Review

New therapeutic targets for hepatic fibrosis in the integrin family, α8β1 and α11β1, induced specifically on activated stellate cells

Yasuyuki Yokosaki1*, Norihisa Nishimichi1

1 Integrin-Matrix Biomedical Science, Translational Research Center, Hiroshima University, 1-2-3, Kasumi, Minami-ku, Hiroshima, Japan, 734-8551; yokosaki@hiroshima-u.ac.jp
* Correspondence: yokosaki@hiroshima-u.ac.jp; Tel.: +81-82-257-1523

Abstract: Huge effort has been devoted to developing drugs targeting integrins over 30 years, because of the primary roles of integrins in the cell-matrix milieu. Five αv-containing integrins, in the 24 family members, have been a central target of fibrosis. Currently, a small molecule against αvβ1 is undergoing a clinical trial for NASH-associated fibrosis as a rare reagent aiming at fibrogenesis. Latent TGFβ activation, a distinct talent of αv-integrins, has been intriguing as therapeutic target. None of the αv-integrin inhibitors, however, has been in the clinical market. αv-integrins commonly recognize an Arg-Gly-Asp (RGD) sequence, and thus the pharmacophore of inhibitors for the 5-integrins is based on the same RGD structure. The RGD preference of the integrins, at the same time, dilutes ligand specificity, as the 5-integrins share ligands containing RGD sequence such as fibronectin. With the inherent little specificity in both drugs and targets, “disease specificity” has become less important for the inhibitors than blocking as many αv-integrins. In fact, an almighty inhibitor for αv-integrins, pan-αv, was in a clinical trial. On the contrary, approved integrin inhibitors are all specific to target integrins, which are expressed in cell-type specific manner: αIbβ3 on platelets, α4β1, α4β7 and αLβ2 on leukocytes. Herein, “disease specific” integrins would serve as attractive targets. α8β1 and α11β1 are selectively expressed in hepatic stellate cells (HSCs) and distinctively induced upon culture activation. The exceptional specificity to activated HSCs reflects rather “pathology specific” nature of these new integrins. The monoclonal antibodies against α8β1 and α11β1 in preclinical examinations may illumine the road to the first medical reagents.

Keywords: Fibrosis; Integrin; TGFβ; Therapeutic target; Drug; Inhibitor; Monoclonal antibody, α8β1, α11β1, Hepatic stellate cell

1. Introduction

Liver fibrosis, also known as cirrhosis, is an intractable disease with high morbidity. There is a serious unmet medical need because no availability of drugs in concert with increasing incidence of non-alcoholic steatohepatitis (NASH)-associated liver fibrosis, i.e., 2 million deaths per year in the world [1-3]. Drugs for NASH-associated fibrosis are aiming at processes of steatosis/hepatitis and following fibrogenesis. Currently, most drugs in clinical trials are the former and the latter are limited especially after simtuzumab (anti-LOXL2) [4] and seronsertive (ASK-1 inhibitor) [5] fell in phase II and III, respectively. In this situation, integrin inhibitors have an emerging therapeutic opportunity in fibrosis [6]. Integrins are a receptor for matrix proteins that essentially consists of fibrosis tissues, and some integrins show activation potential for latent-TGFβ [7]. In fact, antagonists for αvβ1 [8] and αvβ6 [9,10] showed considerable inhibition in experimental animal models for liver, lung, and kidney fibrosis. Encouraged by the discovery of the TGFβ activation in 1999, pharmacological enthusiasm appears to converge into αv-containing integrins. However, many other members of the integrin family have not been examined each for aptitude to the therapeutic target and no reagents against αv-
integrins has been approved for fibrosis and other diseases. We revisit the developmental history of αv-inhibitors, and show alternative new integrins, which contrastingly to αv-integrins exhibit pathology-specific expression in fibrosis, α8β1 [11] and α11β1 [12].

2. Leading three players in fibrosis are each related to integrins

Tissue fibrosis is substantially characterized by deposition of excessive extracellular matrix proteins [13]. The matrix proteins are secreted from activated fibroblasts/myofibroblasts [14]. The fibroblast activation and differentiation to myofibroblast are regulated by TGFβ [15]. Matrix proteins, fibroblasts, and TGFβ, these 3 diverse players in fibrosis, are each functionally dependent on integrins to play their role in development of fibrosis. Integrins are committed as a sensor for the cell-matrix environment [16] [17], a signaling receptor of fibroblasts [18,19], and an activator of the latent TGFβ complex [20].

Integrins are a family of cell surface α and β heterodimeric receptors for various extracellular matrix proteins. There are 18 α and 8 β subunits that form 24 heterodimers. Cell adhesion by engaging with matrix proteins or cell surface immunoglobulin superfamilly members, including ICAM-1 and VCAM-1, is the classical role of integrins [21], which is typically illustrated by hematopoietic integrins to adhere to vascular endothelial cells [22]. As a signaling receptor, integrins mediate fundamental cellular behavior such as cell migration, proliferation, or survival [23]. There are many matrix protein species, which are recognized by multiple integrins. One integrin engages with multiple matrix proteins and one matrix protein interacts with multiple integrins. Ligand repertoire of each integrin thus overlaps one another but, of note, the repertoires of each of 24-integrins are unique. Tissues resident cells, such as epithelial and mesenchymal cells, recognize their matrix environment through integrin receptors and integrins are thus a sensor for the cellular environment including tissue injury. In healthy tissues, integrin plays a role in tissue homeostasis to keep tissue integrity, for example epithelial cells know their position on the basement membrane by recognizing signals from components of the basement membrane such as laminins and collagen type IV via integrin receptors (Figure 1). Once the basement membrane is injured, however, cells notice changes in underlying matrix proteins to those normally present in the subepithelial tissue, like collagen type I and

![Image](image.png)

**Figure 1. Recognition of tissue injury by epithelial cells via integrin receptors.** At the healthy tissue (upper panel), epithelial cells know their peaceful circumstance recognizing components of the basement membrane such as laminin via integrins (red). Upon tissue injury, cells recognize contact with unusual matrix proteins such as collagen type I and fibronectin, which are normally in sub epithelium, and notice the emergent condition.

fibronectin. The recognition of tissue injury by integrin is the case in interstitial cells including fibroblasts. A primary biological role of otherwise quiet fibroblasts is to watch on,
migrate, and repair tissue injury, secreting matrix proteins [24] and the recognition for the tissue architecture could be an initiation for tissue repair. In these contexts, integrins have been predicted to play a vital role in development of fibrosis. However, the close relationship of integrins with TGFβ was not predicted until the discovery.

3. Latent TGFβ activation by integrins

The fact that integrin controls TGFβ, the master regulator of fibrosis [25], is one of the strongest evidences for the commitment of integrins to fibrosis. In 1999, integrin β6 subunit knockout mice (lacking integrin αvβ6 heterodimer) were found to be protected against bleomycin induced pulmonary fibrosis [26]. The β6-knockout mice were previously found infiltrated with inflammatory cells in the lungs by bronchoalveolar lavage and were thought to exhibit exaggerated lung fibrosis by bleomycin [27]. This seemingly contradictory result was resolved by the discovery, αvβ6 mediated-TGFβ activation. Due to anti-inflammatory and pro-fibrotic bilateral nature of TGFβ, lack of TGFβ activation in the β6-knockout lead to both the inflammation and the protection from fibrosis. Unlike to many other cytokines, TGFβ is stored in the matrix milieu encapsulated with pro-domain of the TGFβ protein as an inactive homodimer (Figure 2), so called latency associated peptide (LAP) This manner of storage allows TGFβ to act at once on demand without de novo protein synthesis. How matured TGFβ is released form pro-TGFβ was in long controversy and the discovery of a regulatory system was a big innovatin to understand how interstitial milieu is regulated to be fibrosis controlled.

There is an RGD (Arg-Gly-Asp) tripeptide, that αv-containing and other integrins preferentially bind (Figure 2). Binding of cell surface αvβ6 to the RGD in pro-TGFβ complex was found to elicit tensile force with contraction of the αvβ6-expressing cells, as the other end of the complex is anchored to latent transforming growth factor β binding protein (LTBP) cross-linked to the extracellular milieu [28]. Following release from the complex, TGFβ is allowed to bind to its receptors. This is the process so called “TGFβ activation”. This discovery owes much to a bioassay, where luciferase TGFβ signal reporter cells and β6-transfected cells are co-cultured. None of the biological roles of integrins may have been more attractive for these 2 decades.

**Figure 2. Integrin mediated TGFβ activation.** TGFβ is stored in the extracellular milieu anchoring to LTBP that is fixed to matrix proteins, as a pro-protein, also termed as LAP. The pro-TGFβ protein forms homodimer holding TGFβ by the pro-domains. There is a RGD sequence in the pro-domain. To release TGFβ, RGD-recognizing integrins binds to the RGD sequences and pro-domains are removed from the matured TGFβ by cellular tensile force initiated by contraction of the cell expressing the integrins.
4. Trends of target-integrins for fibrosis

An RGD-tripeptide is the first amino acid sequence found as a motif that integrins recognizes [29] and is present in many matrix proteins such as fibronectin, vitronectin, and tenascin-C. It is an RGD-peptide that helped the discovery of the first heterodimer by eluting a fibronecton affinity column in 1985 [30]. As many as 8 of 24 members of the integrin family recognize RGD (Figure 3) including all of 5 αv-integrins. The RGD has been a keyword in integrin mediated matrix biology. It seems, therefore, natural that an RGD-based pharmacophore has been central to design integrin inhibitors. In addition, expectations for inhibitors of αvβ3 [31] and αvβ5 [17][23] [32] [33] as anticancer agent due to their angiogenic potential raised the prospect of RGD-peptide and boosted the pharmacological enthusiasm for the medical drugs [7].

Currently, there are three therapeutics venture companies in US that exclusively targeting the integrin family and reached clinical trials [6]. Two of these companies have been invested over $200 M from pharmaceutical companies including Novartis, AbbVie, in total and both listed on NASDAQ after 5 years of foundation. Morphic targets each αvβ1 and αvβ6 and Pliant developed dual inhibitor of αvβ1/αvβ6 for idiopathic pulmonary fibrosis [34] and a selective inhibitor on αvβ1 for NASH-associated fibrosis. The other company Indalo brought the pan-αv into a clinical trial. These concentrated developments on anti-αv-inhibitors are based on many animal experiments and in vitro mechanistic studies, especially αvβ6. A profibrotic role of αvβ6 and an anti-fibrotic effect of the anti-αvβ1 antagonists have been described in lung [9] [10], biliary [35], and kidney fibrosis [36] [37]. Anti-fibrotic treatment targeting αvβ6 has been effective across organs. However, expression of β6 subunit is restricted to the epithelial cells, not central to fibrosis, and the profibrotic role was found irrelevant to some fibrosis models such as CCl4-induced liver fibrosis [35], where fibrosis develops in distance from epithelium. In addition, the pro-TGFβ activation was found to be performed by any of the other αv-integrins, αvβ1, αvβ3, αvβ5 and αvβ8, [38], [39] [40] [41] depending on conditions. To explore further profibrotic effects of the αv-integrins, first, ligav was deleted selectively in myofibroblasts in pdgfrb-Cre driven conditional αv-knockout mice [42]. The mice lacking all myofibroblast αv-integrins were protected from liver fibrosis. Next, to determine which integrin was important to fibrosis, individual integrins were deleted for αvβ3, αvβ5, αvβ6

---

**Figure 3. The integrin family.** Twenty-four heterodimers and the combinations of 18 α and 8 β subunits are indicated. Eight RGD-recognizing integrins are paired with red lines. Orange lines indicate leukocyte integrins. An alternative name for αIIbβ3 is GPIIb/IIIa, and for αLβ2 are LFA1 and CD11a/18.

(global) and αvβ8 (HSC selective), but no protection was found. Therefore, either a combinatorial effect of the αv-integrins or an independent effect of αvβ1 (not deleted due to technical limitation) was hypothesized to drive fibrosis. Shortly, a specific small molecule blockade against αvβ1, C8, was developed and showed an excellent inhibitory effect on murine fibrosis models in multiple organs [8], despite off target effects of C8 on α4β1
found later [43]. The above history of RGD peptide starting from the use in the elution buffer, and the established pharmacophore may explain why general interests in integrin-inhibitors converge on αv-integrins. Inhibitors of integrins αvβ1 and αvβ6 is the leading pack in 2021[6].

5. What the history of αv-integrin inhibitors tells us

αv-integrins activate TGFβ and protect against fibrosis when deleted. Nevertheless, no approved αv-inhibitor for fibrosis and other diseases is in the clinic, despite drugs against integrins, αIIbβ3 [44], α4β1 [45], α4β7 [46], and αLβ2 [47] [48] are making big market [6]. It has been difficult to give specificity to a small molecule inhibitor to individual αv-integrins, with the same pharmacophore shared. The initial αvβ3 inhibitor relied rather on the differential expression in cancer endothelial cells than binding specificity. Furthermore, ligand repertoires of αv-integrins overlap one another, thus signals from αv-integrins could be redundant in some respects. Under these inherent non-specific circumstances of αv-integrins, an interesting idea is to inhibit pan-αv-integrins [49] [50]. This inversion, leaving the specificity in the second, reveals the technical difficulty. The pan-αv concept may hold true, because always important is the balance of efficacy and toxicity for drug discovery, depending on the medical need. In fact, IDL-2965 [51] underwent the Phase I clinical trial, but we haven’t heard progress, yet. In addition, also known for RGD-mimetic integrin inhibitor, is a paradoxical signal input [52] [53] [54] by binding to the ligand binding pocket of integrin. Therefore, ligand mimetic inhibitors for αv and other integrins should be examined for the signal before entering a clinical trial.

Among αv-integrins, only αvβ6 shows preference for ligands, as the binding sequence in pro-TGFβ is RGDLxxL/I [55] [56]. For αvβ6, target specific inhibitor has been obtained [57]. On the other hand, αvβ1 [58] [59] is a peculiar integrin in terms of the combination of the subunits, both of which are promiscuous, and most cells store a considerable amount of αv and β1 proteins in the cytoplasm to form various heterodimers. However, αv and β1 do not always form the heterodimer in all cells, by unknown mechanisms. Unlike most other integrins, expression of either of the subunits does not identify the heterodimer, and there is no mAb or labeling reagent that binds to both subunits together. It is, therefore, impossible to define the site of αvβ1 accumulation by immunohistochemistry in the fibrotic and healthy tissues. What if αvβ1 is concentrated in liver fibrosis? The αvβ1 selective compound, PLN-1474, or others [60] may be closest to the goal, otherwise the pharmacokinetics and pharmacodynamics may need to be analyzed carefully to avoid possible off target effects. Alternatively, αvβ6 is a principal integrin to activate TGFβ in epithelial cells, which could be a disease-specific integrin if upregulated in epithelial cells selectively in fibrosis tissue. Progress of these drugs in clinical trials holds a key to future direction of the development of integrin inhibitors.

Specificity to the target is the fundamental critical requirement for molecular target drugs. It is difficult, however, to obtain the specificity for the inhibitors against αv-integrins. The absence of the drugs in the clinic may reflect the significance of the specificity.

6. Disease specific integrins

Integrins αvβ6 and αvβ1 are leading targets for fibrosis, but holds concerns such as epithelial cell restricted expression of αvβ6 and unclear systemic distribution of αvβ1. Herein, one should be aware that there are 19 non-αv containing integrins. Most of the non-αv integrins, excluding leukocyte and platelet integrins, play similar biological roles with αv-integrins in view of cell adhesion, tissue integrity and tissue repair as matrix protein receptors. In addition, three of the integrins, αIIbβ3, α5β1 and α8β1, engage with RGD-containing ligands. It is, therefore, not surprising if some non-αv integrins play roles in fibrosis comparably or more innately than αv-integrins. Is there any subunit, unlike to αv-integrin, that fulfills “disease-specific” expression? Integrin α8β1, unlike to most other integrins, shows characteristic restricted expression in mesenchymal cells [61]. A comprehensive gene expression data for 150 primary cells from various tissues [62] reveals α8
α mesenchymal fibroblasts stimulation is selective in manner, These expression subunit 4 (red). 

α5β1 is one of the 4 collagen receptor integrins and, of note, selectively expressed in fibroblasts, just like α8β1 (Figure 5). We will refer to the data by us and others for these 2 integrins to evaluate their suitability as therapeutic targets of fibrosis. It appears that backgrounds why these 2 integrins have been unattended is not because of their functional limitation but substantially by the absence of specific inhibitors.

Figure 4. Selective expression of integrin α subunits in fibroblasts. Four α subunits, α1, α8, α11 and αv are upregulated by fibrotic stimulation in HSCs (Figure 5). Expression of the subunits in primary cultures of various cell types are compared focusing on fibroblasts (red). These 4 subunits are expressed in primary cultured fibroblasts from various tissues, and α8 and α11 are in fibroblast selective manner, while αv subunits are ubiquitously expressed across cell types. α1 is expressed in fibroblasts but also in non-mesenchymal cells such as leukocytes and endothelial cells. α8 and α11 are principally expressed only in mesenchymal cells and α8 is the most specific subunit to fibroblast comparing with α11.

7. Pathology specific integrin α8β1 with TGFβ-activating potential

Three non-αv integrins, αIIbβ3, α5β1 and α8β1, recognize RGD sequence. αIIbβ3 (GPIIb/IIIa) is exclusively expressed on platelets. α5β1 interacts with narrow spectrum of ligands and specifically recognize RGDsequence in the 10th type III repeat of fibronectin and does not recognize RGD<sub>L</sub> in TGFβ pro-protein. Integrin α8β1 more promiscuously engages with RGD in many proteins including, nephroectin, fibronectin, osteopontin, tenascin-C, Mfge-8, and, of note pro-TGFβ, protein.
7.1 Proposed contribution of αβ1 to fibrosis and opposing findings

In 2000, α8 was first reported highly upregulated in lung fibroblasts and HSCs in experimental fibrosis models. Similarly, α8 induction was observed in activated fibroblasts of cardiac fibrosis [64] vascular stenosis [65], gingival over growth [66]. Due to the prominent upregulation in the lung and liver fibrosis tissues in activated fibroblasts/HSCs, integrin αβ1 was proposed to be a therapeutic target of fibrosis [67]. However, the expectation was opposed by two findings. TGFβ activation bioassay using luciferase reporter cells co-cultured with αβ1-expressing colon cancer cell line, SW480, exhibited negative results, unlike αβ6, despite recognition of RGD by αβ1 in pro-TGFβ [68]. Second, expected reduction in fibrosis was not observed in a global Itga8-null mice line in heart [69] and kidney [70] fibrosis.

By our recent experiments, however, αβ1 activates TGFβ, in a cell type specific manner [11]. αβ1 on fibroblasts/HSCs activates TGFβ, in contrast, there is no activation by α8-transfected SW480 as previously reported [68]. A tensile force elicited to release TGFβ between αβ6 and pro-TGFβ protein was demonstrated in a crystal structure-based 3D model [71]. Cell contractility is an important force for integrin mediated TGFβ activation. Interestingly, however, αβ6 expressed on SW480 non-contractile cells activates TGFβ, which is disrupted by cytochalasin D [11]. The distinct TGFβ activation of αβ1 from αβ6 should be more explored for better understanding of the integrin mediated TGFβ activation.

The other set of conflicting results are from the Itga8-null mouse line. This could be attributed to simply differential effects of αβ1 by organs. However, a special phenotype of the Itga8 knockout mouse line [72] used in those experiments need to be assessed carefully. The authentic knockout mouse line was established in 1997 by crossing Itga8−/− heterozygous mice and Itga8+/− mice lacked the whole α8 expression since the fertilization. Interestingly, the line is known for bilateral fatal kidney agenesis, suggesting a role of αβ1 in nephrogenesis. This effect is corroborated by discovery of recessive mutations of ITGA8 in families with kidney agenesis [73]. Of note, the bilateral agenesis in the KO line occurs

Figure 5. Induction of integrin α subunit in HSCs by culture activation. Bars indicate relative expression of day 5 for α subunits indicated by qPCR. All α subunits that are expressed in tissue cells (excluding cells in the circulation) are evaluated. Each bar represents mean ± SE, and statistical significances were calculated by ANOVA. (Adopted from Ref. 11)

only in about half of the mice at birth and the other half are survived with one or two kidney(s). The experiments with negative results were performed using the latter half of survived mice, naturally. As the number of kidneys indicates, effects of Itga8-deletion could be different between the fetal and survived groups at least on nephrogenesis. Besides the mechanism for the kidney agenesis, the effect of Itga8-deletion on fibrosis in the survived mice is likely to be compensated by “a stochastic factor” [72]. Such compensation in genetic models is commonly found in zebrafish, where the compensatory expression was not observed in siRNA knockdown model, but in hereditary genetic loss [74]. The
compensation in zebrafish suggests the same tuning for molecular network in mice and support the idea that the no fibrosis reduction in the Ilga8−/− mice line were biased by the compensation. In addition, the Ilga8−/− mice that survive are fertile and maintained within a Ilga8−/− colony [75], which could concentrate the genetic background related to the stochastic factor. In our Tamoxifen-inducible Ilga8floxclox; Rosa26-Cre mice, α8 expression is preserved until the beginning of the experiment, kidney development is normal, and importantly liver fibrosis is attenuated [11].

7.2 Neutralizing mAb for α8β1

Most integrin heterodimer receptors have been characterized for the function by use of a specific neutralizing monoclonal antibody (mAb), which is generally obtained following molecular cloning of a subunit and identification of the heterodimer [76]. However, no one has successfully generated the neutralizing mAb against α8β1, despite repeated challenges. Therefore, none of evidence for α8β1-mediated fibrogenesis was provided, although literatures were accumulated describing α8 expression in activated fibroblasts. We, therefore, immunized avian species, chicken, with murine α8 protein and screened with human α8β1, consequently obtained 3 neutralizing clones [77]. A following epitope mapping revealed that the epitopes of 3 independent mAb clones overlapped and the shared sequence was totally conserved across mammalian species. The conserved epitope sequence explains why preceded efforts of others in mice, rats, and rabbits have been unsuccessful. The mAb clones, YZ3, YZ5, and YZ26 show potent blocking activity of IC50 < 0.1 μg/ml for cell adhesion, indicating sufficient potency in vitro and in vivo experiments.

7.3 A role of α8β1 in fibrosis

A profibrotic property of α8β1 was confirmed with the mAb, YZ3 [11]. First, we injected the mAb in the liver fibrosis models, biliary duct ligation (BDL), CCl4 and clinically relevant NASH-associated model. The fibrosis in the liver was attenuated in all models. Expression of α8 in clinical liver fibrosis was analyzed in 90 patients undergone hepatectomy and elevated in the fibrotic livers compared to F0 controls. Several reports indicate α8 expression is associated with a contractile phenotype of cells such as arrector pili [78] and sensory hair cells [79]. We, therefore, evaluated contribution of α8β1 to myofibroblast differentiation in HSC culture activation. In three markers upregulated, Acta2, Coll1A1 and extra domain-A of fibronectin (EDA), we found Acta2 was reduced by the anti-α8 mAb. To ensure the α8β1-dependent upregulation, we plated HSCs on a α8β1 ligand, nephronectin. This interaction induces nephrogenesis and is biologically active [80]. As expected, the HSCs induced Acta2 expression dose-dependently on nephronectin, which was abrogated by YZ3. The α8β1-induced myofibroblast differentiation was confirmed by gel contraction assay, where gel contraction induced by nephronectin in collaboration with TGFβ was inhibited by YZ3. Taken the potentials of myofibroblast differentiation and TGFβ activation together, α8β1 is, at least in part, driver of liver fibrosis.

8. Pathology specific integrin α11β1, with a property of collagen receptor

Collagen type I and type III are representative matrix proteins excessively deposit in fibrosis. Changes in collagen density of fibrotic tissue are sensed at least in part by the receptor integrins. There are 4 integrins exclusively serve for collagens [81], α1β1, α2β1, α10β1 and α11β1 [82]. We [11] and others [63] found, unlike the other 3 members, α11 is highly induced during culture activation of HSCs (Figure 5) like α8. Tissue distribution of α1 is relatively selective in the mesenchyme and also covers other cell types such as neurons, leukocytes, and endothelial cells (Figure 4). α2 is expressed ubiquitously in epithelial cells, endothelial cells and fibroblasts and mesenchymal stem cells. α10β1 is a specific receptor for collagen type II that is a predominant constituent for cartilage tissue (50 % <) and expressed largely in chondrocytes. α11 has been known for its mesenchymal specific
expression [83]. Comparing to α8, α11 is expressed more diversely in mesenchyme including chondrocyte, smooth muscle cells, adipocytes, and mesenchymal stem cells (Figure 4).

In all the 24 integrins, α11β1 and α8β1 are the only members, that are limited to the mesenchymal cells and preferentially expressed in fibroblasts. Interestingly, both are also the only subunits that are highly induced by culture activation in HSCs [11]. Since α11 is a receptor for collagen at least type I, III, and V [84] [82], and highly induced in fibrosis, it may not be surprising if α11 modulates development of fibrosis. There are accumulating evidence for myofibroblast differentiation by α11β1 [85] [86] and TGFβ dependent expression of α11 [87,88]. Furthermore, α11 accumulates in fibroblasts/myofibroblasts in the sites of fibrosis, in murine models of the liver, lung and kidney [12] and human gingival overgrowth [66]. In addition, cardiac fibrosis is induced by over-expression of α11 in mice [89]. This set of data strongly suggested a profibrotic role of α11β1. As the last piece, direct inhibition of in vivo fibrosis by a specific antagonist for α11 would be of great value. Alternatively, Itga11 knockout mice with inducible ablation could serve as the remaining piece. One or both ways of the target validation have to be done to begin the development of the clinical reagents targeting α11β1. We have generated a neutralizing mAb against α11β1, clone YW33. The mAb specifically recognizes α11 in the 4-collagen receptor integrins and the recognition is across mammalian species, at least human, mouse and rat. The mAb inhibits cell adhesion of human α11-transfected C2C12 cells to collagen type I and type III. Interestingly, the anti-α11 mAb, YW33 also detaches adhered cells from the plates coated with collagen type I and Type III (Figure 6), showing that the mode of action of the inhibition is allosteric. An allosteric inhibition is proposed as an essential requirement for integrin inhibitors [90] as described later.

9. Future directions for anti-fibrotic integrin inhibitor drugs

Although many anti-fibrotic drugs targeting integrins and other molecules have suc-

![Figure 6. Cell detaching effect of anti-α11 neutralizing mAb. C2C12 cells transfected with Itga11 cDNA were plated on plates coated with indicated collagen. After cells adhered, the anti-α11 mAb, YW33, was added into the culture. Pictures in the left column show cells adhered to the plate, and in the right show cells 90 min after the mAb input. Changes in cell shapes into rounded form indicates weakened attachment of cells.](image-url)
development, and the absence of effective drugs but many candidate drugs are present, the combination therapy is a reasonable option. Finally, there is a mode of action in inhibitors that is potentially critical to disrupt integrins on duty in the tissues, which may have been paid less attention than needed [91] [90].

9.1 Evaluation in animal models

Severity of fibrosis is evaluated relying largely on deposition of collagens in tissue of experimental animals. Several quantification methods of the collagen deposition are established, such as a measurement for hydroxyproline content in the tissue [92] and areas positive for Sirius red or Masson’s trichrome staining in histology sections. A collagen amount, due to its physical existence in fibrosis itself as an essential material, is the gold standard of the evaluation. However, because results from preclinical studies do not assure the consequences in clinical trials at all, one might suspect of the value as gold standard in terms of evaluation of drug efficacy in human fibrosis.

Collagen species, components of fibrosis tissue, is the essential and final product of a fibrogenic pathway, which inversely indicates the production is preceded by changes in and around collagen-producing cells. An important focus of evaluation of anti-fibrotic drugs could be derived from such changes in collagen production. Most of clinical fibrosis, including idiopathic pulmonary fibrosis and liver cirrhosis, progress slowly [93] and steadily sometime over decades. In contrast, animal models are set to develop the pathology in several weeks treated with chemicals or surgical procedures evoking exaggerated conditions. Considering the chronicity in human, the dysregulation in the collagen-producing network in the animal models is distinct from the persistent and intractable collagen production in human liver cirrhosis. Is the pathogenesis of collagen deposition in human reflected in the amount of collagen deposition in animals? What we ought to look into animal experiments may not be the consequence after “weeks” but a marker of pathology that leads to chronic deposition. Because minor changes in gene expression, unable to be found when the gold standard was established [94], are detectable and, moreover, cluster of genes that are related to the disorder are estimated by the expression. The platinum standard that reflects future collagen deposition must be the established.

Nevertheless, the collagen reducing effect within the experimental period appear to be indispensable for a go-no-go decision in a preclinical examination. What is the requirements to be the winner? Since the force of collagen accumulation during the animal experiment is far beyond physiological and even pathological regulation in animals, one winner of the decision is a reagent that abrogates collagen production network, and another that affect viability of HSCs, and a looser could be blockades for a specific pathway of collagen production, which generally display effect in a longer period. Considering the chronic nature of human fibrosis, a reagent that inhibit profibrotic process gently but steadily is an important choice, which is contradictory to the acute collagen reducing efficacy required for the decision.

9.2 Target specificity of integrin inhibitors and pathology specificity of target integrins

Learned from the developmental history, αv-inhibitors have an inherent nonspecific property. In contrast, the integrin inhibitors already in the clinic, such as abciximab [95], natalizumab [96] [97], vedolizumab [98], and lifegrast [99] commonly exhibit; 1) specific binding to the target, 2) the targets, αIbβ3, αβ1, αβ7 and αLβ2, play a role non-redundantly in the target disease, and are 3) expressed on cells important to the pathology, i.e., platelet, and subsets of lymphocytes. These characters of the clinical integrin inhibitors convey that importance of specificity is not only in the drugs but also in the targets. In this respect, for fibrosis an integrin that plays a role specifically in activated fibroblasts/myo-fibroblast is a hopeful candidate. The functional specificity to the effector cells is achieved simply by the specific expression on the effector cells. The integrins, αβ1 and α11β1 are the only integrins that are expressed specifically in fibroblasts and highly induced by the
stimulation mimicking fibrosis. The unusually demarcated expression must be quite favorable as the clinical integrin inhibitors. In addition, the target specificity of drugs are secured in the neutralizing mAbs. Alternatively, integrins mediating the local TGFβ activation could satisfy the conditions as the target. As multiple integrins, αvβ1, αvβ3, αvβ5, αvβ6, αvβ8 and α8β1, appear to activate TGFβ under different circumstances, a requirement for the activation in each integrin could allow pathology specific use of the inhibitors. One such expectation is in the identification of “milieu molecule” [100] [101] [20] required to exert TGFβ activation.

9.3 Combination therapy

There are a lot of diverse pathways to fibrosis, in which where cellular signals influence one another. A single blockade of the pathway in this network could be bypassed through another pathway to develop fibrosis. To evade this issue, clinical trials employing multiple drugs in combination are recently underway. This strategy may also be applied within integrin inhibitors. For example, TGFβ activation was recently found to be performed by α8β1, the panαv inhibitor may not completely block TGFβ activation as expected, depending on the condition for α8β1 to play the role. Shutting down the α8β1-mediated pathway could greatly enhance the effects of panαv to inhibit fibrosis. Apart from the pathways, temporally, as progression of clinical liver fibrosis is gradual and persistent, continuous administration of a safe drug in combination with an intermittent administration of potent drug, such as panαv.

One more conceivable effective combination is targeting the pathology-specific integrins together. The mAbs for α8β1 and α11β1 are both expected to inhibit myofibroblast differentiation [11] [12] [85] through distinct pathways. A signal mediated by α8β1 is initiated by engagement with nephronectin and α11β1 by collagens. Because the expression of these integrins is induced in activated HSCs and activated HSCs with myofibroblastic phenotype appears only in fibrotic tissue, the combination is favorable in terms of safety. Once the differentiation is blocked, fibrotic tissue is starved for myofibroblasts, and matrix proteins are no longer newly deposited. This is, therefore, an attractive application of the combination therapy in terms of effects and safety. Of course, a combination of integrin inhibitors with drugs for NASH-associated fibrosis of different mechanism, such as modulators for lipid metabolism, is also an expected option. Since combination therapy is commonly used in the cancer chemotherapy, the strategy appears to be adequate to apply to fibrotic diseases, where no effective drugs in the market.

9.4 Allosteric inhibition

A unique feature in the approved integrin inhibitors is that the target integrins are all expressed on circulating cells, i.e., platelets and leukocytes. On the contrary, target integrins of anti-fibrotic drugs are expressed in the tissue cells such as epithelial cells and fibroblasts. Importantly, mode of actions of integrin inhibitors may not be the same by cell type expressing the target. Most of circulating cell integrins are not occupied by ligands but prepared for attaching to vascular cells with the ligand binding pocket open, thorough binding to such as VCAM-1, ICAM-1 and MadCAM-1, while integrins expressed in solid tissues are largely engaged with ligands abundant in the milieu, such as fibronectin, vitronectin, tenascin-C, and nephronectin, or laminin and collagen species. In these situations, inhibitor for matrix receptor integrins must dissociate the ligand from integrins to block signal input [90,91], while those for circulating cell integrins just cover the ligand-binding pockets to perform their own tasks. Notably, the dissociation of ligands from cells is achieved by allosteric inhibition, which is clearly illustrated in the action of anti-α4 neutralizing mAb, natalizumab [102]. Allosteric inhibition occurs by binding not directly to the ligand binding pocket but to the site nearby the pocket, as the antibody clashes with the ligand occupying the pocket (Figure 7). Although this mechanism of action needs to be clarified more precisely, there are 2 types of neutralizing mAb for integrins; one detaches the cell and the other does not. If this is the case, the small molecule inhibitors that
occupy the ligand binding pocket would have no effect on integrins expressed on tissue cells. Because some of the neutralizing mAbs exhibit allosteric inhibition (Figure 6) depending on the epitope, mAb may be a prior modality to a small molecule for to really shut down signals from tissue cell integrins. Data demonstrating the requirement for mAbs to disrupt an interaction of tissue-cell integrin from the ligand are in the literature [102].

10. Concluding remarks

Inhibition of integrins attenuates fibrosis in preclinical studies [8] [9] [10] [26] [34] [35] [36] [42] [49] [50] [51] [59] [58] [103]. Integrins are closely related to leading performers of fibrogenesis, matrix proteins, fibroblasts, and TGFβ, and one of few classes of therapeutic targets of fibrosis, a critical process relating to mortality, in NASH. Because the pro-TGFβ activation in situ is so inspiring for design concept of anti-fibrotic drugs, αv integrins have been a central target for the liver fibrosis, leaving many other integrins behind. However, being no αv inhibitor drugs in the clinic, more pathology-specific drugs and targets have to be explored. There are stocks of integrins that fulfills the specificity as targets, α8β1 and α11β1. Their cell-type specific expression in fibroblasts and by far the highest upregulation in activated fibroblasts α subunits are not found in any other members of the integrin family. Both integrins in fact exhibit profibrotic properties such as myofibroblast differentiation. α8β1 activates TGFβ, α11β1 may as well [104]. Furthermore, inhibition of α8β1 and inducible genetic deletion of ligand attenuated liver fibrosis. The neutralizing mAb for each integrin readily allows further validation in vivo and in vitro. Besides the effects, the “pathology specific” induction in the activated HSCs must be a distinctive advantage to eliminate off-target adverse effects. Combination therapy with αv integrin inhibitors may be an expected option.

What is at all the role of integrins that are expressed specifically on activated HSCs? The answer must be within the biological missions of HSCs.

**Figure 7. Schematic of mode of actions for neutralizing mAb with competitive or allosteric inhibition.** In both left and right panels, a ligand binds and occupies the ligand binding pocket of an integrin, encompassing the α and β subunits of integrin. Left panel: The mAb competitive inhibitor binds to in and periphery of the ligand binding pocket. When a ligand is already binding to the pocket, the mAb does not bind or even inaccessible to the epitope. The epitope is completely covered with the ligand. Right panel: The mAb binds to integrin at the epitope that is not close as the competitive inhibitory mAb but localizes closely but around of the ligand binding pocket. The mAb is allowed to bind to the integrin while clashing with the ligand.
Figure 8. Schematic summary of the roles of the integrins on activated hepatic stellate cells in liver fibrosis. During the fibrogenesis, α8 and α11 subunits are induced on activated HSCs/myofibroblasts. In the fibrotic milieu of the tissue, increased matrix rigidity induces intranuclear translocation of YAP1 and initiates transcription of Ilga11 gene. Induced α8β1 and α11β1 both promote α-SMA expression, actin fiber formation, and cellular contractility upon ligand-engagement with such as nephronectin and collagen type I, respectively. Once HSCs acquire the myofibroblast phenotype, the cells contribute more to TGFβ activation on their surface through interactions of integrins including α8β1 and αvβ1 with pro-TGFβ in the surrounding matrix proteins. Released matured TGFβ binds to its receptor and initiates the Smad signaling cascade. The signal promotes production of matrix proteins containing collagen species from myofibroblasts and α11 expression. Crosstalk between TGFβ-initiated and α8β1- and α11β1-mediated signals cooperatively enhance α-SMA expression. These effects by α8β1 and α11β1 on HSCs consequently render myofibroblasts highly contractile and productive for collagens and other matrix proteins, which reinforces tissue stiffness to maintain and enhance α11 expression.

**Patents:** PCT/JP2010/068374 and PCT/JP2013/059368 are patents for the anti-α8β1 mAb, and PCT/JP2019/008202 is for the anti-α11β1 mAb.

**Funding:** This work was supported in part by Japanese Society for Promotion of Research KAKENHI Grant, Exploratory Research 24659367, Scientific Research (B) 26293174, and Scientific Research (B) 17H04161, by the Grant from Agency for Medical Research and Development (AMED) Japan, Translational Research Program, PreB 20334760, via Okayama University.

**Acknowledgments:** This work was in part carried out in part at the Analysis Center of Life Science and Animal Care Facility in Hiroshima University.

**Conflicts of interest:** The authors have no conflicts of interest to declare.

**References**


