 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 $P2Y_2$ (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, TNFα stimulated JNK activation and proliferation of OCs. TNFα receptor antagonist partly inhibited the ALF-P-stimulated proliferation of OCs. Moreover, PPADS significantly inhibited TNFα-stimulated cell proliferation, induced apoptosis, and inhibited the activation of JNK. However, our data showed no significant difference in plasma TNFα levels between the NC-P and ALF-P samples.
Conclusions JNK activation induced by $P2Y_2$ receptor crosstalk with the TNFα signaling pathway is important in mediating the effects of ALF-P on the proliferation and survival of OCs.

Keywords ALF-P · JNK · OCs · P2Y · TNFα

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
TGFβ	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 $P2Y_2$ 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 $P2Y_2$ 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 TNFα-induced programmed cell death in the liver [14, 15, 20–22]. Moreover, TNFα takes a central role in the pathogenesis of ALF. The levels of circulating TNFα are increased in ALF patients and are associated with a poor prognosis [23–25].

Notably, a close relationship exists between extracellular ATP and TNFα in the development of ALF. TNFα 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 TNFα. However, little is known about the roles of ATP, TNFα, and their pathways in the regulation of OCs in ALF.

Here, we demonstrate that one of the P2Y receptors, $P2Y_2$ 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 $P2Y_2$ receptor signaling in ALF-P is ATP-independent and closely linked with the TNFα 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) μM/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 (rmTNFα: R&D systems, USA), or the adenosine triphosphate receptor agonists (ATP: R&D systems, USA; ATPγS 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 TNFα 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-dye™ (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× 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 μg 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 RLUs 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 $P2Y_1$ and $P2Y_2$ 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 ± 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).

$P2Y_2$ 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 $P2Y_{11}$, which is absent in mice and other rodents [34], was analyzed in OCs. Our data showed that all P2Y subtypes, including $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$, were expressed in OCs. However, except for $P2X_2$, 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

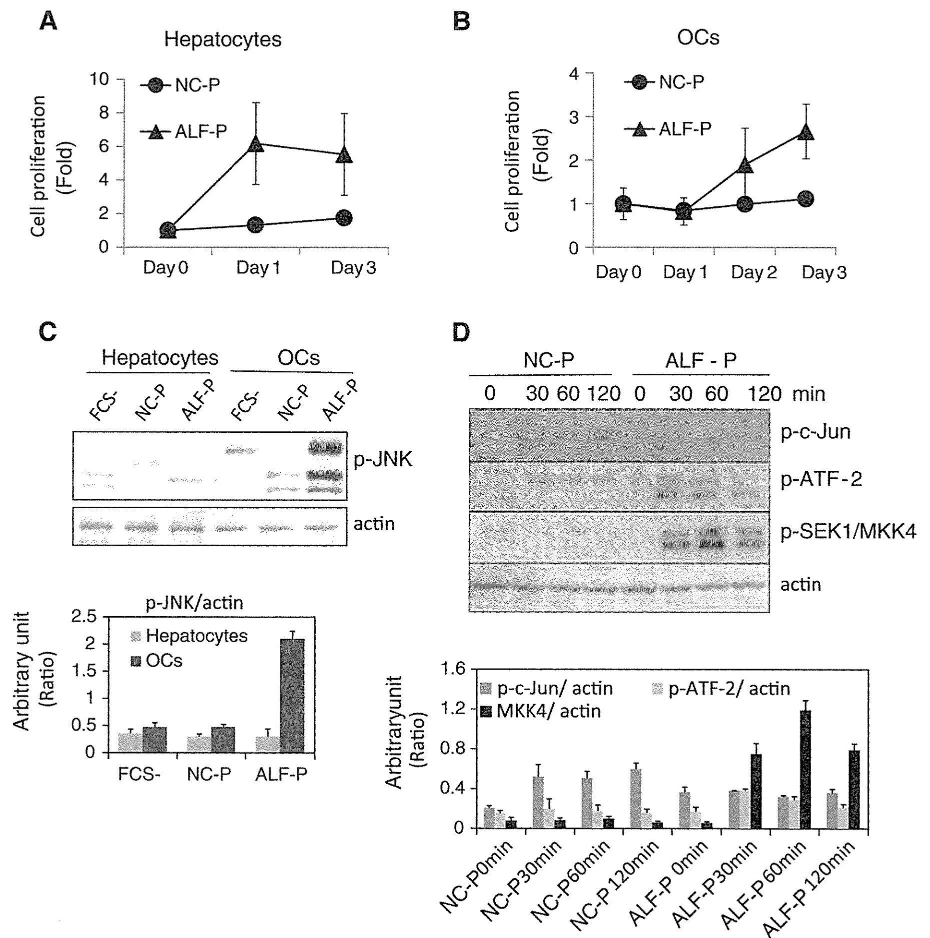

Fig. 1 Effects of ALF-P on cell proliferation and JNK phosphorylation. Mouse primary hepatocytes were prepared as described in "Materials and methods". The hepatocytes (**a**) and oval cells (OCs) (**b**) were stimulated with 30 % ALF-P or 30 % NC-P. The proliferation of the cells was measured as described 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 ± 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 $P2Y_1$ and $P2Y_2$ 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). $P2Y_2$ gene silencing reversed the effects of ALF-P on the phosphorylation of JNK and the proliferation of OCs. By contrast, $P2Y_1$ 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, ATPγS and 2MeSATP (Fig. 3c). Moreover, when cells were treated with apyrase, an ATP diphosphohydrolase 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

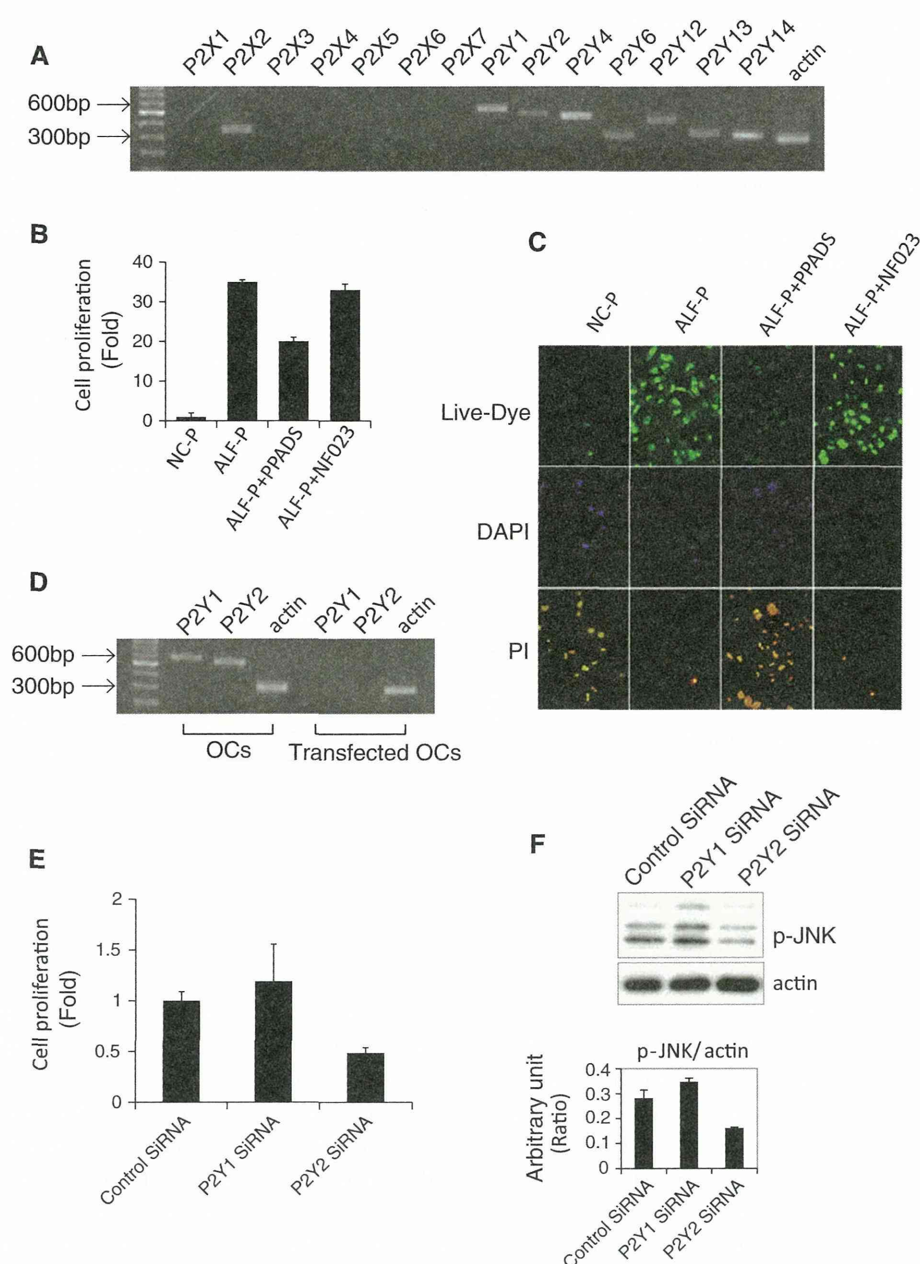

Fig. 2 ALF-P regulates the growth of OCs through $P2Y_2$ receptor. **a** The expression of P2 receptors in OCs was detected using RT-PCR analysis as described in "Materials and methods". **b** OCs were incubated with 30 % NC-P or 30 % ALF-P with or without 250 μM PPADS or 200 μM NF023. The proliferation 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** $P2Y_1$ siRNA, $P2Y_2$ 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 $P2Y_1$ or $P2Y_2$ 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 (TGFβ), and tumor necrosis factor (TNF) α. Similar to ALF-P, EGF, TGFβ1/2, TGFβ3, and TNFα stimulated the phosphorylation of JNK, whereas the other reagents, including HGF, TUDC, insulin, and IFNγ, did not have an effect (Fig. 4a). Furthermore, when we used SB-431542, AG1478, and R-7050, specific inhibitors of EGF, TGFβ, and TNFα, 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 TNFα 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 TNFα 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).

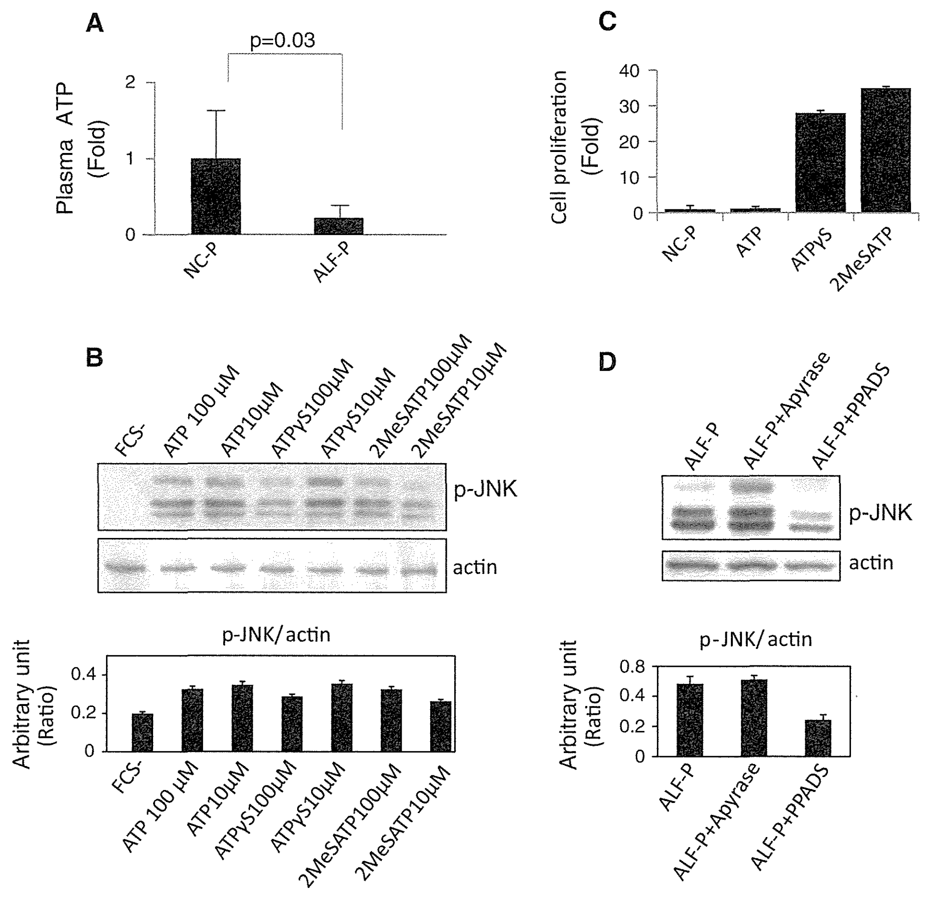

Fig. 3 Extracellular ATP does not contribute to the effects of ALF-P on the growth of OCs. **a** Plasma ATP was measured using samples from patients with ALF and normal control subjects. Data are expressed as the mean ± SD ($n = 3$). **b** OCs were treated with ATP or two other P2 receptor agonists at 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 ± 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

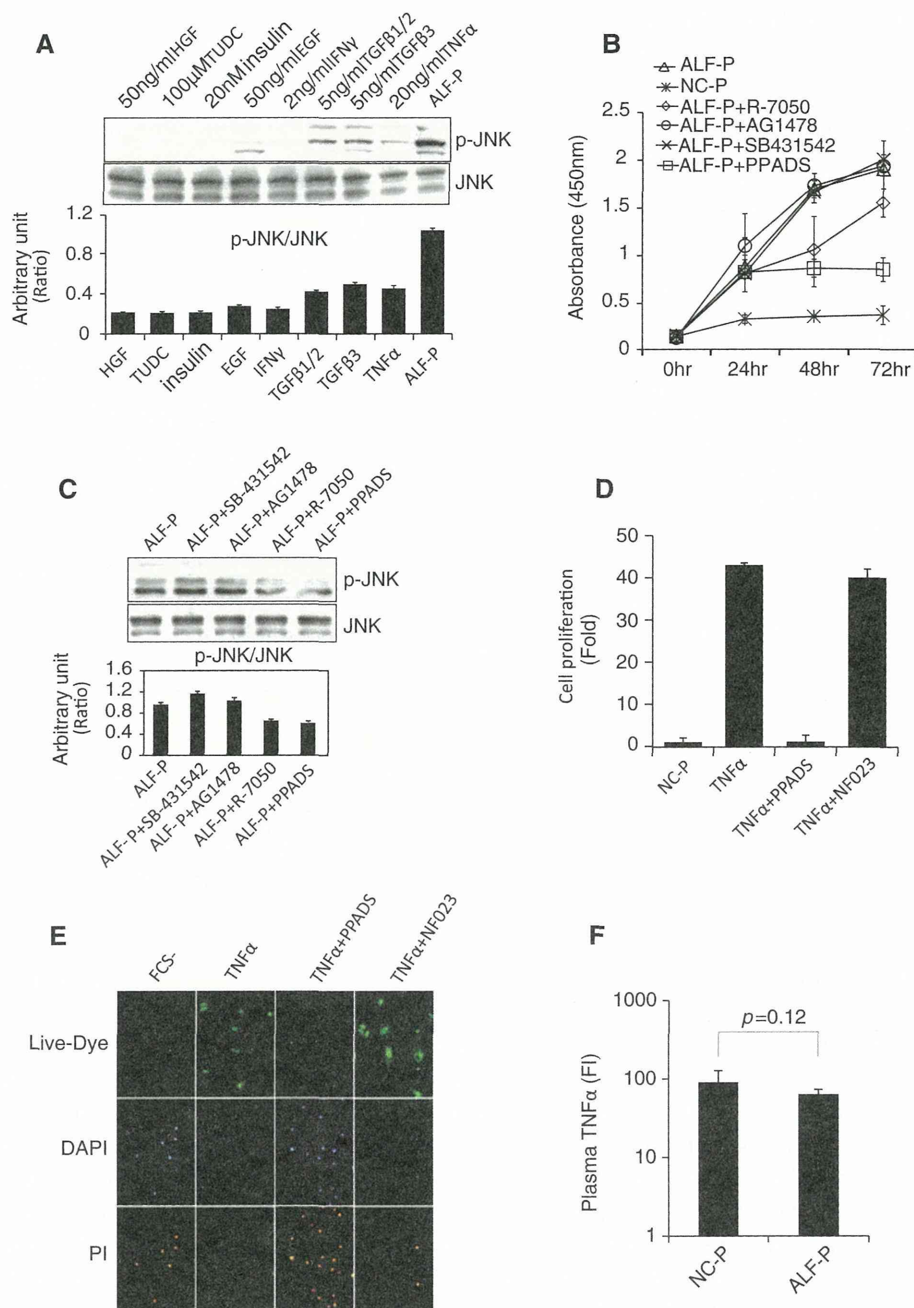

Fig. 4 TNFα receptor signaling mediates the function of ALF-P in a P2 receptor-dependent manner. **a** Cells were incubated with 30 % ALF-P and the other indicated reagents. The phosphorylation of JNK was detected after 3 days of stimulation. JNK was also detected as an internal control. Data are expressed as the mean ± 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 ± SD ($n = 3$). **d** OCs were treated with 10 μg/mL TNFα with or without 250 μM PPADS or 200 μM NF023. Cells proliferation was determined after 3 days of stimulation. Data are expressed as the mean ± 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 TNFα 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 ± SD ($n = 3$). $^{*}p < 0.05$ versus control

reported that $P2X_4$ and $P2X_7$ 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 $P2X_2$ 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 $P2Y_2$ rather than $P2Y_1$ participates in the regulation of OCs by ALF-P. Notably, although the expression of $P2Y_2$ 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 $P2Y_2$, $P2Y_4$, and $P2Y_6$ 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 $P2Y_2$ 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