TIM-3 Is a Novel Therapeutic Target for Eradicating Acute Myelogenous Leukemia Stem Cells

Koichi Akashi

Abstract Acute myelogenous leukemia (AML) is derived from self-renewing leukemic stem cells (LSCs). We found that T-cell immunoglobulin mucin-3 (TIM-3) is expressed on LSCs in most types of primary AML, except for acute promyelocytic leukemia (M3 by the FAB classification). TIM-3 is not expressed in normal hematopoietic stem cells (HSCs). In a xenogeneic transplantation system, we showed that targeting of TIM-3 by an anti-TIM-3 cytotoxic antibody is sufficient to eradicate human AML LSCs without affecting normal human hematopoiesis. These data strongly suggest that TIM-3 is a promising therapeutic target to cure AML patients.

Keywords Acute myelogenous leukemia • Leukemic stem cell • Cancer stem cell • TIM-3 • Hematopoietic stem cell • Xenotransplantation

Introduction

In normal hematopoiesis, human hematopoietic stem cells (HSCs) reside within the $CD34^+CD38^-$ cell fraction of bone marrow cells. They self-renew and differentiate into mature cells to maintain normal hematopoiesis. Similarly, in acute myelogenous leukemia (AML), leukemic blast cells are derived from a small population called leukemic stem cells (LSCs) or leukemia-initiating cells, which also resides within the $CD34^+CD38^-$ cell fraction [1, 2]. LSCs self-renew and give rise to clonogenic leukemic cells, whereas non-LSCs lack the potential to self-renew or maintain leukemia [1, 3, 4] indicating that AML is hierarchically organized initiating from LSCs.

Conventional chemotherapy currently achieves complete remission in ~90 % of AML cases [5, 6]. However, a considerable proportion of AML patients (~60 %) eventually relapse after intensive chemotherapies. The recurrence of AML in these

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patients may be caused by regrowth of surviving LSCs. To selectively kill AML LSCs while sparing normal HSCs, one of the most practical approaches is to target AML LSC-specific surface molecules or molecules required for LSC function. To achieve such specificity, the target molecule should be expressed on LSCs at a high level but not on normal HSCs [7]. The molecule can be expressed in mature blood cells or progenitors, because these cells can anyway be replenished by normal HSCs.

Search for Surface Antigens Specific to AML LSCs

A number of candidate surface molecules for eradicating AML LSCs have been reported mainly by utilizing cDNA microarray analysis of purified LSCs. Figure 1 shows the results of transcriptome profiling of purified LSCs from AML patients and normal adult HSCs [8]. The molecules strongly expressed in AML LSCs, including CLL-1 [9], CSF1R [10], CD96 [11], and CD99 [12], are specifically expressed in LSCs. CLL-1 is a transmembrane glycoprotein [13]. The proportion of CLL-1-expressing $CD34^+CD38^-$ AML cells, however, is highly diversified in cases [9]. CD96 is a member of the Ig gene superfamily. CD96 is expressed on activated T cells [14]. The expression level of CD96 protein is also high enough to clearly distinguish AML LSCs from normal HSCs. T-cell immunoglobulin mucin-3 (TIM-3) is expressed in LSCs of most AML types (except for M3) at high levels, but is not expressed in normal HSCs [8]. The expression level of CD25 [15], CD32 [15], CD44 [16], and CD47 [17] in LSCs was only two- to threefold higher at the mRNA level as compared with normal HSCs, and in some AML cases, LSCs did not express these molecules. CD33 and CD123 [18] proteins are expressed at a high level in normal HSCs and myeloid progenitors, including CMPs and GMPs [19], suggesting that targeting these molecules should harm normal hematopoiesis.

It might also be important to understand the function of these therapeutic target molecules in the development of AML. A previous study has shown that anti-CD44 monoclonal antibodies reduced the leukemic burden and blocked secondary engraftment in a NOD-SCID model [16]. This effect on LSCs was mediated in part by the disruption of LSC-niche interactions [16]. Anti-CD47 antibodies can block LSC reconstitution in a NOD-SCID model [17], and this might be due to the activation of phagocytosis by macrophages through inhibition of interaction of CD47 with SIRPA [20].

Recently, we have reported that TIM-3 is expressed on the cell surface of LSCs in most AML types [8, 21]. TIM-3 is not expressed in normal human HSCs [8] (Fig. 1). Furthermore, a recent study has succeeded in prospectively isolating LSCs from residual HSCs within the $CD34^+CD38^-$ fraction in de novo AML patients by using TIM-3 as a positive LSC marker [12]. Here, we summarize recent progress in studies of TIM-3 and discuss the potential usefulness of TIM-3 for eradicating AML LSCs. TIM-3 has several advantages over other candidate markers. First, TIM-3 protein is not detectable in normal HSCs or in other myelo-erythroid or lymphoid progenitors, although TIM-3 is upregulated in monocyte-lineage committed progenitors.

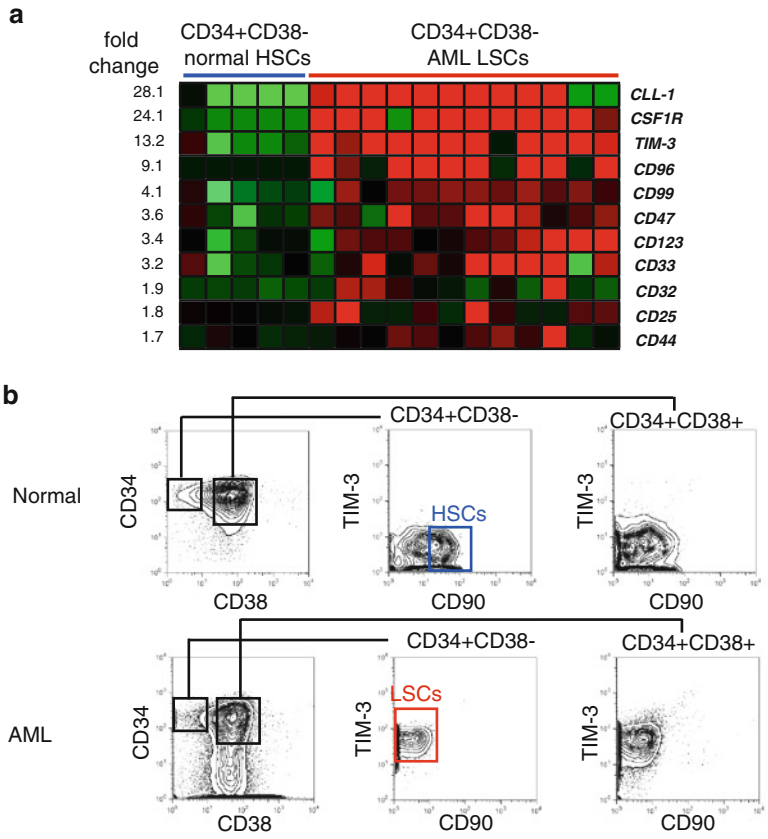

Fig. 1 TIM-3 expression in normal HSCs and AML LSCs. (**a**) Results of gene expression analysis comparing $CD34^+CD38^-$ normal HSCs and AML LSCs. Surface molecules highly expressed in LSCs are shown. (**b**) FACS analysis of TIM-3 protein expression in normal HSCs and AML LSCs. Both $CD34^+CD38^-CD90^-$ LSCs and $CD34^+CD38^+$AML cells express TIM-3, whereas $CD34^+CD38^-CD90^-$ HSCs completely lack TIM-3 expression. TIM-3 expression originates within the $CD34^+CD38^+$ progenitor fraction in normal human hematopoiesis. A representative FACS analysis is shown here

Second, TIM-3 marks all functional LSCs that can reconstitute human AML in immunodeficient mice in the majority of M0, M1, M2, and M4 AML cases, and its expression level is sufficient to eradicate LSCs by antibody-based treatment.

TIM-3 Expression and Functions in Normal Hematopoiesis

TIM-3 was originally identified as a surface molecule expressed in interferon (IFN)- γ-producing $CD4^+$ Th1 cells and $CD8^+$ T cytotoxic type 1 (Tc1) cells [22] in murine hematopoiesis. TIM-3, a type 1 cell-surface glycoprotein, has a structure

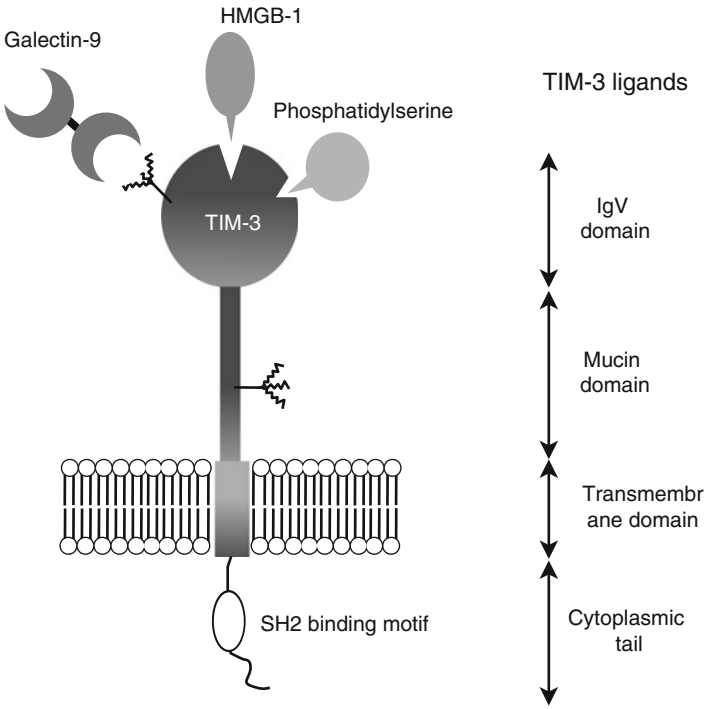

Fig. 2 Structure of TIM-3 molecule and its ligands. TIM-3 is a type 1 cell-surface glycoprotein and has a structure that includes an N-terminal immunoglobulin variable domain followed by a mucin domain, a transmembrane domain, and a cytoplasmic tail with highly conserved six tyrosine residues and a SH2 binding motif. Galectin-9, HMGB1, and PS have been identified as ligands of TIM-3

that includes an N-terminal immunoglobulin variable domain followed by a mucin domain, a transmembrane domain, and a cytoplasmic tail (Fig. 2). In steady-state human hematopoiesis, TIM-3 is expressed in monocytes and in a fraction of NK cells, but not in granulocytes, B cells, or T cells [8]. However, TIM-3 is upregulated in T cells in response to immune reactions. TIM-3 plays an important role in regulation of Th1-dependent immune responses and immune tolerance [22–24]. Galectin-9, an S-type lectin, has been reported as a TIM-3 ligand in lymphocytes. Galectin-9 has two distinct carbohydrate recognition domains and binds to carbohydrate chains on the IgV domain of TIM-3. TIM-3 has highly conserved six tyrosine residues and a Src homology 2 (SH2) binding motif in its cytoplasmic tail, and stimulation of TIM-3 by galectin-9 results in increased phosphorylation of tyrosine residues in T cells [25]. Engagement of TIM-3 by galectin-9 induces apoptosis of Th1 cells and inhibits their IFN-γ production [26]. These data collectively suggest that TIM-3 is a negative regulator of Th1- and Tc1-driven immune responses.

TIM-3 is also known as a marker of "exhausted" $CD8^+$ T cells. Exhausted T cells show impaired proliferation and effector function under antigen stimulation.

One of the major markers for exhausted T cells is the inhibitory molecule programmed cell death 1 (PD-1), and T cell function is partially restored by blocking the interaction between PD-1 and PD-1 ligand in mice [27]. TIM-3 is also expressed on exhausted $CD8^+$ T cells in patients with chronic viral infections, including human immunodeficiency virus (HIV) [28], hepatitis B virus [29], and hepatitis C virus (HCV) [30]. Blockade of both TIM-3 and PD-1 ligation can significantly restore T cell proliferation and effector potential, suggesting that both TIM-3 and PD-1 pathways play a major role in $CD8^+$ T cell exhaustion [31].

TIM-3 can also modulate the immune reaction pathway to regulate innate immunity. NK cells and some myeloid cells, including monocytes/macrophages, dendritic cells, and mast cells, express TIM-3 in both human and mouse hematopoiesis. In NK cells, TIM-3 is induced on their surface on activation [32, 33], but the function of TIM-3 in NK cells remains controversial. It has been reported that TIM-3 is a human NK cell co-receptor to enhance IFN-γ production [32], but another report showed that NK cell-mediated cytotoxicity was reduced by cross-linking of TIM-3 [33].

In terms of the myeloid lineage, TIM-3 is expressed in monocytes/macrophages, dendritic cells (DCs), and mast cells [34–37]. In human bone marrow, $CD34^+CD38^-CD90^+$ normal HSCs and the majority of $CD34^+CD38^+$ progenitor cells do not express TIM-3. Within the $CD34^+CD38^+$ progenitor fraction, human myeloid progenitors can be divided into three subpopulations, such as common myeloid progenitors (CMPs), granulocyte/macrophage progenitors (GMPs), and megakaryocyte/erythrocyte progenitors (MEPs) [38]. TIM-3 is expressed only in a fraction GMPs, but not in CMPs and MEPs. Purified $TIM\text{-}3^+$ GMPs give rise mainly to colony-forming unit-macrophage (CFU-M), whereas $TIM\text{-}3^-$ GMPs can generate various types of myeloid colonies, suggesting that upregulation of TIM-3 occurs in concert with monocyte lineage commitment at the GMP stage in humans [8].

In mature monocytes or dendritic cells, TIM-3 signaling synergizes with that of Toll-like receptors to promote secretion of tumor necrosis factor-α (TNF-α) inflammatory responses [34]. In addition, TIM-3 on macrophages and DCs recognizes phosphatidylserine (PS) in apoptotic cells through its IgV domain. Binding of PS to TIM-3 does not interfere with that of galectin-9 to TIM-3, as the binding sites of these molecules are located on opposite sides of the IgV domain. In TIM-3-expressing DCs, recognition of PS by TIM-3 induced enhancement of phagocytosis of apoptotic cells and cross-presentation of apoptotic cell-associated antigen to $CD8^+$ T cells [35]. TIM-3 expression and functions in hematopoietic cells are summarized in Fig. 3.

TIM-3 Is a Marker of Malignant Stem Cells in Human AML

We have identified TIM-3 as an AML LSC-specific surface molecule. We first compared the gene expression profiles of $CD34^+CD38^-$ AML cells and normal HSCs by using cDNA microarray analysis (Fig. 1a). As shown in Fig. 1b, TIM-3 protein is not expressed in $CD34^+CD38^-CD90^+$ normal HSCs, but the vast majority

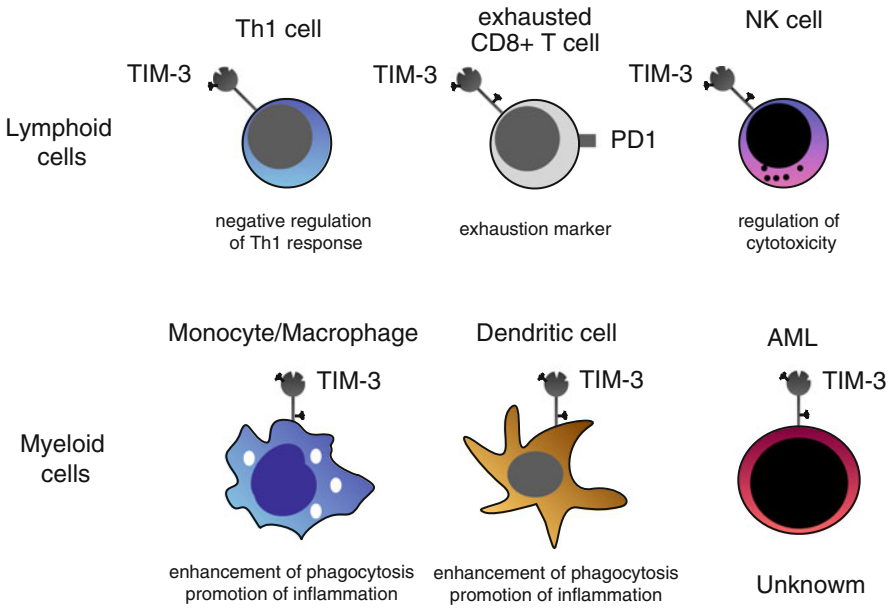

Fig. 3 TIM-3 expression and functions in normal hematopoietic cells. TIM-3 is expressed in Th1 cells, exhausted $CD8^+$ T cells, NK cells, monocytes, and dendritic cells in normal hematopoiesis. The functions of TIM-3 differ by cell type and context

of the $CD34^+CD38^-$ LSCs and the $CD34^+CD38^+$ cells expressed TIM-3 at a high level in patients with most types of AML except for acute promyelocytic leukemia (M3) [8, 21]. Another group has also reported that the expression level of TIM-3 is especially high in immature AML cells with core-binding factor translocations or mutations in *CEBPA* [21].

It is important to note that the TIM-3^+ fraction in AML patients contained all functional LSCs. We separated AML cells into the TIM-3^+ and TIM-3^- populations and transplanted each population into sublethally irradiated immunodeficient mice, and found that only TIM-3^+ AML cells, but not TIM-3^- cells, reconstituted human AML in these mice [8]. These data suggest that targeting TIM-3^+ cells is sufficient for eradication of LSCs in AML patients.

Targeting AML-LSCs by Monoclonal Anti-TIM-3 Killing Antibodies in a Xenograft Model

To utilize TIM-3 to target AML LSCs, it is critical to establish anti-human TIM-3 antibodies that can kill TIM-3-expressing cells in vivo. To achieve successful antibody-based treatment, antibody-dependent cellular cytotoxicity (ADCC) and

complement-dependent cytotoxicity (CDC) activities are critical to eliminate target cells [39]. Additionally, recent studies have suggested that antibody-dependent cellular phagocytosis (ADCP) could play an important role in killing target cells in vitro [40] and in vivo [41].

An anti-TIM-3 monoclonal antibody (IgG2b) was obtained by immunizing Balb/c mice with L929 cells stably expressing human TIM-3 and soluble TIM-3 protein [8]. In this antibody, the variable portions of the VH regions of the cloned hybridoma that recognize TIM-3 were grafted onto IgG2a Fc regions, because the IgG2a subclass is most efficient to induce ADCC activity in mice [42, 43]. The clone called ATIK2a was established, and it was effective in killing TIM-3-expressing cell lines by its CDC and ADCC activities [8].

We then tested the effect of ATIK2a on the growth of AML LSCs or normal HSCs in xenograft models. NOD-SCID mice transplanted with 10^5 $CD34^+$ cord blood cells were treated with ATIK2a. These mice were reconstituted with normal hematopoiesis with nearly equal percentages of human cell chimerisms, although human mature monocytes were depleted. In contrast, in mice reconstituted with human AML, ATIK2a exerted profound effects on leukemia development. The mice were transplanted with human AML of M0, M1, and M4 types, and after confirmation of AML development in these mice, ATIK2a was injected six times over 2 weeks. Strikingly, human AML cells disappeared in mice treated with ATIK2a but not in those with control IgG treatment. These data strongly suggest that targeting of AML LSCs by utilizing anti-TIM-3 killing antibodies is a practical approach to cure human AML.

TIM-3 Is a Functional Molecule for AML LSC Maintenance

Since TIM-3 has a tyrosine residue and SH2 domain that can activate Src family proteins, we hypothesized that TIM-3 signaling has some function to maintain AML-LSCs. We found that the serum levels of galectin-9, a TIM-3 ligand, were significantly (>10-fold) elevated in AML patients but not in normal individuals on an ELISA assay. Furthermore, $TIM\text{-}3^+$ AML cells had abundant galectin-9 protein in their cytoplasm, and they secreted galectin-9 in the sera of mice transplanted with human AML. Mice reconstituted with normal human HSCs or B cell acute lymphoblastic leukemia did not have detectable levels of serum galectin-9. These results collectively suggest that AML cells secreted galectin-9 in an autocrine manner. Furthermore, TIM-3 stimulation by galectin-9 in AML cells in vitro induced significant gene expression changes including NF-κB target genes (unpublished data). Collectively, it is suggested that AML LSCs had growth and survival advantages through an autocrine stimulation loop of the TIM-3/galectin-9 system.

Conclusion

TIM-3 has been shown to play pivotal roles in modulating immune reactions. By transcriptome analysis, we newly identified TIM-3 as a surface molecule specific to AML LSCs but not expressed in normal HSCs. Our in vivo xenogeneic transplantation analysis directly showed that targeting TIM-3 could be an efficient, useful therapeutic approach to eradicate AML LSCs.

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TGF-β LAP Degradation Products, a Novel Biomarker and Promising Therapeutic Target for Liver Fibrogenesis

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Abstract While there are many blood and/or tissue biomarkers as well as algorithms clinically used to assess hepatic fibrosis, a good biomarker and therapeutic target of hepatic fibrogenesis, which reflects prefibrotic changes, has not been established. The most fibrogenic cytokine, transforming growth factor (TGF)-β, is produced as a latent complex, in which TGF-β is trapped by its propeptide. On the surface of activated hepatic stellate cells, plasma kallikrein activates TGF-β by cleaving latency-associated protein (LAP) between the R^{58} and L^{59} residues, releasing active TGF-β from the complex. We made specific antibodies that recognize neo-C-terminal (R^{58}) and N-terminal (L^{59}) ends of LAP degradation products (LAP-DPs) and found that LAP-DPs may serve as a novel surrogate marker of TGF-β activation—namely, generation of active TGF-β—and is thus a therapeutic marker for TGF-β-mediated liver fibrogenesis in patients and can also be used to monitor effects of anti-fibrogenic factors or compounds for discovery of a novel anti-fibrosis drug.

Keywords Biomarkers • Hepatic fibrogenesis • TGF-β • LAP • Latent TGF-β activation • Hepatic stellate cells • Plasma kallikrein • LAP-DP • Drug discovery • Anti-fibrosis drug

Abbreviations

ECM	Extracellular matrix
HSCs	Hepatic stellate cells
αSMA	α smooth muscle actin
TGF-β1	Transforming growth factor-β1

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LAP	Latency associated protein
SLC	Small latent complex
LTBP	Latent TGF-β binding protein
LLC	Large latent complex
PLN	Plasmin
PLK	Plasma kallikrein
LAP-DP	LAP degradation products
LAP β1	TGF-β1 LAP
BDL	Bile duct ligation
HBV	Hepatitis B virus
HCV	Hepatitis C virus
NASH	Non-alcoholic steatohepatitis

Introduction of Liver Fibrogenesis

Hepatic fibrosis is the excessive accumulation of extracellular matrices (ECM; mainly collagen) in the perisinusoidal space (or space of Disse) in the liver, and an important pathological step developing from chronic hepatitis to liver cirrhosis irrespective of etiologies [1], whereas hepatic fibrogenesis means fibrosis progression or an ongoing reaction producing excessive ECM, sometimes nonsymptomatic, in the liver [2]. While there are many blood and/or tissue biomarkers as well as algorithms clinically used to assess hepatic fibrosis [3–7], the gold standard is still scoring of stained collagen fibers in the biopsy sample [3]. However, biopsy is invasive and risky. Imaging techniques including ultrasound elastography have been developed [3]. In contrast, a good biomarker and therapeutic target of hepatic fibrogenesis, which reflects prefibrotic changes, has not been established [2,3]. Therefore, development of a noninvasive biomarker for hepatic fibrogenesis, which will lead not only to establishment of a novel diagnosis useful to prevent liver fibrosis/cirrhosis, but also to acceleration of drug discovery and development against liver fibrosis, is in high demand [3].

Activation of Hepatic Stellate Cells

Hepatic stellate cells (HSCs) play a central role in the pathogenesis of hepatic fibrosis by virtue of their ability to undergo a process termed “activation” [1,2]. During this process, HSCs transform into myofibroblast-like cells accompanying several key phenotypic changes, which collectively increase extracellular matrix accumulation [1–3]. These include (1) cellular proliferation caused by upregulation of mitogenic cytokines and their receptors; (2) morphologic changes with loss of stored

vitamin A droplets; (3) contractility caused by increased α smooth muscle actin (αSMA), which may constrict sinusoidal blood flow; and (4) fibrogenesis mainly caused by increased synthesis and release of collagen.

TGF-β and Its Activation Reaction

Among many cytokines and growth factors related to fibrogenesis, the most potent—and therefore the most "fibrogenic"—cytokine is the 25 kD homodimeric cytokine, transforming growth factor (TGF)-β [8]. The TGF-β family is composed of three subtypes (TGF-β1, TGF-β2, and TGF-β3), with biological properties that are nearly identical [8]. TGF-β is produced as an inactive latent complex, in which active TGF-β is trapped by its propeptide, latency-associated protein (LAP), and to exert its biological activities, it must be released from the complex [9]. This reaction is called activation of TGF-β (Fig. 1). TGF-β1 is produced as a 390-amino-acid

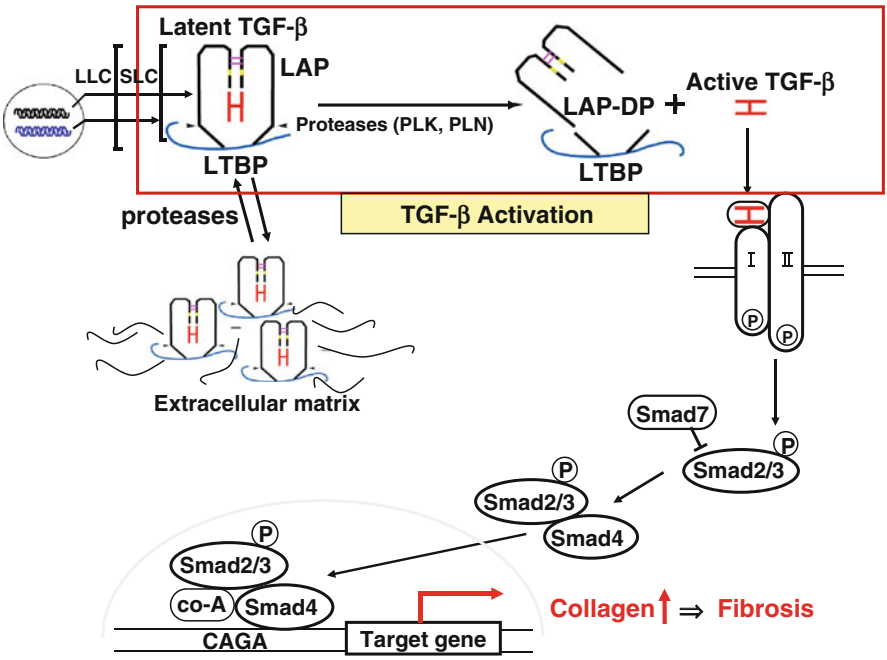

Fig. 1 TGF-β activation and signaling pathway. TGF-β is produced as a latent complex (LLC) composed of active TGF-β trapped by its propeptide LAP (SLC) and a matrix protein LTBP. Upon cleavage of LAP by proteases such as PLK, active TGF-β is released from the complex (this reaction is called TGF-β activation) and exerts fibrogenic activity (stimulation of collagen synthesis) via binding to its receptors and Smad signaling

precursor protein consisting of a signal peptide of 29 amino acids, an N-terminal LAP, and a C-terminal region that becomes the active TGF-β1 molecule, and each region is dimerized through S–S bonds. After processing by cleavage at R^{278}–A^{279} by a furin-like protease, the LAP still non-covalently captures the active TGF-β1, forming small latent complex (SLC) and preventing active TGF-β1 from binding its cognate receptors [9]. The active TGF-β1 and the LAP homodimers are 25 kD and 75 kD, respectively. SLC is S–S bonded to another gene product, the latent TGF-β binding protein (LTBP), via C^{33} residues, forming the large latent complex (LLC). This complex can be sequestered in the ECM (Fig. 1) [10] because LTBP is a member of an ECM protein family, fibrillin [11].

Activation of latent TGF-β is performed through different mechanisms depending on the tissue and cell types and experimental conditions, and several molecules are known to activate TGF-β1 in animal models [12–21]. These include integrins [12–15], thrombospondin [16], and proteases, such as matrix metalloproteinases and serine proteases [17–21]. The integrin αvβ6 binds to and activates latent TGF-β and plays a role in regulating pulmonary inflammation and fibrosis as well as biliary fibrosis [12–15]. Thrombospondin 1 is another major activator of latent TGF-β, especially in the lung and pancreas, by binding to a defined site within LAP and inducing a conformational change in the latent complex [16]. In the normal liver, TGF-β is produced and secreted from sinusoidal endothelial cells and Kupffer cells (KCs, resident macrophages in the liver) at low levels. Elevated production of TGF-β was seen first in all cell types and then mainly in hepatocytes and HSCs after partial hepatectomy, whereas elevated production of TGF-β was seen solely in HSCs after inflammation and fibrosis [22]. TGF-β secreted from HPCs is entirely in the latent form, whereas TGF-β secreted from HSCs is 50–90 % in the active form [22]. Thus, HSCs are recognized as the major source of active TGF-β, namely the site of TGF-β activation, particularly in the damaged liver [22,23].

We have addressed a potential proteolytic mechanism for latent TGF-β activation in HSCs by surface plasmin (PLN) and plasma kallikrein (PLK) during the formation of hepatic fibrosis [20,21]. PLN releases latent TGF-β from the extracellular matrix and activates it by cleaving LAP from latent TGF-β molecules on the HSC surface [9,20]. Lyons et al. first reported that PLN digests LAP and activates TGF-β1 in vitro [24]. Using a protease inhibitor, camostat mesilate, we demonstrated that PLN and PLK are involved in the TGF-β1 activation associated with liver fibrosis and impaired liver regeneration in animal models [20,21]. However, it remained to be elucidated whether PLN- and/or PLK-dependent TGF-β1 activation also occurs during the pathogenesis of liver fibrosis in patients, as there was no good biomarker reflecting protease-dependent TGF-β1 activation reaction. To answer this question, we determined cleavage site within LAP and made specific antibodies that recognize LAP degradation products (LAP-DPs) bearing a neo-amino or carboxyl terminus [25].

TGF-β LAP-DP Serves as a Surrogate Marker for Its Activation Reaction

To identify the cleavage sites in LAP during latent TGF-β1 activation by PLN and PLK, recombinant human LAP β1 was digested with these proteases, the resultant fragments were separated by SDS-polyacrylamide gel electrophoresis (PAGE), and the N-terminal sequence of each LAP-DP was determined using a pulsed liquid protein sequencer Precise 494cLC, which revealed that PLN and PLK primarily cleave LAP β1 between the K^{56} and L^{57} residues, and the R^{58} and L^{59} residues, respectively, during proteolytic activation of latent TGF-β1 (Fig. 2) [25].

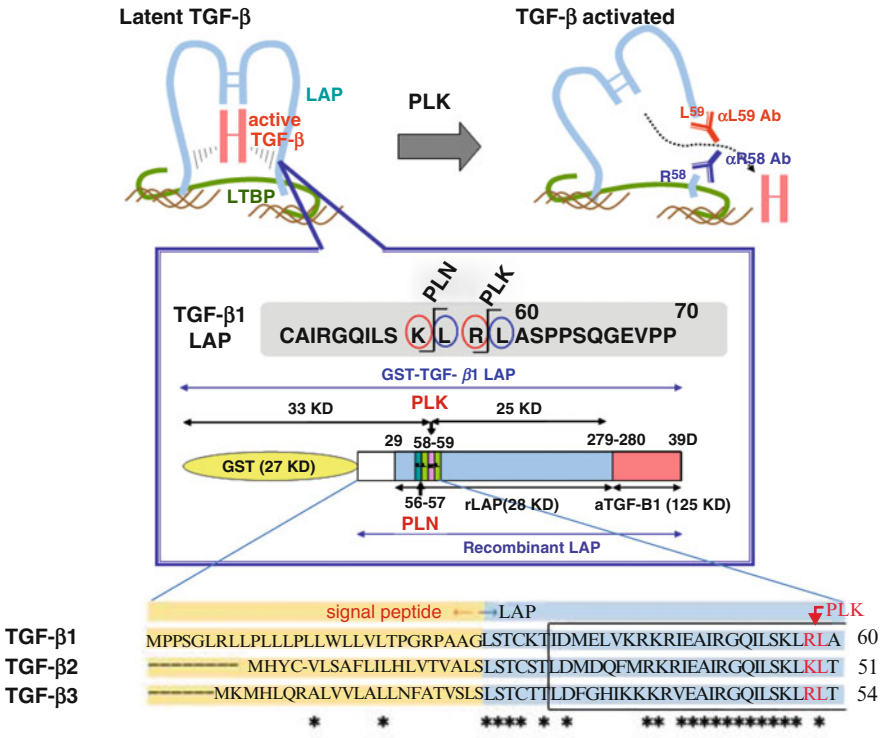

Fig. 2 Cleavage of $K^{56}LRL^{59}$ within LAP activates TGF-β. PLN and PLK cleave LAP between K^{56}–L^{59} and R^{58}–L^{59} residues, respectively, causing release of active TGF-β1 from the latent complex. The amino acid sequences around the PLN and PLK cleavage sites are illustrated. Antibodies that specifically recognize the cutting edges of LAP-DPs were produced. The *dark blue "Y"* labeled R58 represents antibodies recognizing the C-terminal or N-terminal side LAP-DPs, whereas the *red "Y"* labeled L59 represents antibodies recognizing the N-terminal or C-terminal side LAP-DPs. A comparison of amino acid sequences from the N-terminus until the PLK cleavage site among three isoforms of TGF-β is presented at the bottom

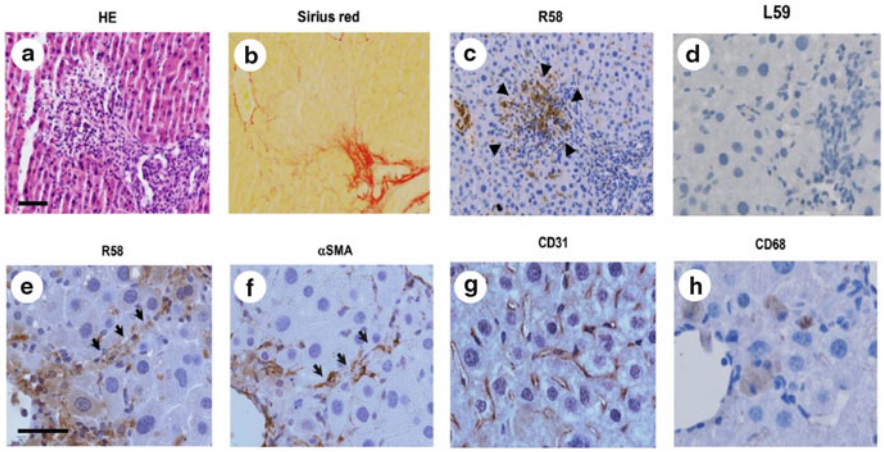

Fig. 3 Emergence of TGF-β LAP-DPs in activated HSCs within pre-fibrotic areas in BDL models. Liver sections from BDL-operated mice were stained by HE (**a**) and Sirius red (**b**), and immunostained with R58 (**c**) and L59 (**d**) antibodies (scale bar = 50 μm), and were immunostained with R58 (**e**), anti-αSMA (**f**), anti-CD31 (**g**), and anti-CD68 (**h**) antibodies (scale bar = 25 μm). More detailed results are provided elsewhere [25]

To detect PLK-produced LAP-DPs, we made two monoclonal antibodies. One is the R58 antibody detecting N-terminal side LAP-DPs terminating on the R^{58} residue (R^{58} LAP-DPs), and another is the L59 antibody detecting C-terminal side LAP-DPs starting from the L^{59} residue (L^{59} LAP-DPs). We established techniques to detect each LAP-DP using these antibodies [25]. The R^{58} LAP-DPs remaining in tissues or cell surfaces through S–S bonded LTBP can be detected mostly in αSMA-positive activated stellate cells in liver tissues from both fibrotic animals and patients by immunostaining with the R58 antibody, whereas the L^{59} LAP-DPs were not detectable by immunostaining with the L59 antibody [25]. Figure 3 shows the results obtained from bile duct ligation (BDL) mice. These mice often exhibited granulomatous lesions (*panel a*), in which fibroblastic cells infiltrated and started ECM production (*panel b*). Importantly, the R^{58} LAP-DPs were detected in granulomatous lesions prior to Sirius red positivity, namely before collagen accumulation (arrowheads in *panel c*). In contrast, L59 antibody failed to stain the L^{59} LAP-DPs, although various antigen unmasking procedures were treated (*panel d*). We found that the L^{59} LAP-DPs were released into the blood and could be measured by an ELISA using the L59 antibody (Hara et al., unpublished data). In panels *e–h*, non-parenchymal regions were recognized by antibody R58 (arrowheads in *panel e*), and mostly overlapped with αSMA-positive HSCs (arrowheads in *panel f*), but not with CD31-positive liver sinusoidal endothelial cells (*panel g*) nor with CD68-positive KCs (hepatic macrophages) (*panel h*). We further found that the R58 antibody detected TGF-β1/3 LAP-DPs but not TGF-β2 LAP-DPs because of the similarity and difference of the R58 side sequence, respectively (Fig. 2). Finally, we succeeded in detecting R^{58} LAP-DPs in patients with chronic hepatitis B and C virus

(HBV and HCV, respectively) infection categorized as A1F2 and A2F2, as well as in patients with non-viral hepatitis, such as autoimmune hepatitis and non-alcoholic steatohepatitis (NASH) [25]. A specific cell shape called a "crown-like structure" (CLS) has been referred to as a biomarker for NASH in both an animal model and patients [26]. Recently, we found that R^{58} LAP-DPs positivity well matched the emergence of CLS [27].

These data suggest the occurrence of a PLK-dependent TGF-β activation reaction in patients and indicate that the LAP-DP may be useful as a surrogate marker reflecting PLK-dependent TGF-β1/3 activation and subsequent fibrogenesis in the fibrotic liver both in animal models and in patients.

Conclusion and Future Subjects

The most fibrogenic cytokine, TGF-β, is produced as a latent complex, in which TGF-β is trapped by its propeptide, LAP. On the surface of activated HSCs, PLK activates TGF-β by cleaving LAP between the R^{58} and L^{59} residues, releasing active TGF-β from the complex. We made specific antibodies that recognize the neo-C-terminal (R^{58}) and N-terminal (L^{59}) ends of the LAP-DP, and found that the LAP-DP may serve as a novel surrogate marker of TGF-β activation—namely, generation of active TGF-β—and is thereby a therapeutic marker for TGF-β-mediated liver fibrogenesis in patients [25].

Utilizing LAP-DP antibodies, we are developing techniques to visualize the fibrogenic area by positron emission tomography (PET), planning to eliminate activated HSCs with pertussis toxin, and undertaking the challenge to solve the co-crystal structure of LAP and a LAP-DP targeting inhibitor, which binds to the LAP cleavage site, thereby inhibiting TGF-β activation and liver fibrosis in HBV-infected chimeric mice (Hara et al., unpublished data). The effectiveness of an inhibitor against the TGF-β activation reaction has been reported in the integrin-mediated activation of TGF-β [3, 15, 28]. LAP-DP is also used to monitor the effects of anti-fibrogenic factors or compounds for discovery of a novel anti-fibrosis drug. For example, we recently found that HCV NS3 protease mimics TGF-β2 and enhances liver fibrosis via binding to and activation of the TGF-β type I receptor, and that an anti-NS3 antibody raised against the predicted binding sites attenuates liver fibrosis in HCV-infected chimeric mice [29]. In this study, R58 LAP-DP staining nicely showed the anti-fibrogenic potentials of the anti-NS3 antibody.

The technique developed accelerates drug discovery targeting TGF-β-dependent fibrogenesis in patients suffering from chronic hepatitis.

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