APOA4

Subjects: Biology

Contributor: Sotaro Motoi

APOA4, which has been previously known as one of the apolipoproteins, was found to be a soluble PD marker and can be secreted into the blood as a result of HGF-c-Met signaling. Recently, it was reported that APOA4 is not just a constituent of high-density lipoprotein, but it also exerts anti-oxidant and anti-inflammatory effects to protect from liver damage in mice.

rh-HGF APOA4 ALF c-Met Fas

1. Introduction

Hepatocyte growth factor (HGF) was originally discovered in the serum of patients with fulminant hepatitis due to its strong capacity to induce hepatocyte proliferation [1][2][3]. Since its discovery, vigorous research has been conducted on HGF world-wide, which revealed its pleiotropic activities [4][5][6], among which its strong and rapid anti-apoptotic effects against murine liver injury led to the dynamic investigation into a fatal and disastrous condition, acute liver failure. Although, to date, liver transplantation is the only curative treatment for the disease, the constant need for a novel drug that can help avoid or at least delay the need of organ transplantation is still unmet [7][8][9]. With the support of the Japan Agency for Medical Research and Development (AMED), we successfully manufactured a recombinant human HGF (rh-HGF) that is currently under evaluation in a phase I study with healthy Japanese volunteers (ClinicalTrials.gov Identifier: NCT03922633). During the process of drug development, it is valuable to evaluate the pharmacodynamic activity of new investigational drugs in healthy volunteers prior to evaluating its clinical efficacy or benefits for diseased conditions. Therefore, we aimed to identify a pharmacodynamic marker for rh-HGF that directly and biologically reflects exogenously administered rh-HGF, in terms of HGF-mediated c-Met receptor activation. DNA microarray analyses of the livers of rh-HGF-administered normal mice showed an induction of various genes as candidate PD markers. Among those, APOA4, which has been previously known as one of the apolipoproteins, was found to be a soluble PD marker and can be secreted into the blood as a result of HGF-c-Met signaling. Recently, it was reported that APOA4 is not just a constituent of high-density lipoprotein, but it also exerts anti-oxidant and anti-inflammatory effects to protect from liver damage in mice [10][11]. Therefore, validation of the utility of APOA4 as a PD marker of rh-HGF in clinical trials would help pharmacodynamic evaluation of rh-HGF, and furthermore, may give clues to elucidate its mechanism of action in humans, not just by direct effect on hepatocytes but also via APOA4 protein.

2. Induction of Hepatic mRNA Expression after rh-HGF Exposure

The murine livers were harvested to collect hepatic mRNAs at 8 h or 24 h after the single rh-HGF injection (1.5 mg/kg), and the prepared cDNAs were applied to the DNA microarray according to the manufacturer's instructions. The obtained gene list was narrowed down by filtering with molecular annotations such as "Extracellular" and/or "Plasma membrane"; thereafter, they were sorted in descending order of change in expression at 24 h (Table 1). Among them, we chose to investigate *Apoa4* due to its sustained expression up to 24 h. While the signal values of *Apoa4* at baseline and with PBS injection were around 2500, it was significantly enhanced by rh-HGF with more than a 6.46- or 5.52-fold change at 8 h and 24 h, respectively. Although there were other soluble proteins, such as *Pcsk9*, *Tnfrsf9*, and *Il6ra* (Table 1) and other growth factors such as *Ctgf* (data not shown), none of them showed a good expression magnitude and sustained expression compared to *Apoa4*.

Table 1. Genes highly induced by rh-HGF in the murine liver. Signal values at each timepoint show the average obtained from 3 mice which were sacrificed at designated timepoint before or after rh-HGF administration (1.5 mg/kg).

Gene Symbol	Signal Value (Average)			Expression Change (versus 0 h)			
				8 h		24 h	
	0 h	8 h	24 h	Ratio	*p* Value	Ratio	*p* Value
Apoa4	2436	15,738	13,453	6.46	0.0002	5.52	0.0016
Prss8	37	123	198	3.32	0.0955	5.35	0.0903
Defa1	34	37	106	1.09	0.8609	3.11	0.2846
Tff3	56	132	167	2.35	0.0623	2.97	0.1078
Hmgb2	701	553	1679	0.79	0.0879	2.40	0.0009
Dnase1	46	59	109	1.29	0.4958	2.39	0.2253
Apoc3	408	1059	938	2.60	0.0002	2.30	0.0004
Afp	71	349	148	4.89	0.0197	2.07	0.0170
Spink3	83	66	166	0.80	0.2707	2.01	0.2758
Tnfrsf9	38,813	112,574	74,826	2.90	0.0043	1.93	0.0192
Il15ra	44,941	118,655	83,474	2.64	0.0092	1.86	0.0151
Pcsk9	567	1458	1036	2.57	0.0187	1.83	0.0431
Gast	37	357	63	9.54	0.0005	1.69	0.1393
Nrg4	75	1724	112	22.85	0.0001	1.48	0.0421
Il6ra	2912	14,047	3742	4.82	0.0005	1.29	0.1408

Gene Symbol	Signal Value (Average)			Expression Change (versus 0 h)			
				8 h		24 h	
	0 h	8 h	24 h	Ratio	*p* Value	Ratio	*p* Value
Fhad1	124	1212	143	9.79	0.0003	1.16	0.0674
Cgref1	113	1350	128	11.96	0.0013	1.13	0.6870
Alpl	411	1499	456	3.64	0.0097	1.11	0.5636
Fam3c	1807	5240	1962	2.90	0.0002	1.09	0.2547
Serpina7	373	1636	283	4.38	0.0018	0.76	0.1721
Creld2	11,476	34,924	8179	3.04 [6]	0.0433	0.71	0.3436
Cxcl14	34	161	21 [6]	4.81	0.0073	0.62	0.3495

F, primary
e 1A) and
nanner. A
as indeed
GF in the

presence of the c-Met inhibitor, PF-04217903 (100 nM). Inhibition of c-Met signaling significantly suppressed the induction of *APOA4* mRNA (Figure 1A) as well as production of APOA4 protein into the supernatants (Figure 1B,C). Considering the inter-species consistency in response of APOA4 to rh-HGF, APOA4 is strongly assumed to represent the pharmacodynamic nature of rh-HGF.

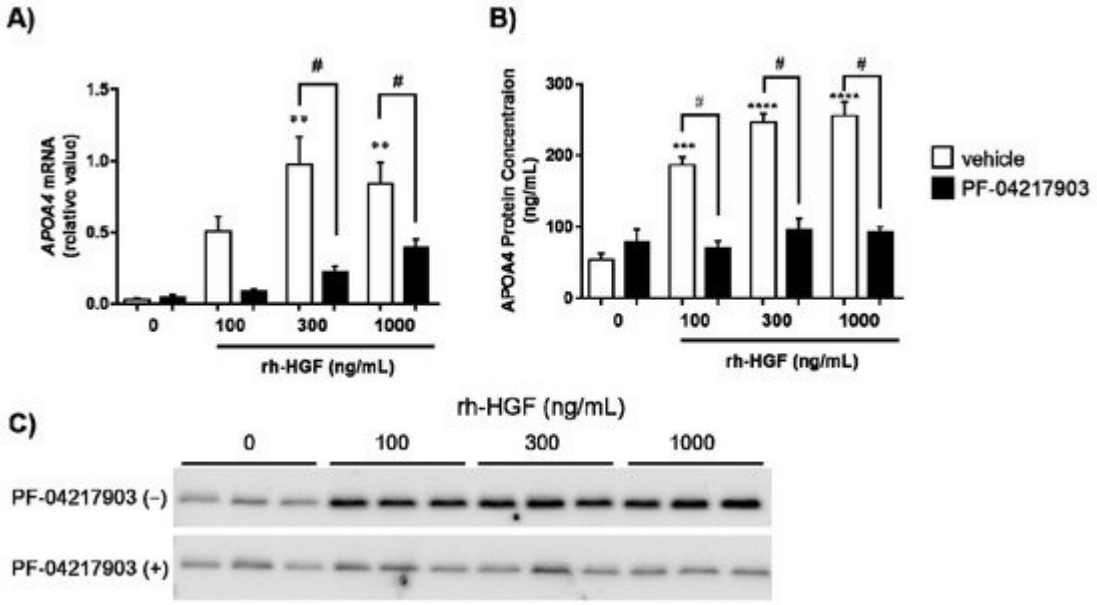

Figure 1. Induction of human APOA4 by rh-HGF, and its c-Met dependency. (**A**) Relative expression of *APOA4* mRNA compared to the expression of 18S rRNA in human hepatocytes, (**B**) Relative concentration of APOA4 protein in supernatants ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$: comparison between vehicle control (incubation medium) or rh-HGF alone-treated group (Dunnett's test). $^{\#}$ $p < 0.05$: comparison between rh-HGF alone-treated group and PF-04217903-rh-HGF co-culture group (unpaired *t* test). The data are presented as mean ± SEM ($n = 3$). The median value of the triplicate analyses was used for calculation. (**C**) Analysis of APOA4 protein levels in the supernatants of the culture using Western blot. The picture shows a representative batch of hepatocytes in triplicate.

4. Discussion

There has been substantial progress in medical practice for the care of ALF patients by incorporating an etiology-based approach and artificial liver support. Among these, liver transplantation is the most reliable measure to rescue potentially fatal cases, especially comatose patients with ALF [8][12][13]. However, it is also true that transplantation is not always applicable for the patients who have an indication of liver transplantation, primarily due to the shortage of donors. Therefore, a new treatment option is needed to prevent progressive liver damage, preserve residual mass of functional liver, and promote the repair and regeneration of hepatocytes. HGF, due to its strong anti-apoptotic effect, stimulation of tissue repair, and regeneration activities, is the most promising hope to address this long-standing problem [4][5][14]. We successfully manufactured rh-HGF that pre-clinically showed substantial inhibition of liver damage with the strong preservation of prothrombin time in a Fas-triggering murine ALF model [15]. Prior to conducting a clinical trial with ALF patients, we tried to identify a PD marker of rh-HGF, which could allow us to evaluate the pharmacological outcome of rh-HGF besides the conventional clinical parameters for ALF care, such as AST, ALT, and PT. Moreover, the availability of such a PD marker for healthy subjects may help to determine appropriate dosing regimens of rh-HGF in patients with ALF.

We firstly chose live animals rather than an in vitro cell system to discover PD markers because it might mimic the clinical setting in humans well, considering the bio-distribution of rh-HGF in the body. rh-HGF is known to preferentially distribute to the liver and with much less amount to the kidney and lung [16]. Therefore, we first harvested the murine livers to identify the candidates for PD markers after rh-HGF administration, and, at the same time we pre-filtered candidates by limiting these to the molecules which were present extracellularly or on the plasma membrane. In addition, we selected molecules which showed sustained expression from the early (8 h) to the late (24 h) timepoint to heighten their likelihood of being detected in the serum independent of their serum half-lives. As a result, murine *Apoa4* was found to be the highest (Table 1). Besides *Apoa4*, other genes such as *Pcsk9*, *Tnfrsf9*, and *Il6ra* that were identified using DNA microarray, were remarkably induced by rh-HGF, although the induction was only transient at 8 or 24 h in most cases, or the signal values, which may impact the protein levels, were relatively low. Since rh-HGF impacts cell growth and regeneration, the induction of cell-cycle- or growth-related genes such as *Ccnd1*, *ccne1*, and *Myc* was very obvious and they showed upregulation (data not shown). While these intracellular molecules may be less efficient as soluble PD markers, their upregulation confirmed the in vivo biological impacts of administered rh-HGF [2][17].

Thereafter, the induction of APOA4 was validated by using an in vitro human system utilizing primary cultured human hepatocytes (Figure 1). Consistent with the previous report that human APOA4 is expressed primarily in the small intestine [18], the baseline levels of *APOA4* mRNA were quite low in human hepatocytes. However, we found that rh-HGF induced *APOA4* mRNA in hepatocytes and stimulated the cells to produce APOA4 protein in a dose-dependent manner, mediated by c-Met (Figure 1). In this culture system, rh-HGF enhanced the APOA4 protein production by four- to five-fold compared to that of the control. Therefore, APOA4 is assumed to have high signal-to-noise ratio when human hepatocytes are treated with rh-HGF. If this observation is proven to be true in clinical settings, APOA4 will be a promising PD marker because of its assay sensitivity.

In order to confirm the adequacy of APOA4 as a PD marker of rh-HGF, the pharmacodynamic profile of APOA4 was evaluated in live animals; initially in normal mice, followed by mice with liver injury. In mice, APOA4 is known to be expressed in the small intestine and the liver [18][19][20]. This was also observed in our study, and the expression of murine *Apoa4* was detected in both the organs at baseline with relatively lower values in the liver (Figure 2). However, rh-HGF strongly induced *Apoa4* mRNA only in the liver at 8 h in a dose-dependent manner and the induction was sustained throughout the 24 h (Figure 2A) and such an induction of *Apoa4* mRNA was not detected in the small intestine (Figure 2B). This discrepancy might be due to preferential bio-distribution of rh-HGF to the liver and not to the small intestine [16]. Even though we cannot deny the possibility that intestinal *Apoa4* is a downstream molecule other than c-Met in the intestine, the observed finding at least proves that the liver is the primary organ impacted by administered rh-HGF in vivo. Thereafter, we focused on the induction of hepatic mRNA and serum APOA4 levels, particularly addressing the c-Met dependency. Notably, rh-HGF induced *Apoa4* mRNA (Figure 3A) in the liver was associated with a dose-dependent increase in serum APOA4 protein concentration (Figure 3B,C). Importantly, the induction of APOA4 was inhibited by a c-Met inhibitor PF-04217903 both at mRNA and protein levels (Figure 3A–C), therefore, it strongly suggested that APOA4 directly represents the pharmacological effects of rh-HGF as the downstream molecule of c-Met signaling.

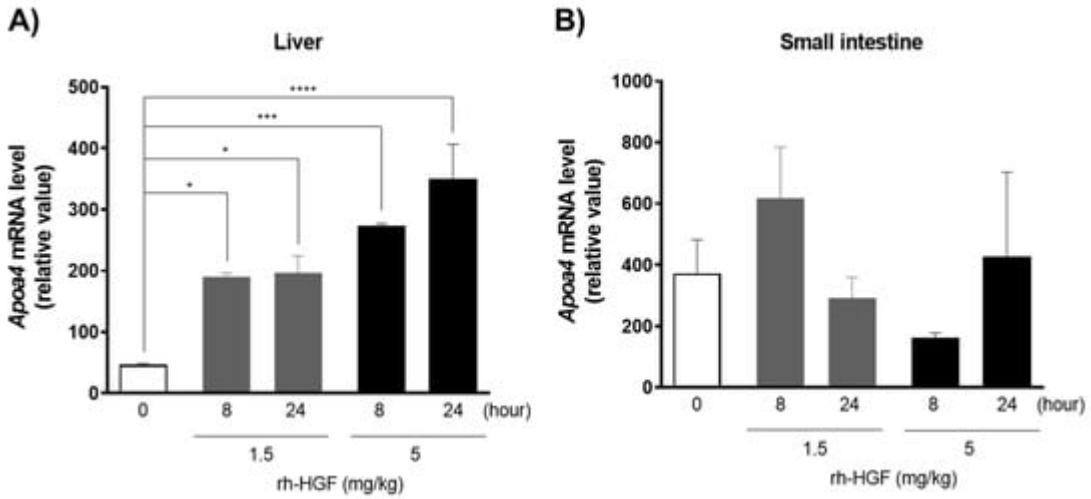

Figure 2. Expression of *Apoa4* mRNA in the liver and small intestine after rh-HGF administration in mice. (**A**) Liver, (**B**) Small intestine. Relative expression of *Apoa4* mRNA against the expression of *Hprt* mRNA was calculated using the median value of the triplicate analyses * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$: comparison between the vehicle control (PBS)- and rh-HGF-treated groups (Dunnett's test). The data are presented as mean ± SEM ($n = 3$).

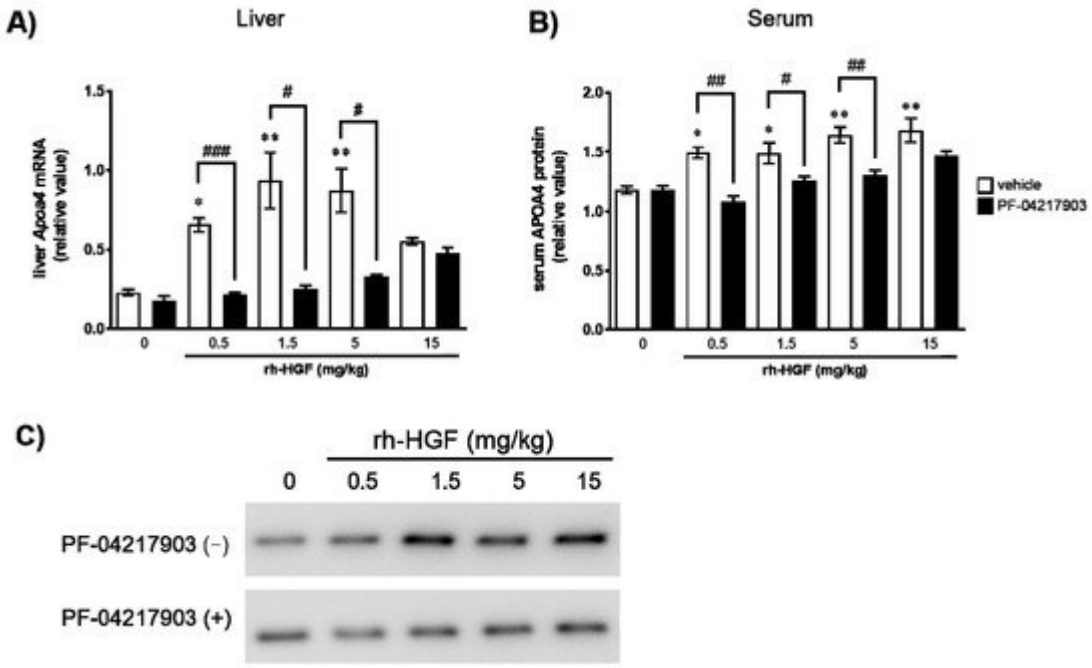

Figure 3. c-Met dependent induction of murine APOA4. Twenty-four hours after rh-HGF administration with or without PF-04217903 treatment, the mice were sacrificed, and their livers and bloods were collected for the analyses. (**A**) Relative expression of *Apoa4* mRNA in the liver compared with the expression of *Hprt* mRNA in mice, (**B**) Relative value of APOA4 protein in serum * $p < 0.05$, ** $p < 0.01$: comparison between vehicle control (PBS) and rh-HGF alone-treated groups (Dunnett's test), $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$: comparison between rh-HGF alone-treated group and PF-04217903- rh-HGF co-administered group (unpaired *t* test). The data are presented as mean ± SEM (n = 3). The median value of the triplicate analyses was used for calculation. (**C**) Analysis of APOA4 protein levels in serum using Western blot. The picture shows a representative image of lanes showing individual animals.

Finally, the adequacy of APOA4 as a PD marker was assessed in murine ALF model, which was developed by Fas-mediated cell death using an anti-Fas antibody [5]. Consistent with the observation in normal mice, rh-HGF drastically induced serum APOA4 protein in a dose-dependent manner in mice with ALF (Figure 4). We previously reported that the anti-apoptotic effects of rh-HGF on hepatocytes were significantly associated with the suppression of intrahepatic hemorrhage in which preserved PT was the best parameter that strongly correlated with the decrease in intrahepatic hemorrhages [15]. Considering the PT kinetics after administering rh-HGF [15], the inductive increase in murine APOA4 protein in serum by rh-HGF (Figure 4) reciprocally correlated with the suppression of PT prolongation [15], which might further exhibit the feasibility of APOA4 as a PD marker of rh-HGF.

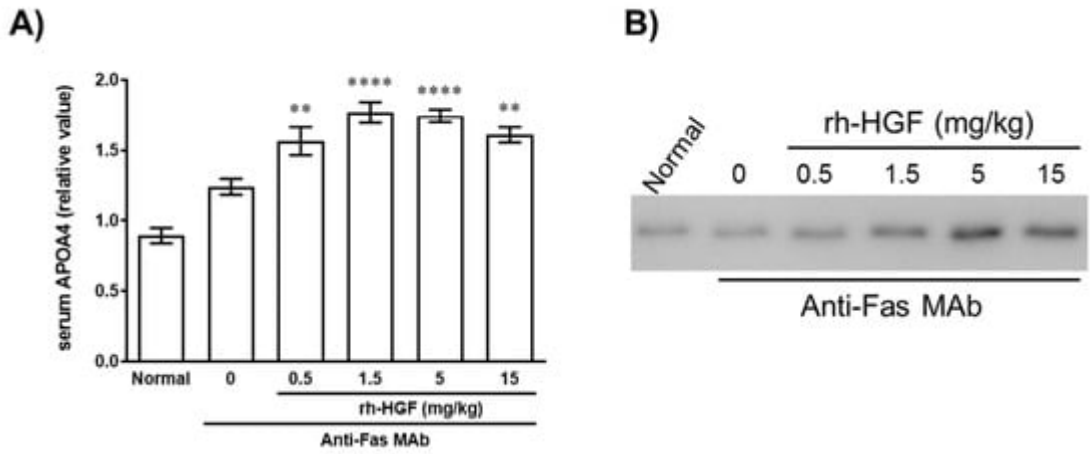

Figure 4. rh-HGF-mediated induction of APOA4 protein in anti-Fas-induced murine ALF model. rh-HGF was injected intravenously one hour prior to the anti-Fas injection. Five hours after anti-Fas administration, the mice were sacrificed and their blood collected for the analyses. (**A**) Relative value of APOA4 protein in serum. PBS was administered in the vehicle control group (rh-HGF, 0 mg/kg). ** $p < 0.01$, **** $p < 0.0001$: comparison between the vehicle control- and rh-HGF-treated groups (Dunnett's test). The data are presented as mean ± SEM (n = 5–8). (**B**) A representative image of serum APOA4 levels by Western blot. Each lane shows individual animals.

Here, we primarily identified APOA4, one of the downstream molecules of HGF-c-Met signaling, to be a soluble PD marker of rh-HGF. Interestingly, it has been demonstrated that APOA4 is not just a constituent of high-density lipoprotein but it exerts anti-oxidant and anti-inflammatory effects to protect liver damage in mice [10][11]. Notably, serum APOA4 seemed to increase in murine ALF over normal mice without exogenous rh-HGF (Figure 4A; rh-HGF, 0 mg/kg). Endogenously induced HGF may be responsible for this, although there may be other molecular events regulating APOA4 synthesis. Taken together, it is essential to investigate the clinical utility of serum APOA4 in future clinical trials with healthy subjects and ALF patients at least to evaluate the pharmacodynamic effect of the rh-HGF, E3112. In addition, such human data may help further understanding of how rh-HGF exerts its therapeutic action in clinical settings, not just by direct effect on liver cells but also via APOA4 protein.

References

1. Gohda, E.; Tsubouchi, H.; Nakayama, H.; Hirono, S.; Takahashi, K.; Koura, M.; Hashimoto, S.; Daikuhara, Y. Human hepatocyte growth factor in plasma from patients with fulminant hepatic failure. Exp. Cell Res. 1986, 166, 139–150.
2. Gohda, E.; Tsubouchi, H.; Nakayama, H.; Hirono, S.; Sakiyama, O.; Takahashi, K.; Miyazaki, H.; Hashimoto, S.; Daikuhara, Y. Purification and partial characterization of hepatocyte growth factor from plasma of a patient with fulminant hepatic failure. J. Clin. Investig. 1988, 81, 414–419.
3. Tsubouchi, H.; Hirono, S.; Gohda, E.; Nakayama, H.; Takahashi, K.; Sakiyama, O.; Miyazaki, H.; Sugihara, J.; Tomita, E.; Muto, Y.; et al. Clinical significance of human hepatocyte growth factor in

blood from patients with fulminant hepatic failure. Hepatology 1989, 9, 875–881.

4. Strain, A.J.; Ismail, T.; Tsubouchi, H.; Arakaki, N.; Hishida, T.; Kitamura, N.; Daikuhara, Y.; McMaster, P. Native and recombinant human hepatocyte growth factors are highly potent promoters of DNA synthesis in both human and rat hepatocytes. J. Clin. Investig. 1991, 87, 1853–1857.
5. Kosai, K.-I.; Matsumoto, K.; Nagata, S.; Tsujimoto, Y.; Nakamura, T. Abrogation of Fas-Induced Fulminant Hepatic Failure in Mice by Hepatocyte Growth Factor. Biochem. Biophys. Res. Commun. 1998, 244, 683–690.
6. Moumen, A.; Ieraci, A.; Patanè, S.; Solé, C.; Comella, J.X.; Dono, R.; Maina, F. Met signals hepatocyte survival by preventing Fas-triggered FLIP degradation in a PI3k-Akt-dependent manner. Hepatology 2007, 45, 1210–1217.
7. Williams, R.; Schalm, S.; O'Grady, J. Acute liver failure: Redefining the syndromes. Lancet 1993, 342, 273–275.
8. Bernal, W.; Hyyrylainen, A.; Gera, A.; Audimoolam, V.K.; McPhail, M.J.; Auzinger, G.; Rela, M.; Heaton, N.; O'Grady, J.G.; Wendon, J.; et al. Lessons from look-back in acute liver failure? A single centre experience of 3300 patients. J. Hepatol. 2013, 59, 74–80.
9. O'Grady, J.G. Acute liver failure. Postgrad. Med. J. 2005, 81, 148–154.
10. Li, X.; Liu, X.; Zhang, Y.; Cheng, C.; Fan, J.; Zhou, J.; Garstka, M.A.; Li, Z. Hepatoprotective effect of apolipoprotein A4 against carbon tetrachloride induced acute liver injury through mediating hepatic antioxidant and inflammation response in mice. Biochem. Biophys. Res. Commun. 2021, 534, 659–665.
11. Wang, Y.; Yang, Z.; Wei, Y.; Li, X.; Li, S. Apolipoprotein A4 regulates the immune response in carbon tetrachloride-induced chronic liver injury in mice. Int. Immunopharmacol. 2021, 90, 107222.
12. Bernal, W.; Wendon, J. Acute liver failure. N. Engl. J. Med. 2013, 369, 2525–2534.
13. Oketani, M.; Ido, A.; Nakayama, N.; Takikawa, Y.; Naiki, T.; Yamagishi, Y.; Ichida, T.; Mochida, S.; Onishi, S.; Tsubouchi, H.; et al. Etiology and prognosis of fulminant hepatitis and late-onset hepatic failure in Japan: Summary of the annual nationwide survey between 2004 and 2009. Hepatol. Res. 2013, 43, 97–105.
14. Ishii, T.; Sato, M.; Sudo, K.; Suzuki, M.; Nakai, H.; Hishida, T.; Niwa, T.; Umezu, K.; Yuasa, S. Hepatocyte growth factor stimulates liver regeneration and elevates blood protein level in normal and partially hepatectomized rats. J. Biochem. 1995, 117, 1105–1112.
15. Motoi, S.; Toyoda, H.; Obara, T.; Ohta, E.; Arita, Y.; Negishi, K.; Moriya, K.; Kuboi, Y.; Soejima, M.; Imai, T.; et al. Anti-apoptotic effects of recombinant human hepatocyte growth factor on

hepatocytes were associated with intrahepatic hemorrhage suppression indicated by the preservation of prothrombin time. Int. J. Mol. Sci. 2019, 20, 1821.

16. Ido, A.; Moriuchi, A.; Kim, I.; Numata, M.; Nagata-Tsubouchi, Y.; Hasuike, S.; Uto, H.; Tsubouchi, H. Pharmacokinetic study of recombinant human hepatocyte growth factor administered in a bolus intravenously or via portal vein. Hepatol. Res. 2004, 30, 175–181.
17. Fujiwara, K.; Nagoshi, S.; Ohno, A.; Hirata, K.; Ohta, Y.; Mochida, S.; Tomiya, T.; Higashio, K.; Kurokawa, K. Stimulation of liver growth by exogenous human hepatocyte growth factor in normal and partially hepatectomized rats. Hepatology 1993, 18, 1443–1449.
18. Wang, F.; Kohan, A.B.; Lo, C.-M.; Liu, M.; Howles, P.; Tso, P. Apolipoprotein A-IV: A protein intimately involved in metabolism. J. Lipid Res. 2015, 56, 1403–1418.
19. Gallagher, J.W.; Weinberg, R.B.; Shelness, G.S. apoA-IV tagged with the ER retention signal KDEL perturbs the intracellular trafficking and secretion of apoB. J. Lipid Res. 2004, 45, 1826–1834.
20. Weinberg, R.B.; Gallagher, J.W.; Fabritius, M.A.; Shelness, G.S. ApoA-IV modulates the secretory trafficking of apoB and the size of triglyceride-rich lipoproteins. J. Lipid Res. 2012, 53, 736–743.