α2β1 Integrin

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Abstract

The α2β1 integrin, also known as VLA-2, GPIa-IIa, CD49b, was first identified as an extracellular matrix receptor for collagens and/or laminins [55, 56]. It is now recognized that the α2β1 integrin serves as a receptor for many matrix and nonmatrix molecules [35, 79, 128]. Extensive analyses have clearly elucidated the α2 I domain structural motifs required for ligand binding, and also defined distinct conformations that lead to inactive, partially active or highly active ligand binding [3, 37, 66, 123, 136, 137, 140]. The mechanisms by which the α2β1 integrin plays a critical role in platelet function and homeostasis have been carefully defined via in vitro and in vivo experiments [76, 104, 117, 125]. Genetic and epidemiologic studies have confirmed human physiology and disease states mediated by this receptor in immunity, cancer, and development [6, 20, 21, 32, 43, 90]. The role of the α2β1 integrin in these multiple complex biologic processes will be discussed in the chapter.

Keywords

α2β1 integrin · Collagen · Disease models

3.1 Collagen Receptors-Structure and Ligand Binding

The α2β1 integrin consists of an obligate heterodimer formed from the α2 integrin subunit non-covalently associated with the β1 subunit. It is one of four 'I domain' integrins, named for the presence of a highly conserved, extracellular, (inserted) I domain, which mediates specific binding of ligands including, most prominently, collagens [30]. The α2 subunit I domain is an autonomously folding domain of approximately 220 amino acids [30]. The I domain found in the collagen receptors is shared with the alpha subunits of the leukocyte β2 integrins and is highly homologous to the A domain found in Von Willebrand factor, in cartilage matrix protein, in some collagen subtypes and in components of the complement system. The crystal

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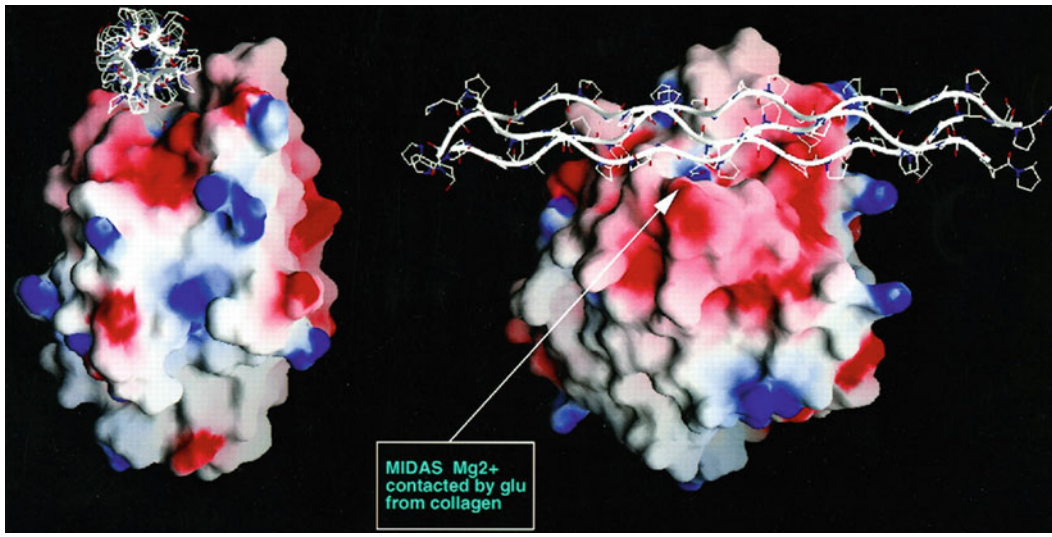

Fig. 3.1 A hypothetical model of an I-domain·collagen complex. A collagen triple helix (*white spiral*) is shown in a possible fit a groove on the MIDAS face. A glutamate side chain from the collagen coordinating the metal ion as indicated by arrow. The I domain is colored according to surface charge distribution (*blue* positive, *red* negative, *white* neutral. Two orthogonal views are shown (Reprinted from Fig. 5, Emsley et al. 1997)

structure of the α2 integrin I domain was first defined in 1997 (Fig. 3.1) [20]. The α2 subunit shares many similarities in structure and ligand binding with the other I domain integrins, including the Mg2+ dependence for binding, and enhancement of integrin function by Mn2+ [36, 60, 116, 118]. The I domain contains a conserved cation binding site, the metal ion-dependent adhesion site (MIDAS) with clear preference for Mg2+/Mn2+. The MIDAS motif is critical for collagen recognition [69].

Structural and other studies of the α2 I domain have identified an inactive or closed conformation, an intermediate or low-affinity conformation, and an active or high-affinity conformation [3, 37, 66, 123, 136, 137, 140]. Experimental approaches have characterized the role that distinct I domain residues play in receptor conformation and ligand binding capability. Mutation of the Mg2+ binding site at T221 disrupts the MIDAS site and inactivates I domain function [112, 135]. Insertion of a disulfide bridge between helices locks the I domain into a high affinity conformation [124]. Within the α2 integrin I domain, amino acid E318 forms a salt bridge with amino acid R288, thereby maintaining the α2 integrin I domain in a closed conformation. Recent reports by Carafoli et al. indicate that mutation of E318 to alanine causes disruption of this salt bridge and promotes the transition to the open, high affinity conformation which enhances α2 integrin I domain binding to low-affinity ligands [19].

Crystal structures of the active α2 I domain E318W complexed with the GFOGER peptides revealed two domains bound to a single triple helix [19], suggesting that a single GxOGER motif in the heterotrimeric collagen V or the FACIT collagen IX, may support binding of the activated integrin. Similarly, a crystal structure of the analogous E317A mutant of α1 I domain also resulted in an opening of the helices [89], and modelling of a similar peptide, GLOGEN, onto E317A [25] allows similar conclusions to be drawn for $\alpha 1\beta 1$.

The $\alpha 2\beta 1$ integrin has high affinity for collagen Type I. Evaluation of the role of the $\alpha 2\beta 1$ integrin structure and function has led to the identification of a number of novel ligands. The other ligands can be subdivided into other

collagens, non-collagenous molecules with collagen-like triple helical structures, laminin and molecules with laminin domains, proteoglycans, as well as infectious organisms, primarily viruses, and other potential non-matrix ligands.

Among collagens, the α2β1 integrin preferentially binds fibrillar isoforms (I-III, V and XI). Integrin α2β1 also recognizes the network forming collagen IV [78], the beaded-filament forming collagen VI, and the transmembrane collagen XIII when in an active, high-affinity conformation [67]. Modulation of integrin conformation by cytoplasmic signals provides an integrin-specific mechanism for adjusting ligand affinity known as 'inside-out' signaling. However, the binding of purified recombinant α2 integrin I domain to collagen type I or IV reflects the same relative affinity for the ligand as does the parent integrin; indicating that differences in the integrin-binding motifs of these isoforms most likely account for the differential recognition by the integrin [18]. The development of overlapping sets of collagen-derived peptides, termed Toolkits, facilitated systematic mapping of motifs for integrin binding and identified the collagen sequence GFOGER as the major high-affinity binding motif for the α2β1 integrin [82, 83, 112]. The GFOGER motif, found in Type I, II and XI, is uniquely able to bind platelet integrin α2β1 without prior activation [124], suggesting the ability to induce the active conformation without the inside-out signals needed for lower-affinity motifs.

More recently, other collagens were defined as α2β1 integrin ligands. Collagen XVI, a member of the fibril-associated collagens with interrupted triple helices (FACITs), binds to the α2β1 integrin, as well as to the α1β1 integrin [33]. The α2β1 integrin ligand, collagen XXIII, a transmembrane collagen, has been reported as the primary apical binding partner for the integrin in keratinocyte adhesion in the epidermis [47, 53, 141].

Many molecules of the immune system contain segments of a collagen triple helix, including C1q. As discussed below, our laboratory showed that α2β1 integrin-mediated stimulation of an innate immune response required α2β1 integrin dependent-adhesion to C1q in an immune complex [34]. The full length α2β1 integrin and the α2 integrin I domain adhere to C1q as well as to members of the collectin family of proteins, including surfactant protein A and mannose binding lectin. The α2 integrin I domain adheres to C1q in the absence of activation. However, the activated E318A mutant of α2 I domain bound to C1q with higher affinity than wild type α2 integrin I domain.

As with collagens, adhesion to laminin isoforms is mediated by the α2 integrin I domain, however laminin binding only occurs in the active, high-affinity conformation [18, 22, 36]. Isolated full-length α2 integrin subunit has been shown to bind to laminin-111 (previously laminin-1) and laminin-332 (previously laminin-5). Netrin-4, a member of the netrin family of guidance signals, demonstrates high homology to the beta 1 chain of laminins and binds to the α2β1 integrin and to the α3β1 integrin [148]. To date, an extensive and detailed molecular analysis to identify the recognition site/s on laminin has not been performed. Laminin-binding has proven to occur constitutively in some cell types, and inducibly in others. However, the role of these adhesive events is not well understood.

Perlecan, a heparin sulfate proteoglycan, and its C-terminal fragment, endorepellin, bind the α2β1 integrin [45, 46]. The terminal globular domain of endorepellin, LG3, interacts directly with the α2 I domain. This interaction has been studied in the context of angiogenesis and shown to be important for α2β1 integrin-dependent angiogenesis.

Decorin, another small leucine-rich proteoglycan modulates α2β1 integrin matrix interactions by playing an important role in regulating extracellular matrix assembly as well as directly interacting with the integrin [13, 40, 52, 143]. Decorin binding to collagen has been shown to affect fibril formation by initially delaying lateral fibril growth and reducing average fibril diameter [142]. Additionally, decorin interacts with α2β1, but not α1β1 integrin, at a site distinct from the collagen-binding domain. Adhesive

interaction between decorin and the $\alpha2\beta1$ integrin was first identified in platelets, and later discovered to be important in angiogenesis.

Single nucleotide polymorphisms in the integrin $\alpha2$ gene, as discussed later in more detail, have an important role in the predisposition of patients to cardiovascular disease. One such minor allele difference (rs1801106; G1600A) has now been shown to attenuate adhesion of platelets to decorin but not to collagen and is associated with increased risk for recurrence of stroke [87]. The non-conservative amino acid substitution E534K, is the basis of the human platelet alloantigen system HPA-5, providing the first evidence of a functional effect of HPA-5 alleles.

The $\alpha2\beta1$ integrin serves as a receptor for many different infectious organisms. In many cases the organisms usurp $\alpha2\beta1$ integrin's routine biology for attachment, cell entry and transmission throughout the body. The best studied interaction of $\alpha2\beta1$ integrin is with echovirus (EV1) [10–12, 31]. EV1, is a human RNA virus which binds directly to the I domain of human $\alpha2\beta1$ integrin. Unlike most viruses that exploit integrin receptors, EV1 does not undergo clathrin-mediated endocytosis, but instead clusters on caveosomes and is internalized via a clathrin- and caveolin-independent macropinocytosis-like mechanism [73, 93]. Additionally, EV1 binding has been demonstrated to activate PKCα, while inhibition of PKCα signaling blocks EV1 internalization [138]. Interestingly, EV1, unlike other $\alpha2\beta1$ integrin ligands, preferentially binds the inactive, closed conformation of the integrin over the active, high affinity conformation [68].

Not only do infectious organisms utilize the integrin as a receptor, lectins that recognize high mannose glycans on viruses are produced from bacteria, algae, plants and animals and bind the $\alpha2\beta1$ integrin. A recently characterized anti-HIV lectin from Pseudomonas fluorescens Pf0-1 exhibited potent antiviral activity against influenza [121]. The lectin induced loss of cell adhesion and viral death that was dependent on binding to the $\alpha2\beta1$ integrin. Following lectin binding to the $\alpha2\beta1$ integrin, the complex was internalized to the perinuclear region and not recycled. The process resembled that described for echovirus mediated cell entry and death.

3.2 Signaling

The $\alpha2\beta1$ integrin plays a unique contribution in regulating cell migration, proliferation and survival. The $\alpha2$, but not the $\alpha1$, integrin cytoplasmic domain mediates p38 MAP kinase pathway activation and a migratory phenotype [80, 81]. Expression of the constitutively active small G protein Rac1 augmented p38 MAP kinase phosphorylation and migration in mammary epithelial cell expressing full length $\alpha2$ subunit. The role of the $\alpha2$-cytoplasmic domain in activation of the p38 MAP kinase pathway was also established in fibroblasts. Fibroblasts grown in three-dimensional collagen gels require the $\alpha2$-cytoplasmic domain for p38 MAP kinase activation that leads to $\alpha2\beta1$ integrin-mediated upregulation of collagen gene expression [62]. Together these results support an important and specific role for the $\alpha2$-cytoplasmic domain in mediating p38 MAP kinase activation. Similarly, the cytoplasmic domain of the $\alpha2$ integrin subunit specifically supports insulin-mediated S-phase entry [81]. The $\alpha2$, but not the $\alpha1$, cytoplasmic domain mediated activation of the cyclin E/cdk2 complex, which allows entry into S-phase in the absence of growth factors other than insulin. These results suggest that the $\alpha2$ integrin cytoplasmic domain and the insulin receptor synergize to regulate cell cycle progression.

More recently, Ivaska et al. suggested that the $\alpha2\beta1$ integrin induced protein serine/threonine phosphatase 2A (PP2A) activity in a collagen-specific manner [63]. In their studies, collagen-induced PP2A activation and resulting dephosphorylation of Akt and glycogen synthase kinase 3β (GSK3β) in Saos-2 cells was $\alpha2\beta1$ integrin-dependent. PP2A is a master regulator of a diverse set of cellular signaling pathways, so its interaction with $\alpha2\beta1$ integrin has the potential to dramatically increase the scope of the signaling activities of the integrin. Careful investigation of these putative signaling

mechanisms is necessary for a clearer understanding of the role for the integrin in various cell types.

3.3 The α2β1 Integrin: Expression and Function

In addition to differences in collagen recognition, expression of the integrin is dependent on cell type and stage of differentiation. The α2β1 integrin is primarily expressed in vivo by epithelial cells, platelets/megakaryocytes, and fibroblasts [146]. In addition, α2β1 integrin expression on T-cells and endothelial cells varies depending on differentiation and the state of activation [29, 55, 56, 144]. The roles and functions of the integrin are therefore highly dependent not only on cell type but on signals from other cells and the associated microenvironment.

The majority of earlier work defined the role and function of the α2β1 integrin by studies of human platelets and in vitro models. These early studies implicated the α2β1 integrin in a wide range of biologic and pathobiologic functions including platelet adhesion required for hemostasis and thrombosis, epithelial differentiation and branching morphogenesis, tumor biology, wound healing, angiogenesis, and inflammation and immunity. Much has been learned over the last 10 years since development of state of the art inhibitory antibodies and gene silencing approaches, novel in vitro culture systems, and new animal models including the global α2 integrin-subunit deficient and the more recent tissue-specific α2 integrin-subunit deficient mouse. These studies and their impact on our understanding of the integrin in human biology and disease will be reviewed.

3.4 Platelet α2β1 Integrin in Ligand Binding

Patient studies first established the link between α2β1 integrin and platelet function. In 1985 Nieuwenhuis identified a deficiency of platelet glycoprotein 1a (α2 subunit) in a patient with abnormal bleeding [106, 107]. Later other patients with either reduced levels of platelet expression of the α2β1 integrin or the presence of autoantibodies to the integrin were also described to exhibit impaired platelet activation by collagen but not by other agonists.

Studies using purified human platelets established the α2β1 integrin-dependent adhesion to collagens I-VIII in a Mg2+-dependent manner. Although the α2β1 integrin is expressed at relatively low copy number on platelets (2000–4000 copies per platelet), the integrin is required for firm attachment of platelets to collagen in the subendothelium after vascular injury [56, 85, 118]. Experiments with purified platelets from genetically modified α2-deficient mice confirmed these results. Platelets from α2-deficient animals fail to adhere to type I collagen under both static and flow conditions [24]. Platelets from animals heterozygous for the α2-null allele adhere to type I collagen to a lesser degree than platelets from wild type animals, consistent with a gene dosage effect.

Platelets however have not one, but two major collagen receptors: the high affinity α2β1 integrin and the lower affinity glycoprotein VI (GPVI)/Fc receptor γ-chain (FcRγ) complex [65, 102, 105]. Despite the significant evidence supporting the role of α2β1 integrin in platelet adhesion to collagen, the relative contribution and precise roles of α2β1 integrin and GPVI/FcRγ in collagen-induced platelet adhesion and activation is still a focus on experimental inquiry. The Santoro group originally proposed a two-step, two-site model of platelet adhesion and activation to collagen, in which the higher affinity α2β1 integrin supports the initial rapid platelet-collagen interaction that mediates platelet adhesion to vessel wall under conditions of flow [103, 116, 118, 128, 134]. This allowed the subsequent engagement of a lower affinity, signal-transducing co-receptor GPVI to bind collagen and mediate collagen-induced platelet activation and aggregation. GPVI, a member of the immunoglobulin superfamily noncovalently and constitutively associates with the FcRγ chain to form a multimeric signaling complex. In this model, the α2β1 integrin mediates strong

adhesion but does not contribute to platelet activation.

Other work raised question about the two-step, two-site model. Studies using a variety of agonists and inhibitors, defined the contributions and mechanisms leading to conformational changes resulting from integrin activation and provided evidence that the $\alpha 2\beta 1$ integrin can mediate GPVI-independent, collagen-induced platelet activation [59, 70, 75, 131]. Collagen-induced phosphorylation of PLC$\gamma 2$ and Syk was inhibited by antibodies that block $\alpha 2\beta 1$ integrin adhesion to collagen or by selective proteases that cleave the $\beta 1$ integrin subunit of the $\alpha 2\beta 1$ integrin. In other studies collagen-induced phosphorylation of c-Src was mediated by the $\alpha 2\beta 1$ integrin [61]. Platelet adhesion to intact collagen stimulated a different response than adhesion to GPVI-mimetics, further supporting distinct signaling from the $\alpha 2\beta 1$ integrin and GPVI/FcRγ [57, 70].

New work attempted to reconcile these conflicting stories. Auger et al. used flourescence video microscopy to monitor increases in intracellular free Ca2+ concentration ([Ca2+]i), an early stage in GPVI/FcRγ-mediated platelet activation, upon platelet adhesion to collagen under flow conditions [5]. In both human and mouse platelets under flow conditions, they identified a population of platelets that displayed an immediate increase in [Ca2 +]i upon collagen contact, as well as a second population of platelets that exhibited a delayed increase in [Ca2 +]i (1–30 s after adhering to collagen). The first population was unaffected by anti-$\alpha 2\beta 1$ integrin antibody blockade suggesting a GPVI/FcRγ-centric mechanism for both adhesion and activation as suggested by Nieswandt et al. The second population conformed to the traditional two-step model. The authors speculated that the apparently heterogeneous mechanism would allow for optimal response to different types of vascular injury. A similar study by Mazzucato et al. used inhibitory antibody-treated human platelets as well as mouse platelets from null animals to link short-lasting α-like and long-lasting γ-like [Ca2+]i oscillation peaks to $\alpha 2\beta 1$ integrin and GPVI signaling, respectively [97]. Interestingly, they found that $\alpha 2\beta 1$ integrin-mediated α-like calcium oscillations occur even in GPVI-null backgrounds indicating that inside-out priming of the integrin may also come from non-GPVI sources. Indeed Majoram et al. reported a role for platelet GPCRs, including protease activated receptor 1 and 4 (PAR1 and PAR4), in PLC-mediated $\alpha 2\beta 1$ integrin activation [94].

Together these studies demonstrated greater synergy between $\alpha 2\beta 1$ integrin and GPVI/FcRγ in mediating these processes than was previously understood. Resting platelets express the integrin in a low-affinity conformation. Activation, downstream of activation of GPVI, PAR1 or PAR4, or another pathway, leads to a conformational change to a high-affinity state which enhances adhesion to Type I collagen and promotes a more permissive binding to other ligands including Type IV collagen and laminin.

3.5 The $\alpha 2\beta 1$ Integrin: Genetic Risk for Hemostasis and Thrombosis and Much More

There is substantial variation in the baseline expression of $\alpha 2\beta 1$ integrin in the population; quantitative measurements of platelet surface membrane $\alpha 2\beta 1$ integrin expression indicate as much as a 10 fold difference among normal patients [64]. The mechanism of genetic regulation of the gene encoding the $\alpha 2$ integrin subunit has been best delineated. The variation is genetically determined and associated with three alleles of the $\alpha 2$ integrin subunit gene, *ITGA2* [84, 86]. The three alleles have been defined by 8 nucleotide polymorphisms in the coding region of *ITGA2* gene at nucleotide 807(C or T) and 873(G or A). Individuals carrying the 807T/873A allele express high levels of platelet $\alpha 2\beta 1$ integrin, whereas individuals carrying the 807C/873G allele exhibit low levels of $\alpha 2\beta 1$ integrin expression. Cheli et al. described another variant in CA repeat length in the *ITGA2* gene promoter that demonstrated linkage disequilibrium with variants in the coding region

[23]. Expression of α2β1 integrin may be similarly regulated in other cell types.

Genetic regulation of α2β1 integrin expression has meaningful biological implications, which have been most widely appreciated in the area of hemostasis and thrombosis. Kunicki et al. reported functional significance of α2β1 integrin expression levels by demonstrating that the number of α2β1 integrin molecules per platelet correlated with the ability of platelets to adhere to Type I collagen [85]. Clinical and epidemiologic studies based on genetic polymorphism analysis demonstrated direct clinical significance of allelic differences in levels of α2β1 integrin expression. The alleles associated with high levels of α2β1 integrin expression were associated with nonfatal myocardial infarction in individuals less than a mean age of 62 years, with an increased risk of developing diabetic retinopathy in patients with Type II diabetes mellitus, and with an increased risk of stroke [95, 119].

The original assumption was that increased integrin expression led to increased platelet adhesion to collagen and subsequent risk of thrombosis. Recently an alternative mechanism for the association was suggested. The level of α2β1 integrin expression correlated with mean platelet volume in humans and during megakaryocyte differentiation and proplatelet formation in mice [88, 126]. Surprisingly, platelet specific deletion of the integrin using the platelet factor 4 promoter-Cre construct and mice with a floxed ITGA2 gene demonstrated that mice lacking platelet-specific α2β1 integrin showed decreased megakaryocyte differentiation, diminished proplatelet formation and decreased mean platelet volume [49]. Since mice with global deletion of *ITGA2* failed to show altered megakaryocytic/platelet differentiation, compensation by alternative integrins, cell types, or pathways was sufficient to prevent this additional phenotype. Epidemiologic data linking levels of the α2β1 integrin expression with risk of pathologic thrombosis and other cardiovascular complications underscore the importance of further clarifying the role for α2β1 in platelet function.

3.6 The α2β1 Integrin During Wound Healing and Fibrosis

Early in vitro studies suggested that the α2β1 integrin was required for wound healing. Studies using skin explants ex vivo showed that keratinocyte-specific α2β1 integrin expression was re-oriented from the basal cell area to the forward-basal aspect of migrating keratinocytes where the integrin is in contact with type I collagen [114]. Keratinocyte migration into the wound was inhibited by antibodies against the α2β1 integrin [110].

In the late phase of wound healing after reepithelialization, tissue contraction of collagen fibers results in a strengthened scar. The scar is the result of extensive fibrosis, a process of tissue replacement by dense extracellular matrix composed of abundant collagen I. The α2β1 and the α1β1 integrins, both expressed by fibroblasts, are key regulators of collagen turnover in the skin, and other organs including the kidney [58, 62]. After binding to collagen, the α1β1 integrin activates a pathway that down-regulates collagen synthesis. In contrast, activation of the α2β1 integrin promotes collagen synthesis [99]. The alignment of the collagen fibers that occurs in healing wounds is recapitulated in three-dimensional collagen gels. The in vitro models provided evidence supporting critical roles for the α2β1 integrin wound healing and fibrosis.

Surprisingly, despite the results of in vitro and explant studies of wound healing, α2-deficient mice demonstrated no defect or delay in wound repair compared to wild-type animals [47, 152]. The morphology of the wounds also failed to demonstrate any difference in keratinocyte migration over exposed dermis at the wound site, suggesting that α2β1 integrin does not play an obligatory role in wound healing. No differences in scar formation or strength were noted.

Differences between the in vitro experiments and α2-null mouse model systems have several possible explanations. First, human and genetically altered mouse models may not be mechanistically equivalent. Acute loss-of-function as observed with use of inhibitory antibodies may

have different effects than the germ-line deletion of α2β1. In addition, antibodies that inhibit integrin binding may produce 'negative signaling' which is distinct from the absence of integrin signaling in the null context.

Interestingly, Zweers et al. and Grenache et al. both reported increased neoangiogenesis in the wound microenvironment of α2-null mice, providing in vivo evidence for an anti-angiogenic role for α2β1 integrin [47, 152]. The increased angiogenesis in the wound healing model was quite surprising. Many studies have focused on understanding the role of the integrin in vascular development and angiogenesis, as discussed below.

Fibrosis also occurs in other tissues; the involvement of α2β1 integrin is particularly well studied in the kidney [16]. Glomerulosclerosis, characterized by excessive collagen deposition in the glomerulus is the most common cause of end stage kidney disease. The specific role of α2β1 integrin in regulating glomerulosclerosis is somewhat controversial. Mesangial cells and podocytes express the α2β1 integrin. One report studying α2-null mice on the C57Bl/6 background suggested that the integrin protected from glomerular injury [44]. In contrast, a study in which α2-null mice were crossed with the COL4A3-null mice, a model of Alport disease demonstrated that α2β1 integrin expression exacerbates glomerular injury, decreased survival, and reduced glomerular matrix deposition and scarring [48].

Consistent with a role for the integrin in promoting collagen synthesis, Miller et al. showed that inhibition of integrin α2β1, using a high-affinity small-molecular weight inhibitor protects mice from glomerular injury [100]. The anti-α2β1 inhibitor also reduced collagen synthesis in wild type but not α2-null mesangial cells, consistent with the α2β1 integrin-dependence of its antifibrotic effect.

In contrast to the kidney, the α2β1 integrin appears to have an anti-fibrotic role in the lung. Xia et al. reported that in idiopathic pulmonary fibrosis (IPF), reduced fibroblast α2β1 integrin levels allowed escape from anti-proliferative signals that normally limit fibroproliferation after tissue injury [147]. Fibroblastic foci in IPF patients were shown to be characterized by low fibroblast α2β1 integrin expression. IPF fibroblasts demonstrated decreased α2β1 integrin-mediated PP2A phosphatase activity. Downstream increases in activity of GSK-3β and β catenin provided the proliferative signals that mark the pathological IPF fibroblast phenotype. Although this work provided an elegant model for how α2β1 integrin downregulation may contribute to the pathogenesis of IPF; the relevant mechanisms for α2β1 integrin loss remain uninvestigated. Additionally, it is unclear how the established role for α2β1 integrin in promoting collagen biosynthesis and ROS production may be involved. Are the disparate elements of α2β1 integrin function somehow context or tissue-specific? Reconciliation of the pro-fibrotic and anti-fibrotic properties of the α2β1 integrin demands further study in light of its potential clinical relevance.

3.7 The α2β1 Integrin and Angiogenesis/ Vasculogenesis

Angiogenesis is coordinated by a complex interplay between endothelial cells and their microenvironment. During VEGF-induced angiogenesis in vivo expression of α2β1 integrin is up-regulated and α2β1 integrin expression has been observed on the sprouting tips of neonatal blood vessels [38, 122]. Together these results suggested an important function for α2β1 in angiogenesis, however the precise nature of the integrin's role is still incompletely understood.

The earliest investigations into the functional role of α2β1 in angiogenesis employed inhibitory antibodies during in vitro studies. Early reports from Gamble et al. indicated that anti-α2β1 antibodies inhibited endothelial cell proliferation on collagen [41]. Soon after, Davis reported that anti-α2 inhibited lumen and tube formation by HUVECs in a 3D collagen matrix [28]. Later studies using planar type I collagen gel angiogenesis assays, confirmed that inhibition of α2β1 integrins with function blocking

antibodies disrupted tube formation [132]. Senger et al. demonstrated in vivo using subcutaneous matrigel plug angiogenesis assays in mice, that inhibition of α2β1 and α1β1 in combination decreased new vessel growth in the implanted plugs. Together these results suggested a pro-angiogenic function for the α2β1 integrin [122].

Studies from α2-deficient mice have yielded contradictory results. Several labs, including our own, reported not only normal developmental angiogenesis, but also increased neoangiogenesis during wound healing in genetically-altered α2β1 integrin-null mice [47, 149]. Similarly, our lab demonstrated that α2β1 integrin-deletion increased tumor angiogenesis in a growth factor-dependent manner via modulation of VEGFR-1 signaling. Additionally studies in the diet-induced obesity model also showed increased angiogenesis in α2-null mice compared to wild type mice [71]. The contradiction between the evidence for pro and anti-angiogenic functions for α2β1 integrin are not totally based of differences in mouse and human endothelial cells or in vivo compared to in vitro models. Cailleteau et al. used an α2 siRNA approach to alter integrin expression in HUVECs. These studies showed that α2β1 integrin engagement by laminin promoted endothelial cell cycle arrest and quiescence [17]. Additionally, α2β1 integrin binding to endorepellin in both human and mouse endothelial cells mediated the angiostatic effects [14, 46, 145].

Based on these inhibitory studies pharmacological inhibitors of α2β1 may have potential anti-angiogenic drug effects (see therapy section). Small molecule inhibitors (SMI) of α2β1 blocked both endothelial tube-formation in vitro and sprouting angiogenesis in zebrafish [115]. A more thorough understanding of the role for α2β1 in angiogenesis promises novel insight into clinical application of α2β1 integrin targeting compounds. Recent studies implicating the α2β1 integrin in notch signaling offer an alternative paradigm for understanding α2β1 integrin in angiogenesis [17, 39, 129]. The notch pathway coordinates sprouting angiogenesis by organizing endothelial cells into migratory 'tip' and proliferative 'stalk' cell conformations with differential capacity to respond to VEGF stimulation [54, 109]. Estrach et al. reported that α2β1-mediated laminin signaling is necessary but not sufficient for induction of the tip cell determinant, Dll4 [39]. Clarifying the functional relationship between α2β1 integrin and notch signaling in the endothelium is a promising avenue of future study.

3.8 The α2β1 Integrin in the Innate and Acquired Immune Response

The α2β1 integrin was initially identified as an integrin expressed at very late stages of T cell activation, thus the designation very late activation antigen-2 (VLA-2)(CD49b) [55, 56]. The α2β1 integrin was then noted on a variety of cells of the inflammatory and hematopoietic system, including activated T cells, but not naïve T cells in chronic inflammatory settings. Early studies showed that α2β1-dependent adhesion to collagen enhanced T cell receptor mediated T cell proliferation and cytokine secretion [120]. Boisvert et al. defined one possible mechanism; they reported that collagen I-stimulated, α2β1 integrin-mediated both activation-independent and T cell receptor-dependent interferon γ expression via the ERK and JNK MAPKs and PI3K/AKT signaling pathways [15].

The α2β1 integrin also influenced T cell activation by inhibiting fas ligand expression and apoptosis in effector T cells in a collagen I dependent manner [2, 42]. In animals, inhibitory monoclonal antibodies directed against the α2β1 integrin significantly inhibited the effector phase of both contact and delayed type hypersensitivity. These early results established a role for the α2β1 integrin in T cell mediated function. The role of the α2β1 integrin in the innate and acquired immune response has been an area of active investigation.

To better the define the role of the α2β1 integrin in T cell function, expression of the α2β1 integrin on T cell subsets and in response to antigenic challenges was investigated. Kassiotis et al. reported that expression of α2β1 integrin

defined two functionally distinct subsets of memory T cells that played a role in the response to infection and immunization [74]. α2β1 integrin expression was stably induced by antigen on approximately 50 % of memory T cells with helper function and stimulated production of tumor necrosis factor-α. The α2β1 integrin expressing, CD49b+, memory Th cells demonstrated enhanced ability to mediate macrophage activation and to kill of intracellular bacteria.

Sasaki et al. demonstrated that mature Th1 and Th2 cells exhibited distinct α2β1 integrin expression profiles [120]. Although naive Th cells did not express α2β1 integrin, Th1 cells acquired high levels of α2β1 integrin expression during maturation in an interferon-γ (IFN-γ) and interleukin (IL)-12-independent manner. This study suggested that high level α2β1 integrin expression on Th1, but not Th2, cells was functionally important, because stimulation of Th1 or Th2 cells with α2β1 integrin ligands caused selective activation of Th1 cells to produce interferon-γ after long-term culture.

Richter et al. studied α2β1 integrin expression during influenza infection in the lung [113]. During the acute phase of infection, the α2β1 integrin was expressed by a significant proportion of both CD4+ and CD8+ T cells in the lung; however, the integrin was expressed less frequently on memory cells, particularly CD8+ T cells. A similar expression pattern for the α2β1 integrin in the spleen was found in a model of lymphocytic choriomeningitis viral infection [1]. The data suggested that α2β1 integrin expression directed localization of CD4+ and CD8+ T cell subsets within the lung and promoted T cell migration within extralymphoid spaces, particularly during the acute phase of infection.

A role for α2β1 integrin expression by Th17 cells has been described. Boisvert et al. showed that human naïve CD4 T cells stimulated toward Th17 polarization preferentially upregulate α2β1 integrin [15]. Th17 cells adhered to collagens I and II, but not IVin an α2β1 integrin-dependent manner. α2β1 integrin-dependent adhesion combined with anti-CD3 antibody co-stimulated the production of IL-17A, IL-17F and IFN-γ by human Th17 cells.

The importance of α2β1 integrin to T cell memory has remained controversial. Work by several groups suggested that professional memory CD4 cells reside and rest in the bone marrow. Recently, Hanazawa et al demonstrated that memory CD4 cells expressed high levels of α2β1 integrin and that antibody-mediated inhibition of α2β1 integrin of memory CD4 cell precursors caused failure to transmigrate from blood through sinusoidal endothelial cells into the bone marrow [50]. These results suggested that the α2β1 integrin was required for the migration of memory CD4 cell precursors into their survival niches of the bone marrow.

In addition to its expression on activated T cells, the α2β1 integrin is expressed at high levels on almost all NK cells and mast cells, and on subpopulations of monocytes and neutrophils [4, 133]. Arase et al. identified the NK cell recognition epitope of the widely used DX5 pan-NK cell monoclonal antibody as CD49b or the α2β1 integrin. These investigators demonstrated that α2β1-expressing and nonexpressing subsets of NK cells are present in the mouse spleen and raised the possibility that α2β1 integrin expression is important in NK cell function. The role of the α2β1 integrin on subsets of neutrophils and monocytes has also been studied. One study found expression of the α2β1 integrin on extravasated neutrophils in human skin blister chambers and in the rat peritoneal cavity following chemotactic stimulation [144]. These studies, as well as others, suggested that the α2β1 integrin on neutrophils is involved in neutrophil migration from the vasculature into extravascular tissue in response to cytokine induction.

Work from our lab has clarified the function of the α2β1 integrin in mast call activation. We initially observed decreased inflammatory responses to Listeria monocytogenes in α2-null mice [34]. This innate immunity defect was determined to arise from a requirement for α2β1 integrin activation on peritoneal mast cells (PMCs) for mast-cell activation and cytokine release in vivo. We also identified C1q complement protein and collectin family members, including mannose binding lectin and surfactant protein A, as novel ligands for the integrin in

mast cell activation in vitro in response to Listeria. Since ligation of the α2β1 integrin alone was insufficient to activate cytokine secretion, we hypothesized that an additional signal emanating from a co-receptor was required to activate mast-cell cytokine secretion. We identified the required co-receptor as hepatocyte growth factor (HGF-R)/c-met [98]. We demonstrated that Listeria induced mast cell activation and cytokine secretion requires costimulatory signals from α2β1 integrin ligation to either type I collagen or C1q as well as c-met activation. The synergistic signal from the two coreceptors resulted in mast cell release of the proinflammatory cytokine IL-6 to trigger the early innate immune response.

3.9 α2β1 in Epithelial Biology

The α2β1 integrin is expressed at high levels on numerous epithelial cells including not only the squamous epithelium, but also ciliated columnar epithelium of the respiratory tract, the epithelial cells of the gastrointestinal tract and urinary tract, and the glandular epithelium of the breast [24]. In contrast to the high α2β1 integrin expression in the normal breast epithelium, markedly reduced or undetectable levels of α2β1 integrin were seen in poorly-differentiated carcinomas. Expression of α2β1-integrin was diminished or lost in a manner that correlated with a loss of epithelial differentiation and tumor progression in mammary carcinoma as well as other adenocarcinomas, including those of the prostate, lung, pancreas, and skin.

Our group's early studies focused on understanding the correlation between α2β1 integrin expression and a differentiated epithelial phenotype and conversely, whether dysregulated α2β1 integrin expression contributed to the malignant behavior of cancer cells. Gain of function and loss of function models in vitro suggested that α2β1 integrin expression contributed to the differentiated epithelial phenotype and branching morphogenesis of mammary and other epithelial cells [130, 150, 151]. These observations were supported by findings from other laboratories. Using a primary human nonmalignant, but immortalized, mammary epithelial cell line, Berdichevsky et al. and D'Souza et al. demonstrated that branching morphogenesis can be blocked by inhibitory monoclonal antibodies directed against the α2 integrin subunit or by altered α2β1-integrin expression mediated by the expression of the c-erbB2 proto-oncogene, respectively [9, 26, 27].

The development of genetically engineered mice with global deletion of *ITGA2* permitted further analysis of the role for α2β1 integrin in vivo. The major changes in branching morphogenesis in vitro were not fully recapitulated in vivo. The α2-null mice have only modest defects in mammary morphology. The in vitro experiments were designed to study a single integrin interaction on epithelial cells with only a small number of matrix molecules. Mammary gland in vivo consists of epithelial cells, fibroblasts, endothelial cells, and immune cells embedded in a complex matrix. The complexity in in vivo systems and compensatory mechanisms may both mitigate the consequences of α2β1 integrin-deficiency.

3.10 The α2β1 integrin Plays a Role in Cancer Progression

Interest in α2β1 integrin in breast cancer began with the observation of a strong correlation between diminished α2β1 integrin expression and a less differentiated phenotype. The α2β1 integrin–deficient mouse model provided our laboratory the opportunity to investigate a role for integrin in the development and progression of breast cancer in vivo. Our group demonstrated that in the spontaneous MMTV-neu mouse model of breast cancer, α2β1 integrin-deletion did not significantly alter the incidence of tumor development or tumor growth, but markedly increased hematogenous metastasis [111]. Increased metastasis in this model resulted in part from increased capacity for cancer cell intravasation.

Detailed in silico examination of publically available data from breast cancer patients supported this finding; expression of the α2 integrin

subunit, but not $\alpha 1$ or $\beta 1$ integrin subunits, was a prognostic indicator of decreased metastasis and better patient outcomes (Fig. 3.2). Similarly, retrospective analysis of lymph node-negative patients from the Wang cohort who relapsed with metastatic disease, revealed an inverse correlation between $\alpha 2\beta 1$ integrin expression and the occurrence of brain lesions; patients with greater than twice the average $\alpha 2\beta 1$ integrin expression suffered no brain metastasis whereas all nearly one third of all other patients suffered brain metastasis ($P = 0.0049$).

Expression of the $\alpha 2\beta 1$ integrin in prostate cancer was also predictive of metastasis and survival. The mouse and human studies supported the in vitro experimental analyses and the reported epidemiologic linkage between the single nucleotide polymorphisms regulating $\alpha 2\beta 1$ integrin expression and poor prognosis in patients with breast cancer [90]. Together these data suggested that $\alpha 2\beta 1$ integrin is a valuable biomarker for risk of metastasis in breast cancer.

Our data clearly showed in an animal model of breast cancer and human breast and prostate cancer that the integrin behaved as a metastasis suppressor. Data from other laboratories suggest that $\alpha 2\beta 1$ integrin's role in prostate and perhaps other cancers may be more complicated. In vitro, $\alpha 2\beta 1$ integrin was required but not sufficient for survival and metastasis of LNCaP prostate cancer cells to bone [91]. $\alpha 2\beta 1$ integrin protein and mRNA expression was enhanced in bone metastases to the level observed in normal, nonmalignant prostate tissue and significantly higher than primary prostate cancer lesions or metastasis to other sites such as lymph nodes [127]. Similarly, $\alpha 2\beta 1$ integrin expression accelerated experimental metastasis or tumor dissemination of melanoma and rhabdomyosarcoma or melanoma, gastric and colon cancer, respectively [7, 8, 51, 92, 96, 139].

Therefore, despite this progress several important questions remain concerning the role of the $\alpha 2\beta 1$ integrin in cancer biology. What is the precise molecular mechanism through which $\alpha 2\beta 1$ integrin loss enables increased intravasation? How does integrin down-regulation during breast cancer progression occur? Many other cancers including prostate, colon and lung cancer also appear to have $\alpha 2\beta 1$ integrin loss associated with cancer progression and metastasis. However, some cancers are associated with high $\alpha 2\beta 1$ integrin expression levels. Answers to each of these questions will provide novel insight into tumor biology, as well as suggesting new avenues for clinical application of the $\alpha 2\beta 1$ integrin as a biomarker or therapeutic target.

3.11 Therapies

Over the past several years there has been increased interest in pharmacological targeting of the $\alpha 2\beta 1$ integrin for treatment of thrombosis and angiogenesis [72]. The $\alpha 2\beta 1$ integrin is viewed as a safe target because although overexpression was associated with pathological clot formations, mice with integrin deletion lack severe bleeding defects, and inhibition causes only minimal increases in bleeding time. Compound 15, a nonpeptide inhibitor of the integrin, has been demonstrated to block platelet adhesion to collagen I under both static and flow conditions [16]. The inhibitor was originally designed to inhibit $\alpha 2\beta 1$ on platelets by locking the integrin $\alpha 2\beta 1$ in the inactive low-affinity conformation [100]. Additionally, in vivo, the compound inhibited thrombus formation in a mouse model and inhibited angiogenesis in a zebrafish model. Other $\alpha 2\beta 1$ inhibitors have shown similar effects; BTT-3016, a sulfonamide derivative prevented platelet aggregation and reduced thrombus formation in a vascular injury model [108]. Another sulfonamide derivative that targets $\alpha 2\beta 1$, E7820, is currently in phase II clinical trials as an adjuvant therapy for metastatic colon cancer [77, 101]. The clinical impact of pharmacological targeting the $\alpha 2\beta 1$ integrin will require further time and experimentation.

3.12 Summary and New Directions

It is increasingly clear that the $\alpha 2\beta 1$ integrin plays a nuanced but important role in critical cell functions in many different cell types. Several

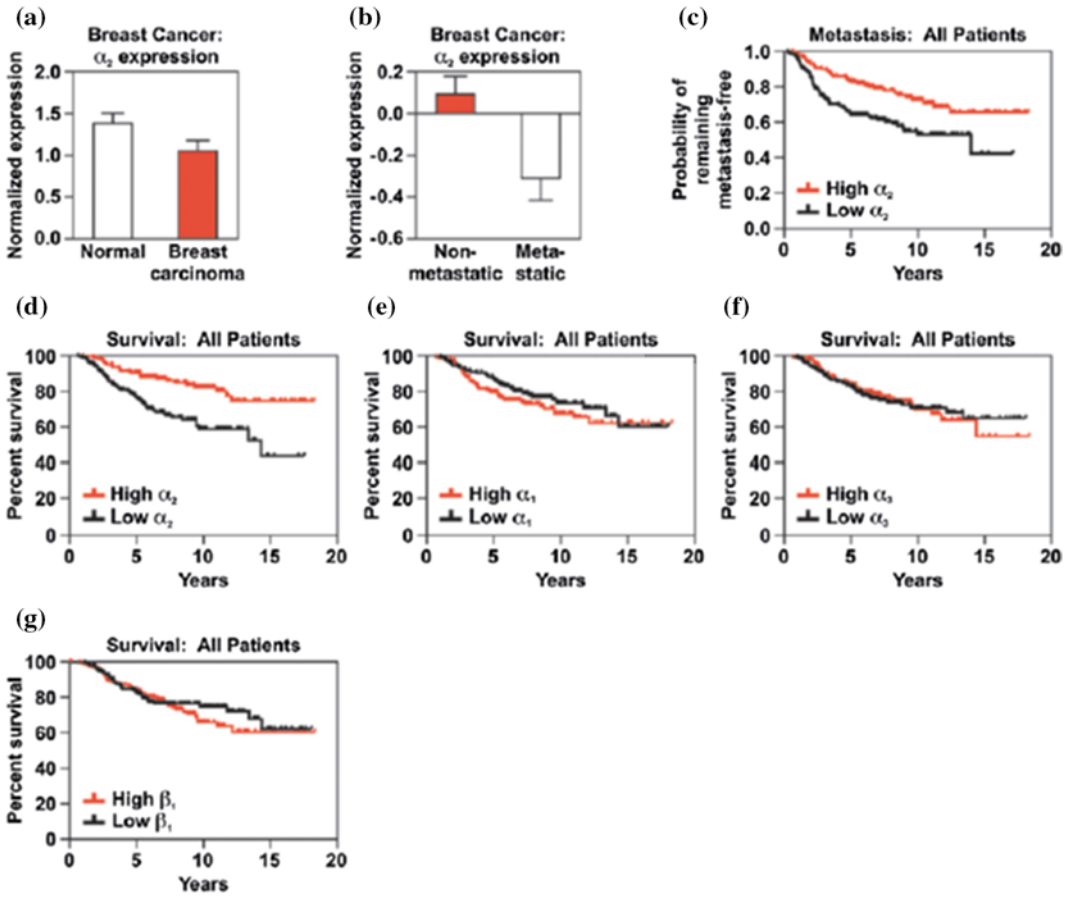

Fig. 3.2 Decreased α2β1 integrin mRNA expression predicts metastasis and decreased survival in breast cancer patients. **a** Expression of the α2 integrin was significantly decreased in breast carcinomas (n = 40) compared with normal breast tissue (n = 7) (P = 0.038). (**b–g**) Analysis of the NKI-295 cohort correlates expression of α2 integrin, but not other integrins, with metastasis (**b** and **c**) and patient survival (**d–g**). The α2 integrin expression was substantially reduced in patients with metastases (n = 101) when compared with non-metastatic patients (n = 194, P = 0.0038) (**b**). Log-rank analysis demonstrates that high-level α2 integrin expression correlates with the probability of both remaining metastasis-free (**c**, P = 0.0022) and with improved long-term survival (**d**, P < 0.0001). In contrast, expression of the α1 (**e**, P = 0.2639), α3 (**f**, P = 0.9509), and β1 (**g**, P = 0.5) integrin subunits did not correlate with patient survival (Reprinted from Fig. 6, [111])

new studies have suggested previously undocumented roles for the integrin in diseases ranging from type 2 diabetes, to dwarfism. In platelets, the combination of animal and in vitro studies have slowly revealed a more nuanced yet equally important role for the integrin than had previously been imagined. The recent development of tissue-specific α2-null mice promises to bring similar clarity and complexity to our understanding of α2β1 integrin function in inflammation, angiogenesis and tumor biology in the years ahead.

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