Induced hepatic stellate cell integrin, α8β1, enhances cellular contractility and TGFβ activity in liver fibrosis

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Abstract

No effective therapy exists for fatal fibrosis. New therapeutic targets are needed for hepatic fibrosis because the incidence keeps increasing. The activation and differentiation of fibroblasts into myofibroblasts that causes excessive matrix deposition is central to fibrosis. Here, we investigated whether (and which) integrin receptors for matrix proteins activate hepatic stellate cells (HSCs). First, integrin α-subunits were investigated systematically for their expression over the course of HSC activation and their distribution on fibroblasts and other systemic primary cells. The most upregulated in plate culture-activated HSCs and specifically expressed across fibroblast lineages was the α8 subunit. An anti-α8 neutralizing mAb was evaluated in three different murine fibrosis models: for cytotoxic (CCl4 treatment), non-alcoholic steatohepatitis-associated and cholestatic fibrosis. In all models, pathology and fibrosis markers (hydroxyproline and α-smooth muscle actin) were improved following the mAb injection. We also CCl4-treated mice with inducible Itga8fl/fl−/−; these mice were protected from increased hydroxyproline levels. Furthermore, Itga8 was upregulated in specimens from 90 patients with liver fibrosis, indicating the relevance of our findings to liver fibrosis in people. Mechanistically, inhibition or ligand engagement of HSC α8 suppressed and enhanced myofibroblast differentiation, respectively, and HSC/fibroblast α8 activated latent TGFβ. Finally, integrin α8β1 potentially fulfills the growing need for anti-fibrotic drugs and is an integrin not to be ignored.

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No conflicts of interest were declared

Introduction

The incidence of non-alcoholic steatohepatitis (NASH) with fibrosis is growing, without any effective therapies. Hepatic stellate cell (HSC) activation and myofibroblast differentiation are central to hepatic fibrosis and occur upon deposition of extracellular matrix [1]. Integrins are major extracellular matrix receptors, so we reasoned that integrins induced on HSCs could play a critical role in this process. In the current study we systematically examined integrin α-subunit expression over the course of activation of HSCs and found that the α8 subunit was most dramatically upregulated. α8 forms a heterodimer with β1 [2] and is expressed as α8β1 in smooth muscle cells and fibroblasts [3]. α8β1 is one of the eight integrins that recognize the tripeptide Arg-Gly-Asp (RGD), an integrin recognition motif that is present in the pro-peptide of TGFβ1 and TGFβ3. Integrin binding to this motif is critical for integrin-mediated TGFβ activation [4]. We therefore evaluated the functional significance of this integrin in multiple in vivo models and provide the mechanisms by which this integrin could modulate the behavior of activated HSCs.

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Materials and methods

HSCs were isolated from C57BL/6 mice and Wistar rats. Murine HSCs were used for expression studies and rat HSCs for the validation. Mechanistic studies employed rat HSCs to avoid possible allogenic effects from a mixture of murine HSCs. Sufficient α8 expression was confirmed before each experiment to avoid potential variability in the time course of α8 induction during activation of HSCs. Animal use and euthanasia protocols were reviewed and approved by the Animal Committees of Hiroshima University or University California San Francisco. For the use of human tissue, informed consent was provided by patients in accordance with the Declaration of Helsinki and approval from the Hiroshima University Institutional Review Board. Chicken neutralizing anti-α8 recombinant mAb, YZ3, which reacts with most mammals, was generated in our laboratory [5] and characterized (see supplementary material, Figure S1). A chimeric form of YZ3 with mouse IgG1 Fc was used throughout this study. All antibodies in this study are summarized in supplementary material, Table S1.

The initial global α8 knockout mouse line made in 1997 was characterized by kidney agenesis and perinatal death [6]. Contribution of α8β1 to nephrogenesis can be modified by ‘stochastic factors’, as the penetrance of the phenotype was only approximately 50% [6]. To reduce the possible compensation for loss of α8β1 function, we employed temporally inducible global deletion, Itga8fllox/flox;Rosa26-CreER, so that expression of α8β1 remains normal before induction of fibrosis. The shorter interval should avoid the effects of life-long genetic compensation that may have affected results in previous studies in other organs [7–9].

Details of cells and culturing, antibodies, animals, RT-qPCR, flow cytometry, experimental fibrosis, hydroxyproline assay, measurement of areas stained by Masson’s

Figure 1. Induction and localization of integrin α8 subunit expression in HSCs/fibroblasts. (A) Itga8 mRNA (RT-qPCR) expression of murine HSCs in 14-day culture. RT-qPCR was repeated three times (n = 9 wells, pooled from three experiments). (B) α8 induction in CCl4-treated liver by western blotting (top) and by immunofluorescence with α8 in red and PDGFRβ in green (bottom). (C) α8 induction in isolated ex vivo HSCs from CCl4-treated liver by RT-qPCR. (D) Expression of integrin subunit mRNAs for α8, β6, and αv in 144 primary cell lines. Data were retrieved from the FANTOM database and sorted by cell types indicated. Each bar represents a cell line.
trichrome or α-smooth muscle actin (αSMA) immunostaining, human liver tissues, recombinant nephronectin proteins, western blotting, immunofluorescence, gel contraction assay, TGFβ activation assay and statistical analyses are provided in supplementary material, Supplementary materials and methods.

Figure 2  Legend on next page.
Results

$\alpha 8$ expression in HSCs and fibroblasts

Mouse HSCs grown on plastic for 14 days showed increased *Itgα8* (Figure 1A; $p = 0.0171$) and *Itgα11* mRNA expression. A similar increase in *Itgα8* expression was observed in rat HSCs up to 27-fold and four-fold at the mRNA (see supplementary material, Figure S2A; 27-fold, $p < 0.0001$) and the protein (see supplementary material, Figure S2B) level, respectively. Notably, $\alpha 8$ protein was not detected in quiescent HSCs at days 1 and 3. Integrin $\alpha 6$, which is constitutively expressed in HSCs [10], was used as a control. As culture-activated HSCs mimic the fibrotic response [11], we explored $\alpha 8$ expression in HSCs in response to *in vivo* CCl$_4$ treatment. Western blotting of whole liver lysate showed marked $\alpha 8$ upregulation. Immunofluorescence showed barely detectable $\alpha 8$ in normal livers and an increase in the perportal area colocalizing with PDGFR$\beta$ (Figure 1B). Furthermore, $\alpha 8$ was upregulated in HSCs isolated from CCl$_4$-treated mice (Figure 1C). Next, we analyzed $\alpha 8$ expression from databases analyzing fibroblasts and related lineages. In 144 primary cells from various human tissues [12], *ITGA8* mRNA was expressed across 15 fibroblast lines but was less abundant in other cell types (Figure 1D), in contrast to integrin $\beta 6$ or $\alpha v$ subunits. FACS analyses showed constitutive $\alpha 8$ expression in fibroblasts from the lung, heart, and kidney (see supplementary material, Figure S2C). Taken together, $\alpha 8$ was found to be expressed in HSCs/fibroblast lineages, and was induced by activation of HSCs *in vitro* and *in vivo*.

In *vivo* role of $\alpha 8$ in fibrosis

The effects of neutralizing anti-$\alpha 8$ mAb YZ3 [5] were evaluated in mouse models of cytotoxic (CCl$_4$ treatment), NASH-associated (choline-deficient, l-amino acid-defined, high-fat diet), and cholestatic (bile duct ligation) liver fibrosis. In all models, morphologic detection of fibrosis was attenuated, and elevated hydroxyproline content and $\alpha$SMa protein or mRNA expression were reduced by inhibition of $\alpha 8\beta 1$ (Figure 2A and supplementary material, Figure S3), which were validated by quantification of the area stained by Masson’s trichrome or $\alpha$SMa (see supplementary material, Figure S4). We used CCl$_4$ treatment in mice with global tamoxifen (Tam)-inducible loss of $\alpha 8$ and found protection against increased hydroxyproline content. Cre-recombination efficiency appeared to be excellent and unaffected by CCl$_4$ treatment in mTmG reporter mice (Figure 2B) and the recombination was confirmed by identifying the expected version of $\alpha 8$ protein truncated by 69 residues by western blotting (see supplementary material, Figure S5). We then analyzed *ITGA8* expression in liver tissues from 90 human patients and found expression increased in fibrotic livers compared with F0 controls ($p < 0.0001$, Figure 2C).

$\alpha 8\beta 1$-mediated myofibroblast differentiation

To investigate the functional consequences of increased $\alpha 8$ for liver fibrosis, we inhibited or induced ligand engagement of $\alpha 8\beta 1$ *in vitro*. HSCs grown on plastic upregulated fibrosis markers, Acta2 ($\alpha$SMa), Col1a1 (collagen type I $\alpha 1$ chain) and EDA (fibronectin extra-domain A), and Acta2 was reduced by $\alpha 8\beta 1$ inhibition (Figure 3A), whereas the inhibition had no effect on Col1a1 and EDA (see supplementary material, Figure S6A). To explore the relevance of the Acta2 induction to $\alpha 8\beta 1$, HSCs were plated on to a ligand for $\alpha 8\beta 1$, naphroectin, a basement membrane protein identified based on its function as an $\alpha 8\beta 1$ ligand. $\alpha 8\beta 1$ binds specifically only to naphroectin [13]. In serum-free 24 h culture, Acta2 was induced on naphroectin, and reduced by $\alpha 8$-mAb, whereas expression of Col1a1 or EDA was unaffected (Figure 3B). The ligand engagement-induced Acta2 expression and its inhibition by the mAb was recapitulated in fibroblasts (see supplementary material, Figure S6B). Plating the fibroblasts on naphroectin also induced $\alpha$SMa immunofluorescence and formation of actin stress fibers (Figure 3C). Furthermore, naphroectin enhanced contraction of collagen gels by fibroblasts, which was reduced by $\alpha 8$ inhibition ($p = 0.0004$; Figure 3D). A collagen gel assay using the fibroblasts showed no contraction in 0.5% FCS medium, regardless of the presence of naphroectin, but with TGFP (10 ng/ml) supplementation, contraction was detected and greatly enhanced by naphroectin, and this enhancement was reduced by $\alpha 8$ inhibition. The naphroectin concentrations used for each cell type were selected to be within the range of those required for specific interaction with $\alpha 8\beta 1$ (see supplementary material, Figure S6C). These results indicate that $\alpha 8\beta 1$-mediated signal enhances fibroblast/HSC contractility and myofibroblast differentiation.
TGFβ activation by α8β1 on HSCs

As several RGD-binding integrins have been reported to activate TGFβ [14] (Figure 4A), we examined α8β1 for this ability. Although β6-transfected SW480 cells clearly activated TGFβ, α8-SW480 did not (Figure 4B, left). As, in contrast to αvβ6, α8β1 is expressed in fibroblasts, but not epithelial cells in vivo (Figure 1D), we employed α8-expressing HSCs. Luciferase activity was detected and, notably, distinctly reduced with α8-mAb by approximately 35% (p < 0.0001; Figure 3B, right). The TGFβ activation required actin polymerization as it was abolished by cytochalasin D, and we suspect is explained...
by much higher expression of Acta2 in HSCs compared with SW480 (p < 0.0001; see supplementary material, Figure S7). Lung and heart fibroblasts expressing high levels of Acta2 like HSCs, displayed α8β1-mediated TGFβ activation (Figure 3C). α8β1 thus activates TGFβ when it is expressed by HSCs and fibroblasts.

Discussion

HSC α8β1 integrin appears to contribute broadly to liver fibrosis as blockade or deletion of this integrin inhibits fibrosis in four different settings. In vitro studies suggest that α8β1 probably contributes to fibrosis by enhancing myofibroblast differentiation and by TGFβ activation of HSCs.

Our findings illuminated previously unknown characteristics of α8β1: de novo expression in activated HSCs in vitro and in vivo, upregulation of αSMA, and TGFβ activation on HSCs and fibroblasts. These findings fit well with previous observations of Itga8 expression in other contractile cells, including airway [15] and gastric [16] smooth muscle cells, arrector pili [17] and sensory hair cells [18]. Recent single-cell RNA-sequencing data from NASH mice revealed exclusive Itga8 expression in HSCs in liver cells [19]. Moreover, another group identified a class of murine HSCs that undergo myofibroblast...
differentiation, in which hierarchical clustering characterized the transition from a quiescent to a collagen-producing phenotype, with upregulation of pro-fibrogenic genes, including Col1a1, Col1a2, Col3a1, and Lox [20]. Interestingly, the upregulated genes include only one integrin, Itga8.

Cell matrix communication is characterized by redundant ligand–receptor interactions. The partial effects of anti-αv mAb found in this study for culture-induced Acta2 expression, gel contraction, and TGFβ activation could be attributed to contributions of other integrins. Nonetheless, inhibition of αvβ1 in vivo potently inhibited liver fibrosis. The molecular mechanisms by which HSC activation leads to induction of Itga8 and αvβ1 engagement induces myofibroblast differentiation should be the focus of future studies.

The increased ITGA8 expression in patients with hepatic fibrosis suggests that our findings are relevant to liver fibrosis in people. Because αv expression is minimal in healthy liver, the relevance of this integrin as a driver of liver fibrosis has been largely overlooked. However, our findings that the specific expression of αvβ1 in activated HSCs is critical for induction of a contractile phenotype and TGFβ activation make this integrin an attractive therapeutic target.

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Author contributions statement

NN, KT and YY designed the experiments. NN wrote the manuscript and conducted many of the experiments. KT generated and evaluated the knockout mice, performed the immunofluorescence experiments in mouse tissues, and wrote the manuscript. KK directed the induction of liver fibrosis in the mouse models. KS performed the pathological evaluation of fibrosis in mice. KC provided most of the human liver specimens and analyzed the patient data. TK provided some of the normal human liver specimens. DS verified the concept and data of this work and revised the manuscript. YY conceptualized this study and performed data analyses and interpretation and wrote the manuscript.

References

1. Friedman SL, Sheppard D, Duffield JS, et al. Therapy for fibrotic diseases: nearing the starting line. Sci Transl Med 2013; 5: 167ra1.
2. Bossy B, Bossy-Wetzel E, Reichardt LF. Characterization of the integrin αvβ1 subunit: a new integrin β1-associated subunit, which is prominently expressed on axons and on cells in contact with basal lamina in chick embryos. EMBO J 1991; 10: 2375–2385.
3. Levine D, Rockey DC, Milner TA, et al. Expression of the integrin αvβ1 during pulmonary and hepatic fibrosis. Am J Pathol 2000; 156: 1927–1935.
4. Munger JS, Huang X, Kawakatsu H, et al. The integrin αvβ6 binds and activates latent TGF-β1: mechanism for regulating pulmonary inflammation and fibrosis. Cell 1999; 96: 319–328.
5. Nishimichi N, Kawashima N, Yokosaki Y. Epitopes in αvβ1 and other RGD-binding integrins delineate classes of integrin-blocking antibodies and major binding loops in α subunits. Sci Rep 2015; 5: 13756.
6. Müller U, Wang D, Denda S, et al. Integrin αvβ1 is critically important for epithelial–mesenchymal interactions during kidney morphogenesis. Cell 1997; 88: 603–613.
7. Hartner A, Cordasic N, Rascher W, et al. Deletion of the αv integrin gene does not protect mice from myocardial fibrosis in DOCA hypertension. Am J Hypertens 2009; 22: 92–99.
8. Hartner A, Menendez-Castro C, Cordasic N, et al. Tubulointerstitial de novo expression of the αv integrin chain in a rodent model of renal fibrosis—a potential target for anti-fibrotic therapy? PLoS One 2012; 7: e48362.
9. Hung CF, Wilson CL, Chow YH, et al. Role of integrin αvβ6 in murine model of lung fibrosis. PLoS One 2018; 13: e0197937.
10. Carloni V, Romanelli RG, Pinzani M, et al. Expression and function of integrin receptors for collagen and laminin in cultured human hepatic stellate cells. Gastroenterology 1996; 110: 1127–1136.
11. Rockey DC, Housset CN, Friedman SL. Activation-dependent contractility of rat hepatic lipocytes in culture and in vivo. J Clin Invest 1993; 92: 1795–1804.
12. Ono H, Ogasawara O, Okubo K, et al. RefEx, a reference gene expression dataset as a web tool for the functional analysis of genes. Sci Data 2017; 4: 170105.
13. Sato Y, Uemura T, Morimitsu K, et al. Molecular basis of the recognition of nephrerinectin by integrin αvβ1. J Biol Chem 2009; 284: 14524–14536.
14. Henderson NC, Arnold TD, Katamura Y, et al. Targeting of αv integrin identifies a core molecular pathway that regulates fibrosis in several organs. Nat Med 2013; 19: 1617–1624.
15. Khalifeh-Soltani A, Gupta D, Ha A, et al. The Mfge8–αvβ1–PTEN pathway regulates airway smooth muscle contraction in allergic inflammation. FASEB J 2018; 32: 5927–5936.
16. Khalifeh-Soltani A, Ha A, Podolsky MJ, et al. αvβ1 integrin regulates nutrient absorption through an Mfge8–PTEN dependent mechanism. Elife 2016; 5: e13063.
17. Fujiwara H, Ferreira M, Donati G, et al. The basement membrane of hair follicle stem cells is a muscle cell niche. Cell 2011; 144: 577–589.
18. Evans AL, Müller U. Stereocilia defects in the sensory hair cells of the inner ear in mice deficient in integrin αvβ1. Nat Genet 2000; 24: 424–428.
19. Xiong X, Kuang H, Ansari S, et al. Landscape of intercellular cross-talk in healthy and NASH liver revealed by single-cell secretome gene analysis. Mol Cell 2019; 75: 644–660.e5.
20. Dobie R, Wilson-Kanamori JR, Henderson BEP, et al. Single-cell transcriptomics uncovers zonation of function in the mesenchyme during liver fibrosis. Cell Rep 2019; 29: 1832–1847.e8.

21. Mederacke I, Dapito DH, Affo S, et al. High-yield and high-purity isolation of hepatic stellate cells from normal and fibrotic mouse livers. Nat Protoc 2015; 10: 305–315.

22. Weinacker A, Chen A, Agrez M, et al. Role of the integrin αvβ6 in cell attachment to fibronectin: heterologous expression of intact and secreted forms of the receptor. J Biol Chem 1994; 269: 6940–6948.

23. Lizio M, Harshbarger J, Abugessaisa I, et al. Update of the FANTOM web resource: high resolution transcriptome of diverse cell types in mammals. Nucleic Acids Res 2017; 45: D737–D743.

24. Wang C, Mochel NR, Christenson S, et al. Expansion of hedgehog activation disrupts stromal identity and induces emphysema. J Clin Invest 2018; 128: 4343–4358.

25. Chan CS, Chen H, Bradley A, et al. α8-integrins are required for hippocampal long-term potentiation but not for hippocampal-dependent learning. Genes Brain Behav 2010; 9: 402–410.

26. Feil R, Brocard J, Mascrez B, et al. Ligand-activated site-specific recombination in mice. Proc Natl Acad Sci U S A 1996; 93: 10887–10890.

27. Matsumoto M, Hada N, Sakamaki Y, et al. An improved mouse model that rapidly develops fibrosis in non-alcoholic steatohepatitis. Int J Exp Pathol 2013; 94: 93–103.

28. Desmet VJ, Gerber M, Hoofnagle JH, et al. Classification of chronic hepatitis: diagnosis, grading and staging. Hepatology 1994; 19: 1513–1520.

29. Yokosaki Y, Matsuura N, Sasaki T, et al. The integrin α9β1 binds to a novel recognition sequence (SVVYGLR) in the thrombin-cleaved amino terminal fragment of osteopontin. J Biol Chem 1999; 274: 36328–36334.

30. Sakata K, Hara M, Terada T, et al. HCV NS3 protease enhances liver fibrosis via binding to and activating TGFβ type I receptor. Sci Rep 2013; 3: 3243.

31. Motulsky HJ, Brown RE. Detecting outliers when fitting data with nonlinear regression – a new method based on robust nonlinear regression and the false discovery rate. BMC Bioinformatics 2006; 7: 123.

References 21–31 are cited only in the supplementary material.

SUPPLEMENTARY MATERIAL ONLINE

Supplementary materials and methods

Supplementary figure legends

Figure S1. Specificity of anti-α8 mAb YZ3

Figure S2. α8 expression in HSCs and fibroblasts

Figure S3. Full gel image of western blot for αSMA in Figure 2A (CCl4)

Figure S4. Measurement of fibrotic area in liver sections from three mouse models stained for collagen fibers and αSMA

Figure S5. Western blotting for the WT and mutant α8 in Tam-inducible α8 knockout mice

Figure S6. Effects of α8β1 inhibition on Col1a1 and EDA, and specificity of nephronectin to α8β1

Figure S7. RT-qPCR for Acta2

Table S1. Antibodies used in this study

Table S2. PCR primer sequences