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Authors
Shai, Shaw-Yung
Harpf, Alice E
Ross, Robert S

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INTEGRINS AND THE MYOCARDIUM

Shaw-Yung Shai[1,2], Alice E. Harpf[1,2] and Robert S. Ross[1,3]

[1]Departments of Physiology, Medicine, and The Cardiovascular Research Laboratories
UCLA School of Medicine
Los Angeles, CA 90095

INTRODUCTION

Cells from both vertebrate and invertebrate species share the ability to adhere to extracellular matrices (ECM). ECM provides cells with a structural, chemical and mechanical substrate that is essential in tissue development, body growth and responses to pathophysiological signals. A group of glycoprotein transmembrane receptors termed integrins are the primary link between extracellular matrix ligands and both cytoskeletal structures and intracellular signaling mechanisms (1-4). Integrins orchestrate multiple functions in the intact organism including organogenesis, regulation of gene expression, cell proliferation, differentiation, migration and death. They are a complex family of heterodimeric transmembrane cell surface receptors composed of α and β subunits (5). In mammals, integrins are expressed in many cell types and one cell type can express a variety of integrin receptors, thus allowing them to interact with many extracellular matrix components.

[2]These authors contributed equally to this work.
[3]To whom correspondence should be sent.

During embryogenesis the heart is the first organ formed. Many cellular components such as the vasculature, blood, neurons, cardiac myocytes and non-muscle cardiac cells are included in the cardiovascular system. Integrins are expressed in all of these cells comprised in the heart. Recent studies have shown the involvement of integrins not only in heart form and function, but also potentially in the development of cardiac diseases. The focus of this review will be on the role of integrins in the myocardium, as their function in the vasculature and platelets has been recently reviewed (6,7). We will discuss how the expression of integrins is critical to the form and function of the heart and offer insights to the future research directions into this important family of ECM receptors in the myocardium.

INTEGRIN STRUCTURE

Integrins are non-covalently associated heterodimeric transmembrane receptors composed of α and β subunits, with α subunits ranging from 120-180 kDa while β subunits are 90-110 kDa (4,8). Each integrin subunit consists of a large extracellular domain (700-1100 amino acids), a single transmembrane segment and short cytoplasmic tails, ranging from 20-60 amino acids (9), with the exception of β4 integrin which has a cytoplasmic domain of 1000 residues (10). The two subunits pair intracellularly as precursors prior to further carbohydrate processing in the Golgi and transport of the mature αβ heterodimers to the cell surface (11). About half of the α subunits have a globular head at the N-terminal end of the chain which contains 7 repeating homologous sequences each folded in a β propeller. In the rest of the α subunits, an I domain composed of about 200 residues is inserted into the β propeller fold. The I domain is homologous to the A domain of the von Willebrand factor protein and therefore is alternatively termed I/A domain. It is in the I domain which shows the metal-ion-dependent adhesion site as the MIDAS motif. The I domain and the MIDAS motif form the ligand-binding site (12,13). Sequences resembling the I domain of the α chain have also been found in β subunits and are believed to form the ligand-binding site for β chains (14,15). It is through the short cytoplasmic domain that integrins signal (see below) and also interact with the cytoskeletal components. The structure of a "generic" integrin is shown in Figure 1. For additional data on detailed integrin structure the reader is referred to the excellent reviews by Humphries (9,16).

THE INTEGRIN FAMILY

Integrins have been found not only in mammals, but in all vertebrates and invertebrates, including flies and nematodes (17). In the *C. elegans* genome, two genes code for α chains (18) and one gene for a β chain (19), thus forming a total of two integrin receptors in this worm. One of them is a putative RGD-binding integrin and the other recognizes laminin. (RGD is Arg-Gly-Asp, an amino acid sequence which forms the recognition site of fibronectin, vitronectin and a variety of other adhesive proteins. This tri-peptide sequence is recognized by several integrins such as α5β1, αIIbβ3 and most of the αVβ integrins.) In the genome of the fruit fly *Drosophila melanogaster*, five genes encode α chains (20-22) and two genes for β chains have been identified. At least five integrin heterodimer receptors are thus formed in the fly and those receptors bind to either RGD or laminin-containing extracellular matrix. In vertebrates, integrin genes coding for the α and β chains have evolved in a more complex manner. Currently, 18 α integrin genes and eight β integrin genes have been identified in mammals. These assemble the

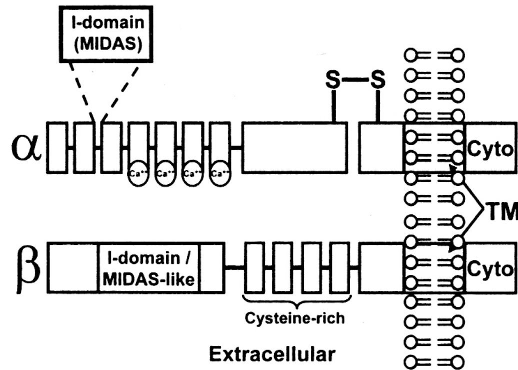

Figure 1. Structure of the integrin heterodimer. Integrin receptors are comprised of non-covalently associated α and β chains as shown. Both of these subunits are single-transmembrane spanning proteins with a long (700–1100 amino acid residue) extracellular ligand-binding domain, and a short cytoplasmic tail of 20-60 amino acid residues which is the principal signaling component of the molecule. The α subunits have seven N-terminal sequence repeats of 60-70 amino acid residues, folded into a β propeller structure. The last 3 or 4 repeats contain divalent cation binding sites and are involved in α–β association. An I-domain which contains the metal-ion-dependent adhesion site (MIDAS) is found as a module of 200 amino acids inserted between the repeat 2 and 3 of some of the α subunits. The MIDAS is critical in ligand-binding activity. In the carboxyl portion of the extracellular domain, some of the α subunits have a proteolytic cleavage site which, when recognized and cleaved, will convert the subunit into heavy and light chain dimers held together by a disulfide bond. Near the membrane is a stalk region which will undergo conformational change when the integrin receptors bind to its ligands.

Like α subunits, the N-terminal of the extracellular portion of the β subunits also contains an I-domain with a MIDAS-like sequence. This region forms the ligand-binding site in β subunits. Four cysteine-rich-domains are found in the carboxyl-terminus of the β chain extracellular portion. They can form disulfide bonds internally and may be related to the ligand-binding affinity, avidity or signal transduction. Sites other than those shown in this diagram have also been identified by numerous techniques and include regions critical for subunit association, integrin-ligand interaction and integrin activation.

TM= transmembrane segment; Cyto=cytoplasmic domain.

24 integrin heterodimers that have been thus far isolated in man (3). Figure 2 shows all α and β integrin heterodimers formed in man, with their possible evolution based on amino acid sequence comparisons (23,24). Phylogenetic analyses of those sequences found in both vertebrates and invertebrates suggested that the α integrin genes have probably evolved from a single α integrin ancestor by gene duplication. Currently, the evolution of the β integrin genes has not been clearly resolved (25). Many extracellular matrix proteins serve as ligands for the integrin receptors. Those include fibronectin, collagen, laminin, vascular cell adhesion molecule-1

(VCAM-1), intercellular adhesion molecule-1 (ICAM-1), vitronectin, tenascin, osteopontin, von Willebrand factor and thrombospondin (8,26,27). As additional integrin receptors are discovered, it is possible that more ligands will be revealed. A majority of integrins can bind several ligands; similarly each ECM protein can serve as a ligand for several different integrin receptors. Thus the relationship between integrin receptors and ECM proteins is quite complex. Moreover, alternatively spliced forms of integrin subunits have been found. This large integrin repertoire allows for even greater alternatives of cells to cope with and adapt to their environmental changes.

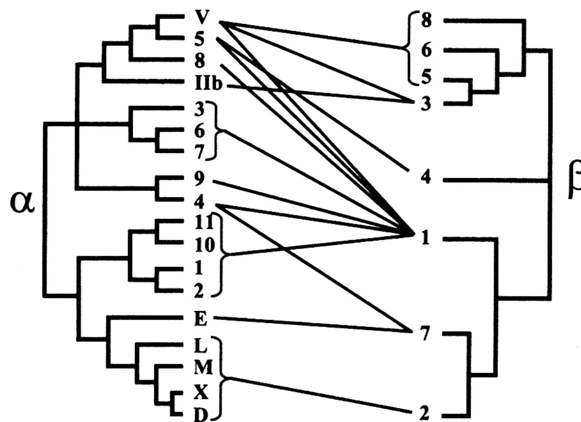

Figure 2. Phylogeny of the integrin family. The putative evolutionary tree for the vertebrate integrin α and β subunit families is shown and heterodimer formation is indicated. Eighteen α integrin genes and 8 β subunit genes were categorized based upon their amino acid sequence analyses. Currently, 24 heterodimers have been identified in man. Thin lines between the α and β subunits represent the heterodimers formed. Alternatively spliced isoforms of several integrin subunits have been identified but are not indicated on this diagram.

INTEGRIN EXPRESSION IN THE HEART

The expression of α1, α3, α5, α6, α7, α10, α11, β1, β3 and β5 subunits are found in the cyocardium (Table 1). Expression of most of these α subunits is temporally modified and developmentally regulated. For example, α1β1 and α5β1 are expressed in the embryonic heart, down-regulated postnatally, and can be re-induced upon mechanical loading of the ventricle (28). β1A and β1D are the two major spliced β1 isoforms found in the myocytes. β1A is expressed in embryonic cardiac myocytes and becomes downregulated at E17 – E18 of the 21-22 day rodent gestation period. Isoform β1D is expressed only in the skeletal and cardiac muscle. In heart, expression of β1D begins during late gestation and becomes the major form in the postnatal heart (29) (Table 1 and Figure 3).

Table 1. Myocardial integrin subunits, primary ligand binding and relative developmental changes in their expression levels.

Subunit	Primary Ligand(s)	Embryo	Neonate	Adult	Hypertrophy
$\alpha 1$	LN, Col	+	+	-	↑
$\alpha 3B$	LN, Col, FN	+	+++	++	NC
$\alpha 5$	FN	+	+	-	↑
$\alpha 6A$	LN	+	+	+	-
$\alpha 6B^{*}$	LN	+	++	+	-
$\alpha 7B$	LN	Late +	+	+	↑
$\alpha 7C^{*}$	LN	-	+	++	?
$\alpha 7D$	LN	-	-	+	?
$\alpha 10$	Col	+	?	+	?
$\alpha 11$	Col	?	?	++	?
$\beta 1A$	LN, Col, FN	Early ++	+	+	-
$\beta 1D$	LN	Late ++	++	++	↑
$\beta 3$	VN, FN, OPN	?	?	+	↑
$\beta 5$	VN	?	?	+	?

LN=laminin; Col=collagen; FN=:fibronectin; VN=vitronectin; OPN=osteopontin; late=late period of gestation; early=early stage of gestation;
"+" = low/basal expression; "-"= no expression; "++" = moderately high expression; "+++"= highly expressed; ↑=increased following hypertrophy; NC = no change; ?= undetermined; *=by polymerase chain reaction only.

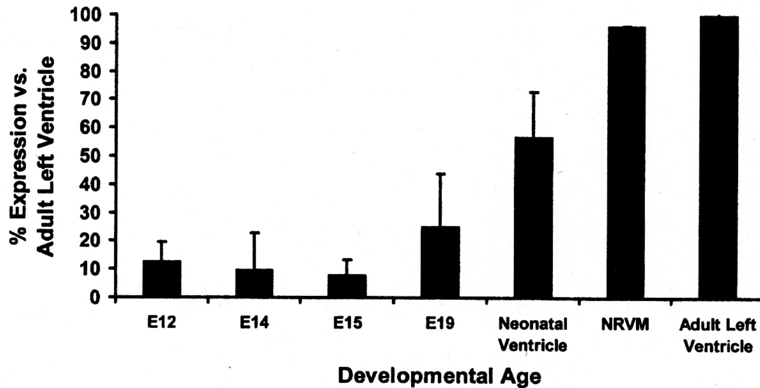

Figure 3. β1D integrin expression in murine heart is developmentally regulated in cardiac myocytes. Whole rat embryos [embryonic (E) days 12, 14, 15 and 19], heart tissue, ventricular cells, and ventricular tissue samples were obtained and used for Western blot analysis with a polyclonal β1D integrin isoform-specific antibody. Expression levels of the total β1D integrin protein (both mature and precursor forms) of each specimen were determined by densitometry and normalized to the total amount present in the adult ventricular sample, which was set to 100%. Because of the small standard error, error bars are not visible in the last two data point of the graph. NRVM=neonatal rat ventricular myocytes.

The expression of β3 and β5 on myocytes, as opposed to their more traditional localization in the vasculature, has been detected by some investigators (30). The varied detection of β1, β3 and β5 expression obtained by different laboratories may be dependent upon the unique antibodies (and their specificities) used by different investigators.

The heart is composed of cardiac myocytes and several non-myocyte cell types, including fibroblasts, smooth muscle cells, and endothelial cells. Fibroblasts constitute almost 2/3 of the cardiac cell population (31,32). Although cardiac myocytes lose their proliferative ability soon after birth, non-myocytes can still proliferate even in the adult heart. When the heart is exposed to stresses such as hemodynamic overload or myocardial infarction, cardiac myocytes increase in size, whereas cardiac fibroblasts increase in number and increase production of ECM proteins, such as collagens and fibronectin (33,34). The abnormal proliferation of cardiac fibroblasts with excessive accumulation of ECM proteins is one of the features of left ventricular remodeling, which ultimately leads to cardiac dysfunction (35). Cardiac fibroblasts express a repertoire of α subunits like that of the cardiac myocyte (36), but they do not express α6 and α7 as these cells have no laminin-containing basement membrane. In contrast, αv and the collagen-specific α2 subunit appear to be uniquely expressed by the cardiac fibroblasts but not by cardiac myocytes (37-39). It is through the integrins that cardiac fibroblasts sense mechanical stimuli and respond by initiating signal transduction cascades and changing their production of ECM (40-45).

INTEGRIN SIGNALING

As transmembrane cell surface receptors, integrins provide dynamic links between cells and ECM molecules (8). In addition to their function as adhesion molecules, integrins also are mechanotransducers (46) and signal bi-directionally across the cell membrane (47,48). As a terminally differentiated cell, the myocyte contains a regular array of actin and myosin filaments. The functional unit in striated muscle is the sarcomere. It is represented by one segment of parallel and well-organized actin and myosin filaments between two adjacent Z lines. Non-sarcomeric actin microfilaments attach the sarcomere to the cardiomyocyte cell membrane. By electron microscopy, the cytoskeleton appears as a dense and seemingly random array of fibers. The cytoskeleton plays a structural role by supporting the cell membrane and by forming tracks along which cell organelles and other elements move in the cytosol. The cytoskeletal array consists of three types of fibers: microfilaments (actin and myosin), intermediate filaments (desmin) and microtubules (tubulin). Three other groups of proteins participate in achieving cell shape, mechanical resistance and morphological integrity of myocytes (49). These are: 1) membrane-associated proteins: dystrophin, talin, vinculin, spectrin and ankyrin, 2) sarcomeric skeleton proteins: α-actinin, titin, C-protein, myomesin and M-protein, and 3) proteins of the intercalated discs: adherens junctions containing N-cadherin, the catenins and vinculin, gap junctions with connexin and desmosomes consisting of desmoplakin, desmocollin, desmoglein and desmin.

In the cardiac myocytes, the cytoplasmic tail of the β1 integrins connects to bundles of actin filaments, *via* bridging proteins like α-actinin, talin, paxillin and vinculin/metavinculin. Because of their co-localization, these proteins are suggested to be participants in linking the actin filaments through the membrane to the ECM. This arrangement is shown in Figure 4. Talin has been shown to bind directly to the β1 integrin cytoplasmic tail, thus linking the integrin receptor to the actin cytoskeleton (50,51). Therefore integrins connect the extracellular matrix to the cytoskeleton and to cytoplasmic proteins (52,53). Interestingly, the striated muscle-specific

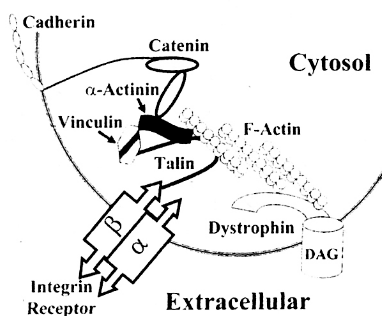

Figure 4. Diagrammatic representation of several key transmembrane proteins which link extracellular matrix to components of the cytoskeleton. Integrins are seen to bind directly to talin, and then through it to the actin-based cytoskeleton. Abnormalities in many of the proteins shown have been linked to either muscular dystrophies or cardiomyopathies. Many of the displayed proteins may interact directly or indirectly with integrins (see text). DAG= dystrophin-associated glycoprotein

β1D integrin has been shown to bind more tightly to talin than the ubiquitously expressed β1A-integrin. β1D may thus provide a firmer attachment to the cytoskeleton than similar attachments by the ubiquitously expressed β1A integrin in non-muscle cells. This arrangement is clearly advantageous in the continuously-contracting cardiac muscle cell.

Once integrins bind to ECM, their interaction with the actin cytoskeleton leads to redistribution of integrins into specific structures known as focal adhesions (54). Vinculin, talin, α-actinin and paxillin all co-localize within the focal adhesions (55). After binding ECM, integrin receptors transmit signals across the membrane to a host of cytoplasmic molecules (56). These events termed "outside-in signaling" regulate cell attachment, survival, proliferation, cell spreading, differentiation, cytoskeleton reorganization and cell shape (57,58)

Similarly, "inside-out" integrin signaling causes modifications in their ligand binding affinity through intracellularly originated signals (59). In addition to their structural role, focal adhesions are also sites of signal transduction. This is important as the integrins themselves do not possess enzymatic activity. Rather, they signal through a host of downstream cytoplasmic molecules. The ability of focal adhesions to form a signal transduction complex was observed by the interaction of the integrin receptors with a tyrosine phosphorylated protein in the focal adhesion, termed focal adhesion kinase (FAK), as well as a host of other molecules (60,61). Crosslinking of integrins leads to increased FAK autophosphorylation by interaction of the β integrin cytoplasmic domain with FAK (62,63). Following activation, FAK combines with Src-family kinases, such as c-Src or c-Fyn. The signaling pathways activated by the FAK-Src family kinase complex appear to play a role in regulating the assembly/disassembly of focal adhesions during cell migration and regulate cell proliferation and protect cells from programmed cell death (64). Upon integrin-mediated activation, Src-family members undergo conformational changes and interact with Shc. Shc is known to link various tyrosine kinases to Ras and once it becomes phosphorylated, it results in the activation of extracellular signal regulated kinase (ERK1/2), which is a known mediator of the cardiac hypertrophic response.

THE ROLE OF INTEGRINS IN HYPERTROPHIC SIGNALING OF CARDIAC CELLS

Many of the features of cardiac hypertrophy are induced by the activation of protein-kinase-C (PKC) and the mitogen-activated protein kinase (MAPK) cascade by G-protein coupled receptors (GPCRs). Tyrosine kinases such as FAK, as well as GPCRs, can both activate MAPK. Previous work in our laboratory, as well as others, showed that in cultured neonatal rat ventricular myocytes (NRVMs), GPCR ligands like phenylephrine, endothelin-1, and angiotensin II promote a hypertrophic response (65,66). Prominent features of cardiac myocyte hypertrophy are an increase in cell size, the rapid expression of immediate-early genes (e.g., c-fos and c-jun), the re-expression of fetal genes like atrial natriuretic factor (ANF), the altered expression of contractile protein genes (such as myosin light chain-2 ventricular (MLC-2v) and α-skeletal actin), as well as an induction of actin-myosin filament reorganization (67). Additional investigations on NRVMs showed that both Rho and Rho-kinase dependent signaling pathways regulate myofibrillar organization and ANF expression in myocardial cells (68).

In contrast to integrin signaling studies in other cell lines, the study of their role in the heart is largely hampered by both the terminally-differentiated nature of the cardiac myocyte and the unavailability of a cardiac myocyte line. Thus *in vitro* studies are commonly performed with the use of short-term cultured primary rat neonatal ventricular myocytes, since they can be easily isolated and manipulated. Our laboratory has examined the regulatory role of β1 integrins in the

adrenergically stimulated NRVM and showed that integrin signaling modulated this hypertrophic response (66) and that the muscle-specific integrin, β1D, was specifically involved (29) (Figure 5). Western blotting showed that the α1-adrenergic receptor (AR) agonist phenylephrine (PE) caused an up to 8.1-fold upregulation of β1D protein in NRVM following 48 hr of PE stimulation, relative to controls. Immunofluorescent microscopy showed that PE caused subcellular redistribution of β1D integrin with a shift in its localization from punctate cytoplasmic staining to one co-localized with actin in the organizing myofibrils, most intensely at the Z-line. Overexpression of β1D augmented the PE response and caused increased cell spreading, suggesting that α1-AR pathways may influence integrin function through an inside-out mechanism. Further, we and others found that the α-adrenergically induced hypertrophy of NRVM is strongly influenced and dependent on an integrin-mediated signaling process requiring FAK (29,69,70). Activation of FAK paralleled this induction of β1D. Thus these studies implicate an important role for integrins and integrin signaling in the *in vitro* hypertrophic response of cardiac myocytes.

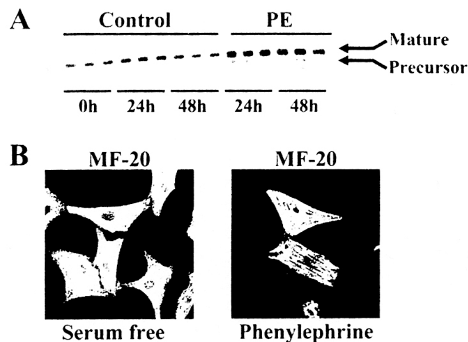

Figure 5. Adrenergic stimulation of NRVMs causes increased β1D integrin expression and cellular reorganization. A. Phenylephrine stimulation causes an increase in β1D protein. A representative Western blot of β1D expression in control and adrenergically-stimulated neonatal rat ventricular myocytes is shown. Phenylephrine (PE) treatment caused an 8.1-fold upregulation of the mature (glycosylated) form of β1D integrin compared to untreated control cells and also caused an 11-fold upregulation of the β1D precursor form, as determined by densitometric analyses (not shown). B. Phenylephrine stimulation causes myofibrillar reorganization in the neonatal rat ventricular myocyte (NRVM). NRVMs were maintained in serum-free medium alone or serum-free medium containing 100 uM of phenylephrine. MF-20 staining was performed to detect sarcomeric myosin.

FAK also binds to the SH3 domain of the GTP-activating protein for Rho. This interaction further suggests a cross-talk between integrin signaling and GPCR-stimulated actin reorganization (71). Recent observations showed that the actin cytoskeleton plays an important role in integrin modulation of signaling through receptor tyrosine kinases (72). Work has also directly linked integrin and RhoA activation to serum response factor dependent gene expression

in NRVM (73). Short et al. showed that integrins regulate the linkage between upstream and downstream events in GPCR signaling to MAPK. These investigators proved that GPCR activation of MAPK is dependent on integrin-mediated cell anchorage (74). They raised the idea of a scaffolding concept, where integrin-mediated recruitment of focal contact and cytoskeletal components form a scaffold to allow efficient assembly of the various components of the signaling pathway. Given this body of data, in combination with the known data that integrin-mediated cell anchorage can regulate the efficiency of signaling from tyrosine kinases, the relationship between integrins and GPCRs needs to be further investigated in the myocardium.

USE OF TRANSGENIC MOUSE MODELS TO ASSESS INTEGRIN FUNCTION IN THE HEART

LaFlamme et al. constructed a dominant-negative β1 integrin molecule, termed Tac β1, by linking the cytoplasmic domain of β1 integrin to the extracellular and transmembrane domains of the small non-signaling (tac) subunit of the interleukin-2 receptor (75). Expression of this chimeric β1 integrin inhibited endogenous integrin function in a variety of cellular processes, including signaling and cell spreading. We used this dominant negative molecule to construct α–myosin heavy chain (MHC)-Tacβ1A transgenic mice, so that the Tacβ1A molecule would be expressed specifically in cardiac myocytes (76). Depending on the extent of the expression of the transgene, the resultant mice have different fates. If the transgene is expressed at a very high level, then the transgenic mice die perinatally. Histological studies on those hearts showed diffused fibrotic replacement of the myocardium. Moderate expression of the transgene caused development of a dilated cardiac hypertrophy and increased expression of the hypertrophic marker genes such as *ANF* and *βMHC*. Mice with low levels of transgene expression showed no obvious basal histological or molecular abnormalities when compared to negative littermate or wild-type background control animals. But when the cardiac function of these "low expressors" was assessed, the ventricular contractility and relaxation were significantly depressed. Despite this, there was no marked change in the ability of the "low expressor" transgenic mice to accommodate hemodynamic loading caused by transverse aortic constriction, suggesting that mechanotransduction was preserved in the face of low-level reduction of integrin function. We would hypothesize that at a discrete level of transgene expression, between the "low and medium" levels we obtained, sufficient disruption of integrin signaling and function would occur, so as to interfere with normal mechanotransduction.

In related studies, Valencik and McDonald have generated transgenic mice that express either the wild-type α5-integrin or a mutated $\alpha_{5\text{-}1}$-integrin (an α5-integrin molecule lacking the carboxyl-terminal cytoplasmic domain), specifically in cardiac myocytes (77). Deletion of this cytoplasmic domain from the wild-type α5-integrin results in an activated α5-integrin with high ligand affinity, and has been thus regarded as a "gain-of-function" mutation. While the overexpression of wild-type α5-integrin had no detectable adverse effects in the mouse, expression of the gain-of-function $\alpha_{5\text{-}1}$-integrin led to fibrosis, electrocardiographic abnormalities, cardiomyopathy and sudden death at a young age in these transgenic animals. The phenotype was hypothesized to be due to the high ligand–binding affinity of the $\alpha_{5\text{-}1}\beta1$ heterodimer to the ECM. Thus the increased ECM binding to the mutated integrin receptors would generate abnormal intracellular signals, leading to the phenotype similar to our Tacβ1A mice discussed above.

INTEGRIN GENE KNOCK-OUT MOUSE MODELS

Studies utilizing integrin knock-out mice and cells derived from these mice have provided considerable and sometimes surprising insights into unique function of individual members of this family. As outlined above, more than 24 integrin heterodimers have been detected to date, as formed from 18 α subunits and 8 β subunits. Knock-out mice for 7 of the 8 β subunits and 13 of the 18 known α subunits have been generated (78). With rare exception, the phenotypes of each of the integrin knock-out lines are quite distinct and include: embryonic lethality (β1 and α2), impaired renal and lung development (α3), skin blistering (α6), muscular dystrophy (α7), small or absent kidneys (α8), or bilateral chylothorax (α9). Only the β5 null mouse had no phenotype.

Ablation of α4 integrin affected cardiac development in the mouse (79). Mice homozygous for the α4 integrin subunit died by days E11-E14 post-coitum and had a dual phenotype. Early in development, failure of allantoic and chorionic fusion was noted. Yet by E12.5, severe hemorrhage was noted around the heart and head. Detachment of the epicardium and myocardium coincided with observation of loss of epicardium and absent coronary artery development. Interestingly, epicardial absence was also noted in vascular cell adhesion molecule-1 (VCAM-1) null embryos, suggesting that VCAM-1 / α4 integrin interaction is required for normal epicardial formation (80).

Inactivation of the murine α5 integrin gene also resulted in early embryonic lethality at E10 – E11 with defects in extra-embryonic vascular development but no obvious cardiac abnormalities (81). α5β1 integrin receptors are one of the prime fibronectin-binding integrins. In contrast to the α5 integrin null mice, fibronectin-null embryos have abnormal cardiogenesis with a bulbous heart tube, a collapsed endocardium, distended aorta and no yolk sac vessel formation (82). Thus these results suggest that other fibronectin-binding integrins are critical for normal cardiac development.

Inactivation of the β1 integrin gene resulted in the early embryonic death of homozygous β1 null embryos at the time of blastocyst implantation (83-85). Chimeric mice were created from combining wild-type and β1 integrin null embryonic stem (ES) cells (84). If less than 25% of the embryonic cells were contributed by the null cells, embryos appeared normal at E9.5. Null cell contribution above this amount resulted in abnormal embryonic development. In adult animals, null cells were not detected at all in liver and spleen, but all other organs including heart contained some null cells, ranging from 2-25%. Still, few β1 integrin null cells colonized the myocardium of the chimeric mice. The differentiation of the β1 null cells into cardiac myocytes was delayed, and the expression of the myofibrils in these null cells was altered. Cell debris was observed surrounding the null cells in the myocardium and null cells were undetectable by 6 months of age. These results suggested the essential nature of β1 integrin in the heart.

Further murine studies were performed where clever knock-in / knock-out techniques were used to ablate either the A or D isoforms of β1 integrin (86). Complete replacement of the β1A isoform with the striated β1D isoform resulted in embryonic lethality. On the other hand, deletion of the β1D exon from the mouse genome only resulted in mild cardiac abnormalities in the β1D null animals, with increased expression of atrial natriuretic factor, a marker gene for the cardiac hypertrophy reported. This study emphasizes the unique roles that β1A and β1D integrin play in the organism.

In order to study the role that the β1 integrin plays in ventricular myocytes more carefully, we generated a cardiac-specific β1 integrin knock-out mouse by employing a Cre-loxP strategy (87). In this procedure, the Cre recombinase recognition sites, loxP elements, were inserted into the gene so that exon 2 (which contains the translational start site) and exon 3 were surrounded. Inactivation of the β1 integrin gene was restricted to cardiac myocytes by expressing Cre recombinase under the control of a ventricular-specific promoter, myosin light chain 2 ventricular (MLC-2v)(88). Crossing of these two genetically-altered mice thus resulted in excision of exons 2 and 3 of the β1 integrin gene only in ventricular myocytes. Similar techniques have been used to ablate β1 integrin expression specifically in keratinocytes (89). In contrast to the embryonic lethality seen in the traditional β1 integrin knock-out mice, our cardiac-specific conditional knock-out mice survived to term and grew into adulthood. They developed progressive fibrosis in the heart and later died of heart failure (Figure 6). Whether this phenotype resulted from inactivation of the gene during cardiac development, or from the reduced expression of β1 integrin in the postnatal ventricular myocytes, is under investigation. This animal model provides strong evidence that β1 integrin plays a very important role in the maintenance of cardiac myocyte and heart function.

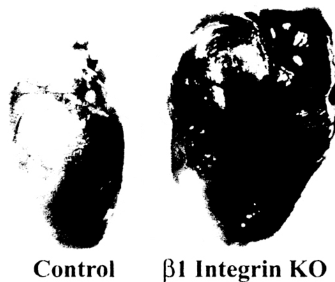

Figure 6. Dilated heart failure develops by 6 months of age in cardiac-specific β1 integrin knock-out mice. The heart on the right is one from a representative 6-month old β1 integrin knock-out mouse, the one on the left from a control littermate. Pleural effusions, ascites and increased lung/body weight and liver/body weight ratios accompanied the cardiac pathology. Note the knock-out heart specimen had a dilated ventricle, enlarged atria and atrial thrombi. No similar specimens were detected in control animals.

STUDY OF INTEGRIN FUNCTION WITH AN EMBRYONIC STEM CELL CULTURE MODEL

In vitro differentiation of ES cells offers a unique approach to examine events that occur during embryonic development and complements gene knock-out studies in whole animals. Upon withdrawal of leukemia inhibitory factor and stromal contact, ES cells form embryoid bodies (EBs), spherical aggregates of differentiated cell types which appear in a well-defined temporal pattern. Myogenesis, formation of cardiac-like cells, as well as hemopoiesis,

vasculogenesis and development of neuron-like cells has been observed (90). Appropriate culture conditions result in portions of the EB that begin to contract spontaneously. Cells derived from these beating areas show structural features like embryonic cardiac myocytes and expression of cardiac myocyte marker genes such as myosin heavy chain, tropomyosin, MLC-2v, or ANF.

Having ES cells with homozygous inactivation of the β1 integrin gene, Fassler et al. and Guan et al. showed that the differentiation of β1-nulls into cardiomyocyte-like cells was severely impaired (91,92). Cells characteristic of atrial or ventricular phenotypes were only transiently seen, with only pacemaker-like cells surviving but still appearing abnormal. Thus appropriate expression of integrin family members appears essential for the ability of cells to arrange into three-dimensional structures and undergo normal organogenesis. Organization of cells into higher-order structures is affected by mechanical and biophysical stresses which likely require normal surface integrin expression.

MECHANICAL STRESS AND CARDIAC DISEASE

Mechanical stress on the heart from either pressure, loss of myocardium or volume loading can cause adaptation of the myocardium, to optimize cardiac performance. Ventricular hypertrophy is an important adaptive mechanism that in the short term allows the heart to maintain or increase its output. Though this initial response is compensatory, frequently it evolves to cause heart failure and increases mortality. Increased pressure and volume work leads to global hypertrophy of the ventricle wall with increased myofibrillogenesis and sarcomere deposition. Similarly, ventricular remodeling may develop after myocardial infarction. Recent data have shown modulation of the extracellular matrix in the hypertrophied or post-infarction myocardium (42,93,94).

Since myocardial hypertrophy is often modulated through mechanical overloading and integrins function as mechanotransducers, it is likely that integrins play pivotal roles in this process (46). In mice, rats and cats, well-established models of transverse aortic constriction (TAC) or pulmonary artery banding are used to create myocardial pressure overload hypertrophy. These procedures allow study of the myocardial adaptation/ remodeling processes leading to pressure-overload hypertrophy, as seen in patients with hypertension or valvular disease. Currently, the transition from hypertrophic compensation to heart failure is not well understood. Kuppuswamy et al. analyzed cytoskeletal protein fractions from a model of feline right ventricular hypertrophy (95). They showed cytoskeletal association of c-Src, FAK and β3-integrin, as early as four hours post pressure overloading, while no similar findings were detected in sham controls. In the normally-loaded ventricles, the majority of c-Src, FAK and β3-intergin were in the membrane fraction. This study showed that c-Src is tyrosine-phosphorylated during pressure overloading. Furthermore, cytoskeletal association of these signaling proteins has been shown to result in the phosphorylation of several cytoskeletal proteins, potentially leading to changes in the cytoskeletal structure. Mechanical load induces movement of the non-receptor tyrosine kinases (c-Src and FAK) to the cytoskeleton. Integrins, in their function as mechanotransducers, may play a critical role in initiating this signaling cascade. Pressure overload of the adult cat myocardium also resulted in accumulation of ECM proteins such as fibronectin and vitronectin, suggesting that multiple integrin-related signaling pathways could be perturbed following integrin engagement with ECM ligands (96). In a model of murine pressure-overload hypertrophy, our laboratory has detected increased expression of α1, α5 and

β1 integrin transcripts, augmented protein levels for α7 and β1 integrin and modulation of FAK, cSrc and ERK phosphorylation (97).

ROLE OF THE CYTOSKELETON IN CARDIOMYOPATHIES

As discussed above, adaptive responses of the heart to processes such as pressure overload may evolve to decompensated heart failure. As the myocardium transitions from compensated to decompensated heart failure, an imbalance in the ECM-integrin interaction may occur. As an example, during the initial phases of pressure overload, fibronectin and its prime integrin receptor, α5β1, increase their expression in parallel. However, in later stages of hypertrophic induction, there appears to be a mismatch of fibronectin and α5β1 expression (98). The disruption of coordinated connection between fibronectin and its integrin receptor may lead to cardiac myocytes being released from their ECM attachment sites, resulting in apoptosis. This process, termed anoikis (99) (Greek for "homelessness"), has been described in epithelial cells and was proposed to be responsible for selective myocyte death in the heart (100). It is likely that changes in the ECM-integrin-cytoskeletal complex could subject the cell to altered mechanical forces that would also be detrimental to survival. Release/shedding of integrins into the extracellular space has been reported during the transition from cardiac hypertrophy to heart failure (100). Thus as the myocyte changes shape, it might release a portion of the extracellular domain of its integrins. The mechanism involved in the release of the integrin is currently poorly understood.

Cardiomyopathies are diseases of the myocardium, which frequently lead to a reduced quality of life because of heart failure and ultimately can result in premature death. When this disease process occurs in the absence of any known underlying etiology, it is referred to as an "idiopathic" cardiomyopathy. Previously classified "idiopathic" dilated cardiomyopathies have begun to be linked to abnormalities in components of the ECM-cytoskeletal system. These include alterations in dystrophin, dystrophin-associated glycoprotein (DAG), calpain-3, laminin alpha-2 chain (merosin), muscle LIM protein (MLP) and metavinculin (101).

Dystrophin is a very large protein that constitutes 5% of the membrane-associated cytoskeleton in muscle cells. Dystrophin crosslinks actin filaments into a supportive cortical network and to a glycoprotein complex (DAG) in the myocyte membrane. Mutations in the dystrophin gene result in neuromuscular disorders like Duchenne muscular dystrophy (DMD), Becker muscular dystrophy as well as X-linked dilated cardiomyopathy. Predominantly the skeletal muscle is affected, although cardiomyopathy is also seen. Dystrophin co-localizes with vinculin and vinculin binds through talin to integrin receptors. Integrin and dystrophin both function to stabilize the cell and link the cytoskeleton to the membrane/extracellular matrix. This function is extremely important for the support of the myocyte cell membrane during the stress of repeated muscle contraction. Mutations in the genes that encode many components of the dystrophin/glycoprotein complex cause a variety of muscular dystrophies. Interestingly, data have been recently published detecting abnormalities in the muscle integrin α7 in muscular dystrophies (102). Further, integrin α7 has been shown to be upregulated in both *mdx* mice (a murine model of muscular dystrophy caused by mutation in its dystrophin gene) and patients with muscular dystrophies (103). Thus, both the integrin- and dystrophin-mediated transmembrane linkage systems contribute to the functional integrity of skeletal muscle. Given this, Burkin et al. (104) postulated that the increased expression of α7 integrin compensates for the loss of dystrophin. They generated transgenic mouse lines in which rat α7 integrin was

overexpressed in the skeletal and cardiac muscles. This group used *mdx / utr*$^{-/-}$ mice as a model of muscular dystrophy. These mice have a double mutation, in both the dystrophin gene and utrophin gene, develop severe progressive muscular dystrophy as that seen in DMD patients and die prematurely. α7 integrin transgenic mice were bred to *mdx/utr*$^{-/-}$ mice and the authors found that *mdx/utr*$^{-/-}$ mice with enhanced expression of the α7BX2 chain isoform showed greatly improved longevity and mobility, as compared to the native *mdx/utr*$^{-/-}$ mice. Overexpression of the wild-type integrin seemingly rescued the skeletal muscle phenotype caused by other mutations. To date, it is not clear if similar results are detected in the cardiac muscle of similarly-affected animal models or patients. Since lack of dystrophins leads to muscular dystrophy and DCM (dilated cardiomyopathy) we hypothesize defects in the integrin receptor may also cause cardiac myocyte instability and DCM in man.

CONCLUSION

Clearly enormous data have begun to be accumulated with regard to the role of integrins in the myocardium. Significant studies remain to be performed in the future which will undoubtedly link this large and complex family of molecules to predictable and perhaps unpredictable functions in the heart. Future questions to be answered include more complete identification of integrin signaling pathways in the heart, how integrin receptors interact with other known signaling pathways in the myocardium, and are integrins the principal mechanotransducers in the heart? Further, do the integrins provide directional cues for cell migration and specifically how does the varied temporal and spatial expression pattern of integrins influence myocardial development? Many of these questions are under current investigation in our own and other laboratories around the world.

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