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L⁵⁹ TGF-β LAP degradation products serve as a promising blood biomarker for liver fibrogenesis in mice

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Abstract

Background: Hepatic fibrosis, which is the excessive accumulation of extracellular matrices (ECMs) produced mainly from activated hepatic stellate cells (HSCs), develops to cirrhosis over several decades. There are no validated biomarkers that can non-invasively monitor excessive production of ECM (i.e., fibrogenesis). Transforming growth factor (TGF)-β, a key driver of fibrogenesis, is produced as an inactive latent complex, in which active TGF-β is enveloped by its pro-peptide, the latency-associated protein (LAP). Thus, active TGF-β must be released from the complex for binding to its receptor and inducing ECM synthesis. We recently reported that during the pathogenesis of liver fibrosis, plasma kallikrein (PLK) activates TGF-β by cleavage between R⁵⁸ and L⁵⁹ residues within LAP and that one of its by-products, the N-terminal side LAP degradation products ending at residue R⁵⁸ (R⁵⁸ LAP-DPs), can be detected mainly around activated HSCs by specific antibodies against R⁵⁸ cleavage edges and functions as a footprint of PLK-dependent TGF-β activation. Here, we describe a sandwich enzyme-linked immunosorbent assay (ELISA) that detects the other by-products, the C-terminal side LAP-DPs starting from residue L⁵⁹ (L⁵⁹ LAP-DPs). We demonstrated that the L⁵⁹ LAP-DPs are a potentially novel blood biomarker reflecting hepatic fibrogenesis.

Results: We established a specific sandwich ELISA to quantify L⁵⁹ LAP-DPs as low as 2 pM and measured L⁵⁹ LAP-DP levels in the culture media of a human activated HSC line, TWNT-4 cells. L⁵⁹ LAP-DPs could be detected in their media, and after treatment of TWNT-4 cells with a TGF-β receptor kinase inhibitor, SB431542, a simultaneous reduction was observed in both L⁵⁹ LAP-DP levels in the culture media and the mRNA expression levels of *collagen type (I) α1*. In carbon tetrachloride- and bile duct ligation-induced liver fibrosis models in mice, plasma L⁵⁹ LAP-DP levels increased prior to increase of hepatic hydroxyproline (HDP) contents and well correlated with α-smooth muscle actin (αSMA) expression in liver tissues. At this time, αSMA-positive cells as well as R⁵⁸ LAP-DPs were seen in their liver tissues.

Conclusions: L⁵⁹ LAP-DPs reflect PLK-dependent TGF-β activation and the increase in αSMA-positive activated HSCs in liver injury, thereby serving as a novel blood biomarker for liver fibrogenesis.

Keywords: Biomarker, Hepatic stellate cells, Fibrogenesis, Liver fibrosis, Plasma kallikrein (PLK), TGF-β activation

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Background

Hepatic fibrosis, the common pathology resulting from chronic liver diseases regardless of etiology, is characterized as the excessive deposition of extracellular matrices (ECMs) produced mainly from activated hepatic stellate cells (HSCs) in injured tissue [1, 2]. Because early fibrosis is often asymptomatic, yet steadily progresses toward cirrhosis, it is important to monitor fibrogenic activity both to track disease progression and eventually to assess response to anti-fibrotic drugs. Liver biopsy, a widely accepted technique, is highly invasive and reflects only already-accumulated ECMs, but not ongoing ECM synthesis. Thus, a non-invasive marker for fibrogenesis in the liver is a major unmet need in the field [3, 4].

A key driver of liver fibrosis is transforming growth factor (TGF)- β [5]. TGF- β is produced as a homodimeric precursor protein consisting of an N-terminal pro-region named latency-associated protein (LAP) and a C-terminal region that becomes the biologically active TGF- β molecule. After processing at R²⁷⁸-A²⁷⁹ by a furin-like protease, the cleaved LAP still remains non-covalently associated with the active TGF- β , forming a small latent complex and preventing active TGF- β from binding to its cognate receptors. Therefore, active TGF- β must be released from the latent complex, a process called TGF- β activation, to induce ECM production [6, 7]. Resultant active TGF- β binds to cognate serine/threonine kinase signaling receptors and via the Smad signaling pathway stimulates the expression of target genes, including ECM proteins and TGF- β itself, in an autocrine manner [5, 8].

We recently found that plasma kallikrein (PLK) cleaves LAP between R⁵⁸ and L⁵⁹ residues to cause TGF- β 1 activation. We further demonstrated that this mechanism underlies hepatic fibrosis in animal models, as well as in patients, by detecting the N-terminal side LAP degradation products (...QILSKLR⁵⁸) ending at R⁵⁸ residue (R⁵⁸ LAP-DPs) in fibrotic livers using a specific antibody we generated [9]. R⁵⁸ LAP-DPs are S-S bonded to latent TGF- β binding protein via C³³ residue. These DPs were detected primarily around α -smooth muscle actin (α SMA)-positive activated HSCs, both in peri-sinusoidal fibrotic regions prior to excessive ECM deposition, suggesting that R⁵⁸ LAP-DPs might be a marker that reflects PLK-dependent TGF- β activation [9]. On the other hand, the C-terminal side LAP-DPs (L⁵⁹ASPPSQ...), beginning from L⁵⁹ residue (L⁵⁹ LAP-DPs), was not detectable in the liver tissues, perhaps because L⁵⁹ LAP-DPs might be released into the blood.

Here, we describe a sandwich enzyme-linked immunosorbent assay (ELISA) using the specific antibody against L⁵⁹ LAP-DPs and examine whether L⁵⁹ LAP-DPs can be used as a blood biomarker reflecting PLK-dependent TGF- β activation that correlates with activated HSCs

and whether they can be used to monitor liver fibrogenesis in mice.

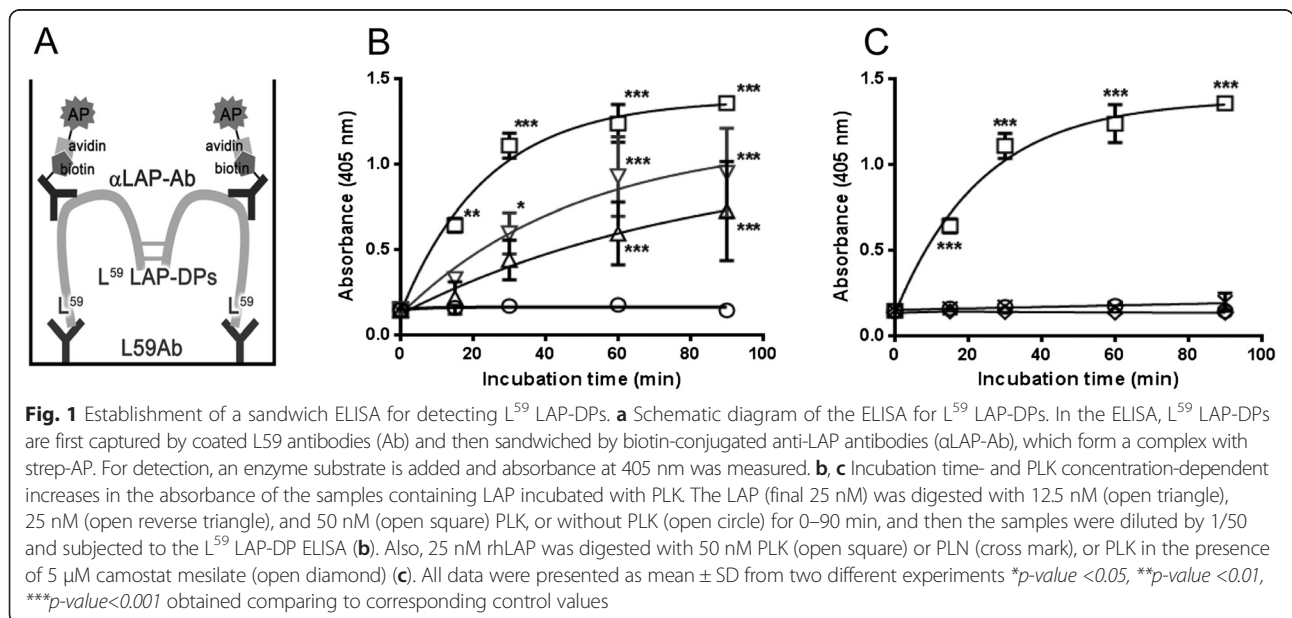
Results

Establishment of a sandwich ELISA for measuring L⁵⁹

LAP-DPs

To quantify the levels of L⁵⁹ LAP-DPs in a culture medium of collagen-producing cells including HSCs or blood from animal models with fibrogenesis, we established a sandwich ELISA using a combination of L59 and commercially available anti-LAP antibodies (Fig. 1a) and examined the specificity of the ELISA for L⁵⁹ LAP-DPs initially in test tube reactions. The absorbance of a sample containing recombinant human LAP β 1 (rhLAP β 1) incubated with PLK, in which L⁵⁹ LAP-DPs are produced, increased proportionately to both the duration of PLK digestion and the concentration of PLK (Fig. 1b). This increase was completely abolished in the presence of a protease inhibitor, camostat mesilate (Fig. 1c, open diamond). In addition, there was no increase in the absorbance in the sample containing LAP incubated with plasmin (PLN) (Fig. 1c, cross mark), in which LAP was degraded between K⁵⁶ and L⁵⁷ residues [9, 10]. These results suggest that the established ELISA is specific for L⁵⁹ LAP-DPs and may reflect PLK-dependent TGF- β activation reaction in vitro, as we reported for the R58 antibody [9]. We made the standard curve by diluting standard L⁵⁹ LAP-DPs obtained after incubating rhLAP β 1 with an equal amount of PLK at 37 °C for 2 h. By a Western experiment, we confirmed that rhLAP β 1 was almost completely digested to L⁵⁹ LAP-DPs under this condition (data not shown). The absorbance linearly increased up to 100 pM (Additional file 1: Figure S1). We were able to measure a minimum concentration of L⁵⁹ LAP-DPs as low as 2 pM. From results of the spike and recovery test, we decided to dilute samples at least by 1:2 in the case of culture media and at least by 1:5 in the case of mouse plasma (Table. 1). Actually, we diluted plasma samples 1:10 in the following experiments. The recovery rate of diluted samples was 91 % (in average) both in culture media and in mouse plasma (Table 2). The mean variations among intra- and inter-assays were 3.9 ± 0.4 % ($N = 12$) and 9.8 ± 5.3 % ($N = 10$) in culture media and 8.7 ± 3.6 % ($N = 6$) and 6.5 ± 5.3 % ($N = 6$) in mouse plasma, respectively. Finally, analytes were stable at least after two freeze-thaw cycles for both culture media and mouse plasma.

Using the ELISA, we examined whether L⁵⁹ LAP-DPs might reflect TGF- β activation and therefore the bioactivity of the resultant active TGF- β . Mink lung epithelial cells stably transfected with a TGF- β -responsive reporter gene ($\times 9$ CAGA-Luc-transformed CCL64 cells) were incubated with human latent TGF- β 1 (hLTGF- β 1) in the presence or absence of PLK. This cell line



produced negligible levels of endogenous LTGF-β1, active TGF-β1, and L⁵⁹ LAP-DPs. As a source of hLTGF-β1, we used the conditioned media (CM) of HEK293T cells transiently transfected with an hLTGF-β1 construct. We incubated the ×9 CAGA-Luc-transformed CCL64 cells with the HEK293T cell CM containing 20 nM LTGF-β1, 10 pM active TGF-β1, and 5 nM L⁵⁹ LAP-DPs. To assess TGF-β1 activation, namely, generation of active TGF-β1, we determined levels of both active TGF-β1 and L⁵⁹ LAP-DPs in the culture media by respective ELISAs. At the same time, to assess the relationship between L⁵⁹ LAP-DPs and cellular signals evoked by the resultant active TGF-β, we also measured increases in the luciferase activity in lysates made from the ×9 CAGA-Luc-transformed CCL64 cells. The levels of active TGF-β1

and L⁵⁹ LAP-DPs increased depending on PLK concentration maintaining a ratio of 1:1300 (Fig. 2a). L⁵⁹ LAP-DP levels also correlated with active TGF-β1 levels (Fig. 2b). Moreover, the luciferase activity in the lysates of the ×9 CAGA-Luc-transformed CCL64 cells also increased and correlated with L⁵⁹ LAP-DP levels (Fig. 2c, d). These data suggested that L⁵⁹ LAP-DPs reflected the activation of TGF-β and the bioactivity of the resultant active TGF-β in cells.

L⁵⁹ LAP-DP levels reflect fibrogenic activity of HSCs in vitro

We examined whether L⁵⁹ LAP-DP levels might reflect collagen production using an activated human HSC line, TWNT-4, that constitutively produces collagen [11].

Table 1 Summarized results of spike and recovery tests

Sample	Added spike (pM)	Dilution rate	Expected (pM)	Observed (pM)	% recovery	
					Average	Range
Culture media	50 pM	Neat	46	32	69.8	69–71
		1:2	46	40	85.8	85–87
		1:4	46	42	91.9	85–87
		1:8	46	44	95.1	93–98
Mouse plasma	200 pM	Neat	189	132	69.8	65–75
		1:5	201	180	89.6	65–75
		1:10	193	184	95.5	92–99
		1:20	190	197	103.5	103–104
	50 pM	Neat	47	29	61.8	58–66
		1:5	46	38	82.7	82–84
		1:10	46	40	88.7	88–89
		1:20	49	43	88.7	87–89

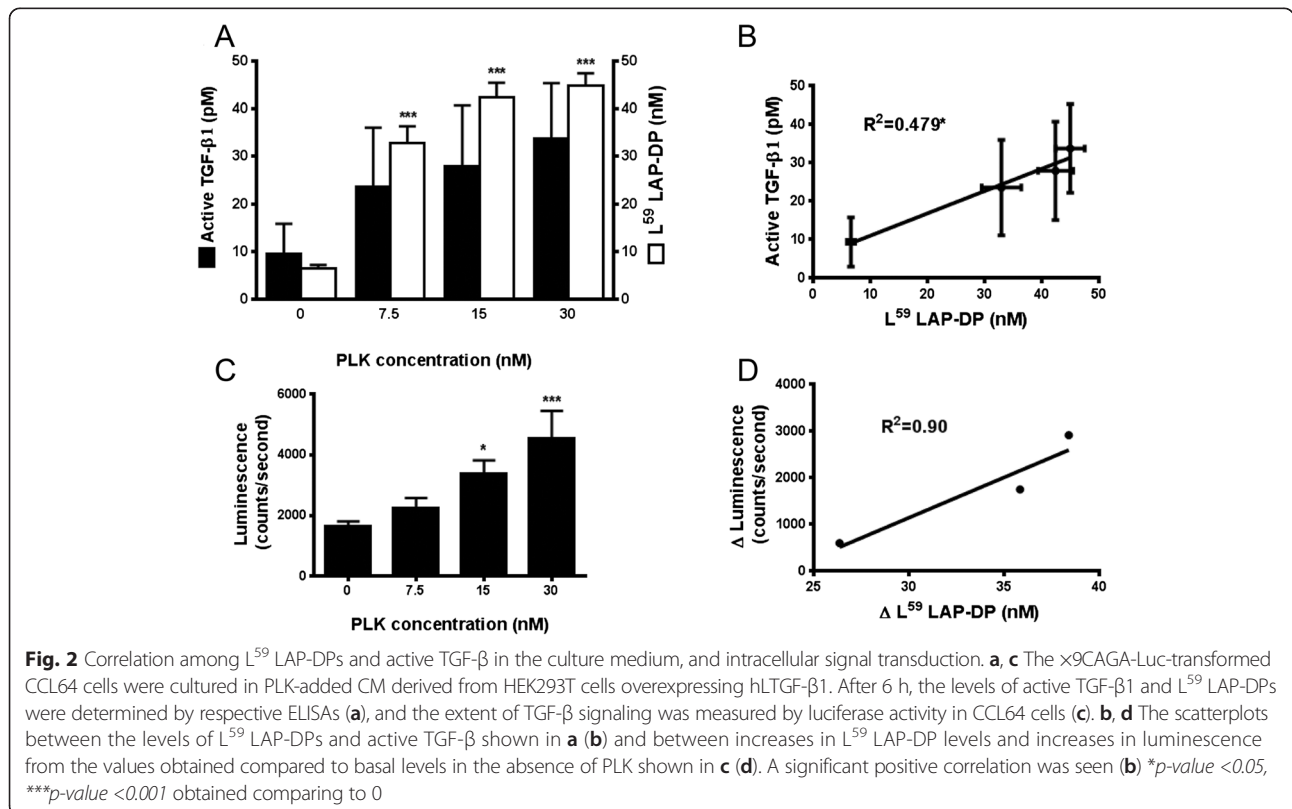
Values exceeding 80 % are presented

Table 2 Summarized results of linearity tests

Sample	Added spike (pM)	Dilution rate	Expected (pM)	Observed (pM)	% recovery	
					Average	Range
Culture media	50 pM	1:2	46	40	85.8	85–87
		1:4	46	42	91.9	90–94
		1:8	46	44	95.1	93–98
		Average 90.9 ± 4.7				
Mouse plasma	200 pM	1:5	201	180	89.6	65–75
		1:10	193	184	95.5	92–99
		1:20	190	197	103.5	103–104
	50 pM	1:5	46	38	82.7	82–84
		1:10	46	40	88.7	88–89
		1:20	49	43	88.7	87–89
		Average 91.3 ± 7.2				

The levels of active TGF-β1 in the media were quite low, less than the detection limit of the assay (~pM), while endogenously detected total TGF-β1 and L⁵⁹ LAP-DPs were present at concentrations as high as 100 pM and 20–40 pM, respectively. After treatment of TWNT-4 cells with 20 μM SB431542 (a TGF-β signaling inhibitor [12]) for 72 h, we measured L⁵⁹ LAP-DP levels in the culture media (Fig. 3b). When *Col 1a1* mRNA levels decreased to

1/30, L⁵⁹ LAP-DP levels were lowered by 50 % and we failed to measure active TGF-β1 in the same culture media. These results suggested that active TGF-β1 disappeared from the culture media during the 72-h incubation after its generation, whereas its by-product, L⁵⁹ LAP-DPs, remained detectable for at least 72 h after incubation, and their levels reflect fibrogenic activity of the activated HSCs.



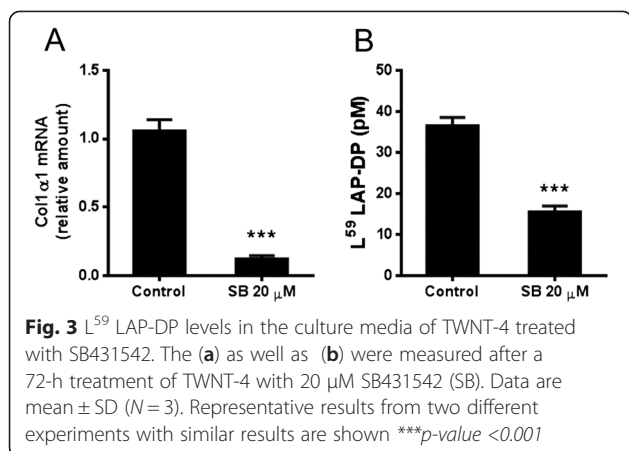


Fig. 3 L⁵⁹ LAP-DP levels in the culture media of TWNT-4 treated with SB431542. The (a) as well as (b) were measured after a 72-h treatment of TWNT-4 with 20 μ M SB431542 (SB). Data are mean \pm SD (N = 3). Representative results from two different experiments with similar results are shown ****p*-value <0.001

Correlation between plasma L⁵⁹ LAP-DP levels and hepatic contents of hydroxyproline in animal models for liver fibrosis

Next, we examined if plasma levels of L⁵⁹ LAP-DPs might reflect hepatic fibrogenesis *in vivo* using two fibrotic animal models (Figs. 4 and 5). We determined the plasma concentrations of L⁵⁹ LAP-DPs in carbon tetrachloride (CCl₄)-treated mice (Fig. 4a, closed columns) and compared them with corresponding hepatic hydroxyproline (HDP) content, which reflects the amount of accumulated collagen or the extent of liver fibrosis (Fig. 4a, open columns). After starting CCl₄ treatment, plasma L⁵⁹ LAP-DP concentrations were significantly higher than in control mice at 4 weeks, before hepatic HDP content increased (Fig. 4a). At 4 weeks, α SMA mRNA expression increased (Fig. 4b), and the levels of plasma L⁵⁹ LAP-DP correlated well with the levels of α SMA mRNA expression in each individual (Fig. 4c). On the other hand, there was no correlation between plasma L⁵⁹ LAP-DP and α SMA mRNA expression at 8 and 12 weeks (Fig. 4d, e, respectively). As shown in Fig. 4f, excessive collagen fibers started to accumulate at 4 weeks after initiating CCl₄ treatment. The α SMA-positive cells appeared, and signals of R⁵⁸ LAP-DPs were also detectable at this time. A similar result was obtained in the mouse bile duct ligation (BDL) model (Fig. 5). Plasma L⁵⁹ LAP-DP concentrations in BDL mice were higher than those in the control animals from post-operative day (POD) 3 to POD 14, whereas hepatic HDP levels gradually increased for up to POD 14. There was no obvious correlation between plasma L⁵⁹ LAP-DP levels and the amounts of hepatic HDP at each mouse (Fig. 5a). On the other hand, pre-fibrotic animals at POD3, in which plasma L⁵⁹ LAP-DP levels were significantly higher, showed a robust increase of α SMA protein expression in liver tissues (Fig. 5b). These results indicated that plasma L⁵⁹ LAP-DP levels reflect ongoing fibrogenesis by the activated HSCs prior to excessive collagen

accumulation, rather than measuring previously accumulated fibrosis.

Discussion

In the present study, we have established a sandwich ELISA using the L59 antibody against the N-terminus of the L⁵⁹ LAP-DPs (Fig. 1) and successfully detected L⁵⁹ LAP-DPs in both human HSC cultures (Fig. 3) and in two mouse hepatic fibrosis models (Figs. 4 and 5). Our ELISA is specific for L⁵⁹ LAP-DPs because it can specifically detect L⁵⁹ LAP-DPs but not non-digested LAP and PLN-generated L⁵⁷ LAP-DPs. The result of a protein-protein Blast (blastp) search showed that only myostatin/GDF8 has a partially similar cleavage sequence with the N-terminal side sequence (...Q⁶⁰ILSKLRLETAP...) [7]. However, we confirmed that the L59 as well as LAP antibodies do not recognize GDF8 incubated with PLK (data not shown).

When \times 9 CAGA-Luc-transformed CCL64 cells were cultured with HEK293T cell CM and PLK, we observed parallel increases in L⁵⁹ LAP-DPs, active TGF- β 1 levels, and resultant luciferase activities (Fig. 2). However, the level of active TGF- β 1 (~33 pM) was 1300 times lower than that of L⁵⁹ LAP-DPs (~43 nM). Because the CM contained about 20 nM dimeric LTGF- β 1, active TGF- β 1 (~33 pM) was quite small (only 0.2 %). It is well known that TGF- β is very sticky and easily adheres to the surface of culture dishes, so levels in the culture media decrease rapidly. Furthermore, *in vivo*, active TGF- β is quickly cleared from the circulation (T_{1/2} = 1.6 \pm 0.71 min) [13], thereby making it difficult to measure accurate levels. We determined that T_{1/2} of L⁵⁹ LAP-DPs is about 8 h (Additional file 2: Figure S2). This T_{1/2} value is comparable to that of aspartate aminotransferase, a well-known blood marker for liver injury [14]. The longer T_{1/2} value for L⁵⁹ LAP-DPs compared to active TGF- β 1 affords significant advantages in terms of being used as a non-invasive biomarker of fibrogenesis.

We speculated that L⁵⁹ LAP-DPs were gradually lost due to either trapping by other proteins or further degradation by other proteases to eliminate their antigenic activity. We are now trying to specify the molecular species detected with our ELISA.

We failed to detect L⁵⁹ LAP-DPs in primary rat HSC cultures, while we did detect them in both the culture media of human HSC line, TWNT-4, and in rat plasma. As we detected almost the same amounts of LTGF- β 1 in the CM of activated rat primary HSCs and TWNT-4, we speculate that LAP-DP levels should be similar. In support of this possibility, we observed an almost similar extent of R⁵⁸ LAP-DP staining in the cultures of activated rat primary HSCs and TWNT-4 (data not shown). Nevertheless, we failed to detect L⁵⁹ LAP-DPs in the culture medium of activated rat primary HSCs. We

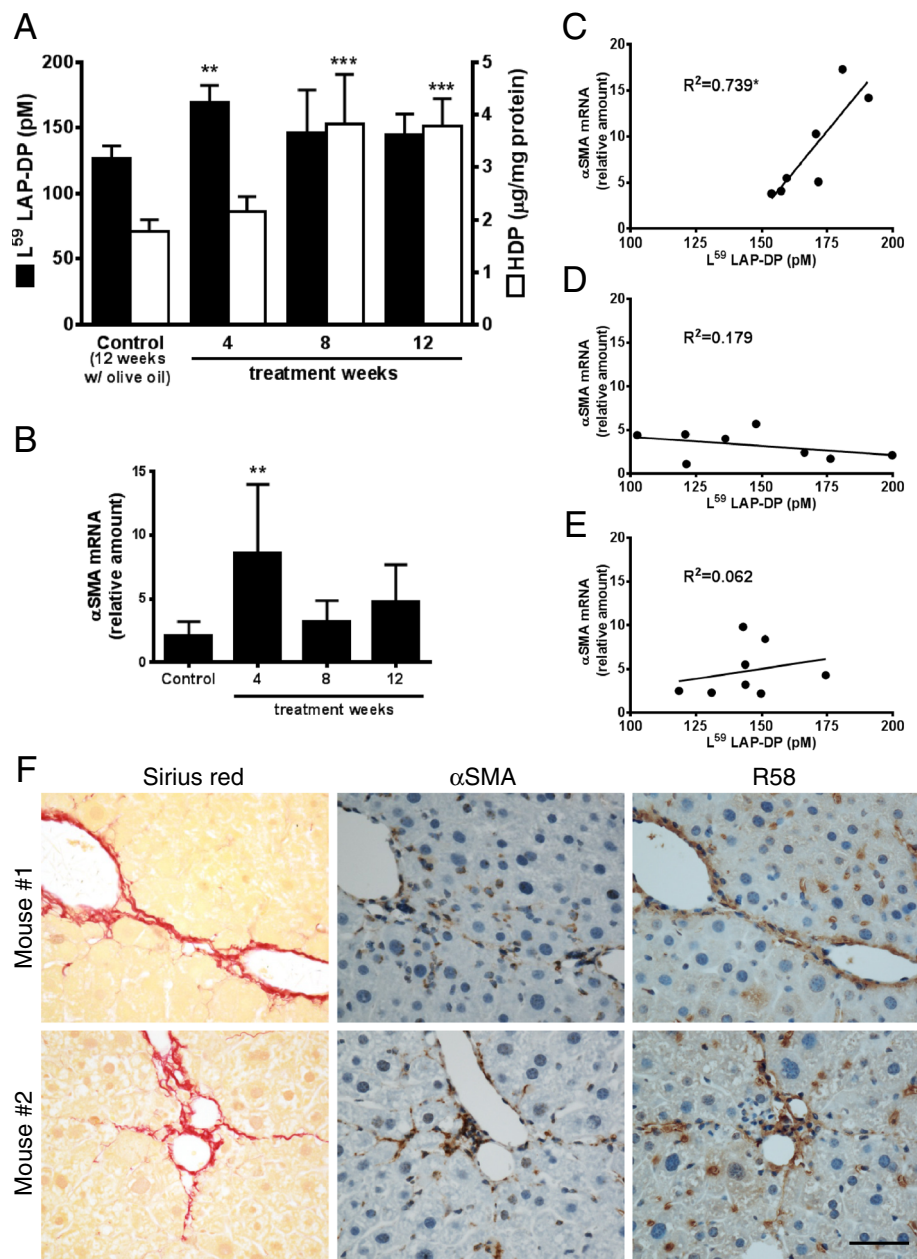
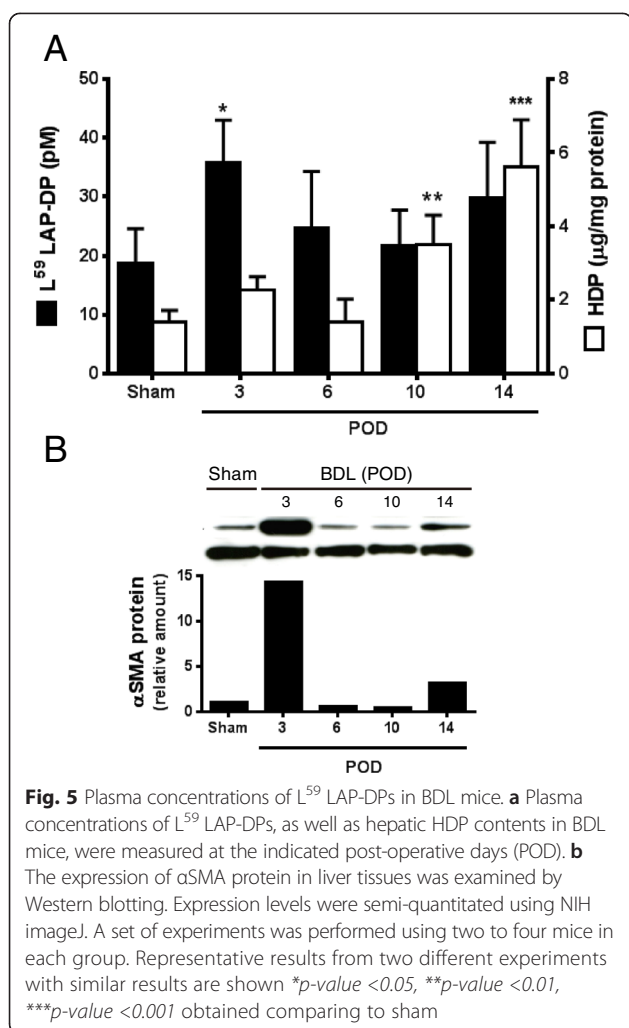


Fig. 4 Plasma concentrations of L⁵⁹ LAP-DPs in CCl₄-treated mice. **a** Plasma concentrations of L⁵⁹ LAP-DPs, as well as hepatic HDP contents, in CCl₄-treated mice (N = 7–8) were measured. **b** The expression levels of αSMA mRNA in the liver tissues from CCl₄-treated mice (N = 7–8) were determined. **c–e** The scatter plots between the plasma concentration of L⁵⁹ LAP-DPs (**a**) and expression level of αSMA mRNA (**b**) in each mouse at 4 weeks (**c**), 8 weeks (**d**), and 12 weeks (**e**) after starting CCl₄ treatment. **f** Immunostaining of liver sections from mice (N = 7–8) treated with CCl₄ for 4 weeks. Fibrotic regions were stained by picro-sirius red (left panels). Their proximal sections were immunostained with anti-αSMA (middle panels) and R58 antibodies (right panels). Representative areas from two randomly selected mice were shown *p-value <0.05, **p-value <0.01, ***p-value <0.001 obtained comparing to control value

speculate that the reasons could include the superior reaction specificity to human L⁵⁹ LAP-DPs than rat L⁵⁹ LAP-DPs as well as the lower concentration of L⁵⁹ LAP-DPs in the culture media compared to those in rat blood (plasma). In vitro, we cultured 3 × 10⁵ rat HSCs using 2 ml culture medium, whereas in vivo, a rat has 5–12 × 10⁷ HSCs in the

liver and 20 ml blood [15], suggesting that since a rat HSC produces the same amounts of L⁵⁹ LAP-DPs both in vitro and in vivo, their concentrations in the culture media should be 1/20–40 of those in blood (namely, 1/40–80 in plasma), which is below the detection limit of the assay. We attempted to concentrate the culture medium;



however, the concentrated contaminant proteins, mostly bovine serum albumin (BSA), interfered with the performance of the ELISA. As a result of these limitations, we could not use primary rat or mouse HSCs. Instead, we used TWNT-4 cells and demonstrated that the levels of L⁵⁹ LAP-DPs in the media correlated with collagen production. A simultaneous reduction was seen between levels of collagen mRNA and L⁵⁹ LAP-DPs in SB431542-treated cells, suggesting that L⁵⁹ LAP-DP levels reflect fibrogenesis in vitro.

In the CCl₄ and BDL models, the plasma levels of L⁵⁹ LAP-DPs significantly increased at the early stage of fibrosis prior to excessive collagen accumulation, when αSMA expression is robustly increased in the liver. At this time, there was a good correlation between the L⁵⁹ LAP-DP levels in the plasma and αSMA expression in the liver. It was demonstrated that the expression of pro-fibrogenic genes oscillated and decreased after robust increase at the early stage, and thereafter increased again at the later stage [16–18]. Supporting these reports, plasma L⁵⁹ LAP-DPs levels as well as αSMA

expressions decreased and were not significantly higher in fibrotic mice than in control mice after the early stage of fibrosis (Figs. 4 and 5). These data suggest that the L⁵⁹ LAP-DPs reflect fibrogenesis by the αSMA-positive cells and might be a predictive marker for fibrogenic activity and thus the accumulation of fibrosis at the early stage of fibrosis. On the other hand, the L⁵⁹ LAP-DP levels in the plasma did not correlate with αSMA expression in the liver at the late stage, and imply that PLK-dependent TGF-β activation mainly contributes to earlier fibrosis. Integrin β6 transcripts are reportedly up-regulated and correlate with the progression/stage of fibrosis in both BDL and Mdr2^{-/-} biliary fibrotic mice [19], indicating that integrin-dependent TGF-β activation occurs in later fibrosis. These observations imply that TGF-β is activated by distinct molecular mechanisms at different stages of fibrosis.

Not only activated HSCs, but also portal fibroblasts, contribute to liver fibrosis [20, 21]. It will therefore be worthwhile to examine PLK-dependent TGF-β activation in portal fibroblasts in future studies.

Recently, Konuma et al. reported that R⁵⁸ LAP-DPs were detected in melanocortin 4 receptor-deficient mice fed a high-fat diet, which exhibit steatohepatitis and fibrosis similar to human non-alcoholic steatohepatitis (NASH) [22]. In this model, positive reactivity for R⁵⁸ LAP-DPs was found around hepatic crown-like structures (hCLS), in which macrophages surround dead or dying hepatocytes containing large lipid droplets. Moreover, both myofibroblasts and fibrosis were observed nearby in areas of hCLS and were reduced when fibrosis was improved by treatment with eicosapentaenoic acid [22]. This result indicated that PLK-dependent TGF-β activation also occurs in the pathogenesis of NASH-related fibrosis in rodents. We are now examining whether plasma L⁵⁹ LAP-DP levels might increase in the high-fat diet-fed melanocortin 4 receptor-deficient mice and decrease in response to eicosapentaenoic acid. If this is the case, plasma L⁵⁹ LAP-DP levels might be used for monitoring the therapeutic efficacy of anti-fibrotic agents in NASH patients.

Because our preliminary studies showed that plasma L⁵⁹ LAP-DPs tend to be higher in patients suffering from hepatitis than in healthy volunteers, we are now investigating whether plasma L⁵⁹ LAP-DPs can be used as a fibrogenesis blood marker in patients with chronic active liver disease.

Conclusions

In summary, we have established a sandwich ELISA to detect by-products, L⁵⁹ LAP-DPs, of PLK-dependent TGF-β activation using the L59 antibody against the N-termini of L⁵⁹ LAP-DPs. In vitro, by treating hLTGF-β1 with PLK, L⁵⁹ LAP-DPs were generated, and their levels

correlate with both active TGF- β and *Col 1 α 1* mRNA levels. In in vivo CCl₄ and BDL models, plasma L⁵⁹ LAP-DP levels increase at the early stage of liver fibrosis prior to collagen deposition and correlate well with hepatic α SMA expression. These results suggest that plasma L⁵⁹ LAP-DPs reflect PLK-dependent TGF- β activation and fibrogenesis in the liver and therefore may serve as a novel blood biomarker for hepatic fibrogenesis.

Methods

Materials

rhLAP β 1 and anti-human LAP β 1 mouse monoclonal antibodies were purchased from R&D Systems (Minneapolis, MN, USA). Streptavidin-conjugated alkaline phosphatase (strep-AP) was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Human PLK was from Calbiochem (San Diego, CA, USA), and human PLN, SB431542, and anti- α SMA antibody for Western blotting were from Sigma Chemical Co. (St. Louis, MO, USA). Anti- α SMA antibody for immunostaining was from Dako (Glostrup, Denmark). Camostat mesilate was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

ELISAs

Active TGF- β 1 was measured using a Promega TGF- β 1 Emax Immuno Assay System ELISA kit (Promega Co., Madison, WI, USA). The sandwich ELISA for L⁵⁹ LAP-DPs was established by combination of the specific antibody against L⁵⁹ LAP-DP [9] and biotin-conjugated mouse monoclonal anti-LAP antibody (BAM2462, R&D Systems). The Nunc F96 Maxisoap Immunoplate (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was coated with 20 μ g/ml anti-L⁵⁹ LAP-DP antibody in Tris-buffered saline (pH 7.3) at 4 °C overnight. After blocking with Tris-buffered saline containing 1 % BSA at 4 °C overnight, samples containing L⁵⁹ LAP-DPs were added and incubated overnight at 4 °C, followed by sandwiching with 1 μ g/ml biotin-conjugated anti-LAP antibody in HEPES buffer for 3 h at room temperature. After another 3 h of incubation with strep-AP, 4-nitrophenylphosphate disodium salt hexahydrate in diethanolamine buffer (pH 9.8) was added and absorbance at 405 nm was measured. The plates were washed by Tris-buffered saline containing 0.05 % Tween 20 between each step. For preparing L⁵⁹ LAP-DP standards, rhLAP β 1 (40 nM) was digested with PLK (40 nM) in 1 % BSA containing phosphate-buffered saline for 2 h at 37 °C.

Evaluation of L⁵⁹ LAP-DP ELISA

The lowest limit of quantification (LOQ) was defined as the value whose lower 2 SD value at absorbance at 405 nm did not overlap with the upper 2 SD value of the blank's absorbance at 405 nm. We examined this for

12 times and choose the highest LOQ value. Certain amounts of the standard L⁵⁹ LAP-DP stock were spiked into culture media and pooled mouse plasma, and then evaluated for the recovery and linearity of this ELISA. Distinct culture media ($N=10$) and pooled mouse plasma ($N=6$) containing different L⁵⁹ LAP-DP concentrations were tested from three to six wells from one plate for intra-assay, and from three wells from two to three plates for inter-assay.

Preparation of LTGF- β 1 containing culture media

HEK293T cells (1×10^6 cells) were seeded on a 60-mm dish. After 24 h, the pcDNA3 vector with an inserted human LTGF- β 1 gene was transiently transfected to HEK293T cells using Lipofectamine 2000. After an additional 24 h, transfected HEK293T cells were cultured in DMEM containing 0.1 % BSA for another 24 h, and the CM were collected and used for the luciferase assay as described below.

Luciferase assay

Mink lung epithelial cells stably transfected with a TGF- β -responsive reporter gene ($\times 9$ CAGA-Luc CCL64 cells) were seeded at 1×10^4 /well in a 96-well plate and cultured overnight [23]. After cells were treated with hLTGF- β 1 in the absence or presence of PLK for 6 h, the luciferase activity in each cell lysate was measured by the Luciferase Assay System provided by Promega Co. (Madison, WI, USA).

HSC culture

Human HSC (TWNT-4) cells [11] were cultured in the stellate cell medium purchased from ScienCell Research Laboratories (Carlsbad, CA, USA) at 37 °C in 5 % CO₂. Cells were seeded at 4×10^4 /well in a 6-well plate, grown until 70 % confluence, and treated with 20 μ M SB431542 for 72 h. The levels of L⁵⁹ LAP-DPs in the culture media were measured by the ELISA developed in this study, and the mRNA levels in each cell lysate were quantitated by real-time reverse transcription-polymerase chain reaction (RT-PCR).

Animals

Male C57BL/6 mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). All animals were maintained on a 12-h light/12-h dark cycle. Food and water were available ad libitum. All animal experiments were performed in accordance with protocols approved by the RIKEN Institutional Animal Use and Care Administrative Advisory Committee.

CCl₄ model

Seven-week-old C57BL/6 mice ($N=7-8$) were given repeated intramuscular injections of 50 % CCl₄ (CCl₄:

olive oil = 1:1) (2 ml/kg twice a week). Control animals were injected with the same volume of olive oil. After 4, 8, and 12 weeks, the animals were sacrificed and samples of plasma (EDTA bleeding) and liver tissues were collected.

BDL model

Ligation of the common bile duct was performed as described previously [24]. The common bile ducts of 8-week-old C57BL/6 mice ($N = 2-4$) were double-ligated and cut. The BDL animals were sacrificed on post-operative days 3, 6, 10, and 14. Sham-operated mice were treated in the same manner except that the bile duct was not ligated. Their samples were collected on post-operative day 14.

Measurement of HDP content

HDP contents were measured as described previously [25].

Staining of tissue sections

Animal tissue specimens were fixed in 4 % paraformaldehyde and embedded in paraffin. Liver sections (4- μ m thickness) were stained by 0.1 % picro-sirius red [26] and immunostained by incubating overnight at 4 °C with anti- α SMA (diluted with 1:100) and R58 antibodies (1 μ g/ml). EnVision/HRP (Dako, Glostrup, Denmark) was used as the second antibody and signal-amplifying system. The tissues were counterstained with hematoxylin.

Quantitative real-time RT-PCT

The total RNA was extracted from HSCs and liver tissues using RNeasy Micro/Mini kits (Qiagen, Hilden, Germany). After synthesis of first-strand cDNA with the Prime Script RT kit (TaKaRa Bio., Japan), qPCR was performed with SYBR Premix (TaKaRa Bio., Japan) in the thermal cycler. Specific primers used are listed in Table 3. The results were normalized for *GAPDH* mRNA levels.

Western blotting

Small pieces of liver tissue were homogenized with RIPA buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 % Triton X, 1 mM EDTA, 10 % sucrose, and cOmplete, a

protease inhibitor cocktail [Roche, Mannheim, Germany]). Homogenates were clarified by centrifugation at 18,000 rpm for 20 min at 4 °C, and the total protein concentration of each supernatant was determined using a BCA protein assay reagent kit (Thermo Fisher Scientific Inc., Rockford, IL, USA). Proteins (20 μ g/lane) were separated by SDS-PAGE and transferred to a PVDF membrane (Millipore, Bedford, MA, USA). Western blot analyses were performed using either anti- α SMA and anti-GAPDH antibodies plus HRP-conjugated anti-mouse antibodies (1:5000) (Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA). The bands were visualized by a Western Blotting Substrate Plus reagent purchased from Thermo Fisher Scientific (Rockford, IL, USA) and semi-quantitated using NIH image].

Statistical analysis

Quantitative data are shown as mean \pm SD. Statistical analyses were performed using GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA, USA). * p value <0.05, ** p value <0.01, and *** p value <0.001 were considered as statistically significant.

Additional files

Additional file 1: Figure S1. The standard curve for the L⁵⁹ LAP-DP ELISA. The absorbance linearly increased up to about 100 pM. The lower limit of quantitation is approximately 2 pM.

Additional file 2: Figure S2. The half-life of L⁵⁹ LAP-DPs in mouse plasma. Exogenously generated L⁵⁹ LAP-DPs were added to mouse plasma and incubated at 37 °C for 0, 1, 2, 4, 8, 16, and 24 h. The remaining levels were measured and plotted against incubation time, and the half-life time was determined.

Abbreviations

BDL: bile duct ligation; BSA: bovine serum albumin; CCl₄: carbon tetrachloride; CM: conditioned media; ECM: extracellular matrix; ELISA: enzyme linked immunosorbent assay; HSCs: hepatic stellate cells; LAP: latency-associated protein; LAP β 1: TGF- β 1 LAP; LAP-DP: LAP degradation product; LOQ: lowest limit of quantification; NASH: non-alcoholic steatohepatitis; PLK: plasma kallikrein; PLN: plasmin; RT-PCR: reverse transcription-polymerase chain reaction; rhLAP β 1: recombinant human LAP β 1; TGF- β 1: transforming growth factor- β 1; α SMA: α -smooth muscle actin.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MH, II, YY, and AK performed the experiments. MH also wrote the manuscript. TM, SLF, DBR, and SK planned the research. SK supervised the entire project. All authors read and approved the final manuscript.

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Table 3 Primers used in quantitative RT-PCR

Target	Primer sequences	
	5'-primer	3'-primer
human <i>GAPDH</i>	GGAGTCAACGGATTGGT	AAGATGGTGATGGGATTTCCA
human <i>Col 1a1</i>	ACGAAGACATCCCACCAATC	AGATCACGTCATCGACAAC
mouse <i>GAPDH</i>	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA
mouse <i>αSMA</i>	ACAGCCCTCGACCCA	GCCACCGATCCAGACAGAGT

the NIH (DK56601 to SLF). Patent of LAP-DP antibodies: JP4653660, US7893553, and US8198412.

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