Cardiac Myocyte–Specific Excision of the β1 Integrin Gene Results in Myocardial Fibrosis and Cardiac Failure

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Abstract—Integrins link the extracellular matrix to the cellular cytoskeleton and serve important roles in cell growth, differentiation, migration, and survival. Ablation of β1 integrin in all murine tissues results in peri-implantation embryonic lethality. To investigate the role of β1 integrin in the myocardium, we used Cre-LoxP technology to inactivate the β1 integrin gene exclusively in ventricular cardiac myocytes. Animals with homozygous ventricular myocyte β1 integrin gene excision were born in appropriate numbers and grew into adulthood. These animals had 18% of control levels of β1D integrin protein in the heart and displayed myocardial fibrosis. High-fidelity micromanometer-tipped catheterization of the intact 5-week-old β1 integrin knockout mice showed depressed left ventricular basal and dobutamine-stimulated contractility and relaxation (LV dP/dt_max and LV dP/dt_min) as compared with control groups (n=8 to 10 of each, P<0.01). Hemodynamic loading imposed by 7 days of transverse aortic constriction showed that the β1 integrin knockout mice were intolerant of this stress as they had 53% survival versus 88% in controls (n=15 each). By 6 months of age, mice with depressed ventricular expression of β1 integrin developed a dilated cardiomyopathy that was not evident in any control animals and had patchy decrease in glucose metabolism as determined by positron emission tomography. Myocyte membrane integrity as determined via Evan’s blue dye staining was disrupted in the β1 integrin knockout mice. This model provides strong evidence for the importance of β1 integrin in cardiac form and function and indicates that integrins can be linked to development of cardiomyopathies. (Circ Res. 2002;90:458-464.)

Key Words: extracellular matrix • homologous recombination • Cre recombinase • heart • positron emission tomography

Integrins are a large family of heterodimeric cell surface receptors composed of α and β subunits. They function in cell–extracellular matrix (ECM) adhesion and cell-cell adhesion, and signal bidirectionally across the cell membrane.1,2 Further, they serve as mechanotransducers, converting mechanical signals to biochemical ones.3 This combination of properties allows integrins to play important roles in cell growth, differentiation, migration, and survival4 and also that dominant-negative disruption of integrin function in transgenic mice resulted in cardiac fibrosis and abnormal cardiac function.5–7 Ablation of β1 integrin expression in all murine tissues resulted in gastrulation defects and death by E5.5 of the 21-day gestation period.8,9 Chimeric mice as well as embryoid bodies constructed from β1 integrin–null cells showed delayed development and differentiation of β1-deficient cells along the cardiac lineage, as well as abnormal sarcomerogenesis of these cardiac-like cells.10 Although a few β1 integrin–null cells were detected in the myocardium of chimeric mice, cellular debris was always detected along with the null cells. These null cells were completely lost from the myocardium of the chimeric mouse heart by 6 months of age.

To more specifically evaluate the role of β1 integrin in the myocardium, we used a Cre-loxP gene targeting approach. Cre recombinase expression driven by the myosin light chain-2 ventricular (MLC-2v) promoter caused β1 integrin gene excision exclusively in ventricular cardiac myocytes.11 Our results in these β1 knockout (β1KO) mice showed the following: (1) the β1 integrin gene was excised only in the heart; (2) β1D integrin protein in the myocardium was...
detected in heart. The lower 330-bp band was detected with a second primer set that amplified a portion of the Cre transgene, indicating that it was present in all tissues. Thus, Cre expression and subsequent Cre-mediated excision only occurred in heart. H indicates heart; L, lung; K, kidney; M, skeletal muscle; S, spleen; and B, brain. C, Western blot analysis of β1D integrin protein to development of cardiomyopathy.

Materials and Methods

Antibodies and Reagents

The anti-β1D integrin antibody was created in our laboratory. Anti-talin and anti-myosin (MF20) monoclonal antibodies were from Sigma and the Developmental Studies Hybridoma Bank, respectively. FITC- and rhodamine-labeled secondary antibodies were from Jackson Immunoresearch Laboratories, Inc. Fluorescein-conjugated wheat germ agglutinin (F-WGA) was from Molecular Probes.

Construction of β1 Integrin–Floxed Mice and Ventricular-Specific Excision of the β1 Integrin Gene

Two clones 14 and 17 kb in length were isolated from a 129/SVJ mouse genomic library (Stratagene) and used for construction of the floxed β1 integrin gene–targeting vector, as shown in Figure 1A. R1 embryonic stem cells were electroporated with the targeting vector, and successful homologous recombination was confirmed by Southern blot analysis using probes derived separately from intron 1 and exon 5 of the β1 integrin gene. (Data are shown in the expanded Materials and Methods section available online at http://www.circresaha.org.) The targeted embryonic stem cells were subjected to a second electroporation with pMC-Cre and selected for resistance to 1-2’-deoxy-2’-fluoro-β-D-arabinofuranosyl-5’-iodouracil (FIAU) (Moravek). Colonies with Cre-mediated type II deletion were confirmed by polymerase chain reaction (PCR) analysis and injected into blastocysts to generate the targeted mice with a floxed β1 integrin gene. Mice with successful germline transmission of the targeted allele were identified and termed β1 integrin–floxed mice.

β1 integrin–floxed mice were mated to ML2v-Cre mice to generate animals with ventricular-specific β1 integrin gene inactivation (β1floxed/ML2vCreov or β1KO). Mice were analyzed in a 129SVJ/Black-Swiss background throughout the study, but results were confirmed in a 129/SVJ background. All animals were housed in an AALAC-approved facility.

Western Blot Analyses

Protein lysate preparation and Western blot were performed via standard methods. Denstometry of samples (n=9 each group) was performed via use of either Alphaease (Alpha Innotech) or Image-Quant (Molecular Dynamics) software.

Hemodynamic Analysis, Transverse Aortic Constriction (TAC), and Echocardiography

Age-matched 5-week-old β1KO and control mice were used for both of these types of studies. Hemodynamic analyses at baseline and after dobutamine infusion were performed using in vivo catheterization with a micromanometer-tipped catheter. For studies assessing survival after aortic constriction or sham operation, animals recovered from operative anesthesia, were returned to their cages, and were checked twice daily. Echocardiography was performed utilizing an Apogee CX echocardiographic machine (ATL Interspec).
Micro–Positron Emission
Tomography (MicroPET)
A microPET scanner developed at the UCLA Crump Institute for Biological Imaging, also available through Concorde Microsystems, was used to image the mice. Animals were anesthetized with 225 mg/kg avertin (2,2,2-tribromoethanol) (IP) and injected 20 minutes later with 200 to 233 mCi of \(^{18}F\)fluorodeoxyglucose (FDG). After an uptake period of 20 minutes, animals received additional anesthesia (ketamine, 100 mg/kg) and were imaged prone, in a long-axis orientation, with the microPET scanner. Scanner bed position was directly over the heart. Images were reconstructed using the maximum a posteriori reconstruction algorithm, which provides a resolution of \(\approx 1.2 \text{ mm}\)

Assay of Membrane Integrity
Six-month-old mice were injected intraperitoneally (50 \(\mu\)L/10 g body weight) with a 2% solution of EBD (Sigma) and euthanized 2 days after injection. Cryosections of myocardial tissue (5 to 7 \(\mu\)m) were examined under epifluorescent optics (as above) and viewed as a red image by using green activation (546 nm) and barrier (590 nm) filters, respectively.

DNA Laddering and Terminal Deoxyribonucleotidyltransferase–Mediated dUTP Nick-End Labeling (TUNEL) Assays
DNA was extracted from 6-month-old \(\beta^1\)KO and control mouse hearts and analyzed by agarose gel electrophoresis for nucleosomal DNA fragmentation. TUNEL assay was performed on heart cryosections by using Cardiotacs kit reagents (Trevisgen), and visualization of TUNEL-positive cells was performed with FITC-ExtrAvidin (Sigma). Cardiomyocytes were detected with an anti-sarcomeric myosin antibody (MP20), and nuclei were localized by staining with DAPI (Sigma). Staining was visualized by immunofluorescent microscopy. TUNEL-positive cells were counted in multiple sections from each heart and recorded as TUNEL-positive myocytes/10 000 myocytes.

Statistics
The Student \(t\) test or ANOVA was used for analyses. \(P<0.05\) was considered significant.

Results
Generation of Ventricular Muscle–Specific \(\beta^1\)KO Mice
To generate mice that carry ventricular myocyte–restricted inactivation of the \(\beta^1\) integrin gene, we used a Cre-loxP strategy. On the basis of previous successful ablation of the \(\beta^1\) integrin gene using a conventional knockout approach, we constructed a floxed \(\beta^1\) integrin gene–targeting vector so that Cre recombinase–mediated excision would delete exon 2 (containing the translational start site) and exon 3, of the \(\beta^1\) gene (Figure 1A). This excision event would prevent expression of all \(\beta^1\) integrin isoforms since splice variation occurs downstream from these exons. We detected appropriate targeting and subsequent excision of the selectable markers in embryonic stem cells and produced gene-targeted mice via standard techniques. Mice homozygous for a floxed \(\beta^1\) integrin gene were phenotypically normal and gave birth to litters of normal sizes.

To excise the \(\beta^1\) integrin gene specifically in cardiac ventricular myocytes, we bred the homozygous floxed \(\beta^1\) integrin mice to \(MLC2v\) Cre recombinase “knockin” mice that had been previously characterized. Analysis of adult animals (ages 6 to 8 weeks) confirmed \(\beta^1\) integrin gene excision only in ventricular tissue derived from homozygous \(\beta^1\) integrin flox\(^{x}\times MLC2v-Cre\) mice (\(\beta^1\)Flox/Flox/MLC2v-Cre\(^{x}\)) (Figure 1B). \(\beta^1\)D integrin protein levels in the 6- to 8-week-old \(\beta^1\)Flox/Flox/MLC2v-Cre\(^{x}\) mice were reduced to \(\approx 20\%\) of the control levels (18.6\% \(\pm 1.9\%\) versus controls, \(n = 9\) each, \(P<0.001\)), which were wild-type mice, homozygous floxed animals (\(\beta^1\)Flox/Flox\(^{x}\)) that had not been crossed with the \(MLC2v\)-Cre mice, or mice doubly heterozygous for the floxed \(\beta^1\) integrin allele and \(MLC2v\)-Cre (\(\beta^1\)Flox/Flox/MLC2v-Cre\(^{x}\)) (not shown) (Figures 1C and 1D). This amount of residual \(\beta^1\)D integrin protein is in agreement with other studies using \(MLC2v\)-Cre–mediated excision.

Conditional Gene Targeting of \(\beta^1\) Integrin Results in Cardiac Fibrosis and Ultrastructural Abnormalities in the Myocardium
We next evaluated the phenotype in the adult \(\beta^1\)KO mice. Histological studies showed that patchy fibrosis developed in the ventricular wall of the \(\beta^1\)Flox/Flox/MLC2v-Cre\(^{x}\) mice (Figures 2A and 2F). No comparable results were detected in age-, sex-, and strain-matched wild-type or \(\beta^1\)Flox/Flox mice. In concert with identical reduction in \(\beta^1\) integrin protein expression, ventricular fibrosis was also detected when the \(\beta^1\)Flox/Flox mice were crossed with a second cardiac-specific Cre mouse, in which the \(\alpha\)-myosin heavy chain (\(\alpha\)MHC) (5.5 kb) promoter drove Cre recombinase expression (data not shown). Transmission electron microscopic analysis of affected areas of the \(\beta^1\)KO myocardium showed focal dissolution of myofibrils and intercalated disks as well as mitochondrial swelling, with disruption and loss of cristae. No similar areas were detected in control animals (Figures 2G through 2H).

Temporal analysis showed that \(\beta^1\) integrin protein expression was reduced to 62.7\% \(\pm 5.6\%\) of control values \((n = 6)\) in the 2- to 3-week-old \(\beta^1\)KO ventricle, to 23.4\% \(\pm 5.7\%\) of control values \((n = 3)\) in 4-week-old ventricle, and to 18.6\% \(\pm 1.9\%\) of control values \((n = 9)\) by 6 to 8 weeks of age (Figure 2I). No histological abnormalities were detected in 3-week-old mouse hearts, whereas 22.1\% \(\pm 6.8\%\) fibrosis developed by age 6 to 8 weeks. These results suggested that \(\beta^1\) integrin is required for maintenance of cardiac myocyte and myocardial integrity.

\(\beta^1\) Integrin Protein Deficiency Leads to Abnormal Cardiac Function, Intolerance to Hemodynamic Loading, and Development of Heart Failure by 6 Months of Age
Cardiac function of the \(\beta^1\)KO mice was evaluated with in vivo cardiac catheterization. Mice were analyzed at 5 weeks of age, when histological abnormalities were not extensive. Contractility and relaxation of the left ventricle in the \(\beta^1\)Flox/Flox/MLC2v-Cre\(^{x}\) mice were significantly impaired as compared with any of the control groups (Figures 3A and 3D). Left ventricular end-diastolic pressure and heart rate did not vary significantly between groups. Because integrins can link transmission of force from the ECM to the cellular cytoskeleton, we challenged the \(\beta^1\)KO mice by hemodynamic loading through TAC (Figure 3E). After 7 days of aortic
constriction, only 53% of the β1KO mice survived, whereas 88% survival was found in hemodynamically loaded β1Flox/Flox/MLC2v+/+ mice. No mortality occurred in sham-operated animals. These data suggested that β1 integrin is required for the murine ventricle to maintain normal function and that excessive hemodynamic demands are not tolerated by the β1 integrin-deficient ventricle.

When the β1 integrin-deficient mice reached ~6 months of age, many of the animals showed clinical signs of congestive heart failure, including pleural effusions and liver congestion, and died spontaneously. Similar findings were evident in many β1KO postpartum female animals. Representative 6-month-old animals were examined pathologically, histologically, and echocardiographically. Histologically, and echocardiographically. Histologically, hearts showed dilation, replacement fibrosis, and even calcification within the ventricular wall and thrombi in enlarged atria (Figures 4A and 4B). M-mode echocardiography showed left ventricular chamber dilation, increased septal and free wall thicknesses, and depressed ventricular function (reduction in both percentage fractional shortening and septum and free wall thicknesses, and depressed ventricular function (reduction in both percentage fractional shortening and septum and free wall thicknesses, and depressed ventricular function (reduction in both percentage fractional shortening and septum and free wall thicknesses, and depressed ventricular function (reduction in both percentage fractional shortening and septum and free wall thicknesses, and depressed ventricular function (reduction in both percentage fractional shortening and septum and free wall thicknesses, and depressed ventricular function (reduction in both percentage fractional shortening and septum and free wall thicknesses, and depressed ventricular function (reduction in both percentage fractional shortening and septum and free wall thicknesses, and depressed ventricular function (reduction in both percentage fractional shortening and septum and free wall thicknesses, and depressed ventricular function (reduction in both percentage fractional shortening and septum and free wall thicknesses, and depressed ventricular function (reduction in both percentage fractional shortening and septum and free wall thicknesses, and depressed ventricular function (reduction in both percentage fractional shortening and septum and free wall thicknesses, and depressed ventricular function (reduction in both percentage fractional shortening and septum and free wall thicknesses, and depressed ventricular function (reduction in both percentage fractional shortening and septum and free wall thicknesses, and depressed ventricular function (reduction in both percentage fractional shortening and septum and free wall thicknesses, and depressed ventricular function (reduction in both percentage fractional shortening and septum and free wall thicknesses, and depressed ventricular function. 8 weeks of age (n=5).

Distribution of Talin and Membrane Permeability Are Disrupted, but No Increase in Apoptosis Is Detected in β1KO Mouse Hearts

Because integrins link the ECM and cellular cytoskeleton and could stabilize cell membranes, we evaluated whether β1 integrin-deficient mice had altered membrane or actin binding protein characteristics. Talin binds directly to the β1 integrin cytoplasmic domain. Immunostaining of heart tissue was performed with anti-β1 integrin and anti-talin antibodies (Figure 5A). Both β1 integrin and talin staining were uniformly visualized in the membranes of myocytes from control mice. In myocardial sections from the β1Flox/Flox/MLC2v+/+ mice, although residual β1D integrin protein was detected in some myocytes, its pattern even in those cells was non-uniform. This result was in agreement with our Western blot analyses (above). Normally even distribution of talin expression was also disrupted in the β1 integrin-deficient myocytes.

We performed EBD and WGA staining to detect membrane abnormalities in the myocardium. None of the cardiac myocytes in control mice were permeated by EBD (Figure 5B). Yet in multiple regions of the β1Flox/Flox/MLC2v+/+ mouse heart, strong red staining indicated that EBD-albumin complexes had entered these myocytes through permeant cell membranes. Similarly, imaging of control mouse myocardium with F-WGA demonstrated intact staining of myocyte cell membranes, whereas β1KO mouse heart sections showed disruption of cell membrane integrity.

Because disruption of cell adhesion can lead to apoptosis and decreased integrin expression can reduce cell adhesion, we evaluated for apoptosis in the β1KO mice. Ventricular DNA from our β1KO mice did not reveal DNA laddering (data not shown), nor were an increased number of TUNEL-positive myocytes detected in hearts of these mice (7 ± 1.7 [β1KO] versus 9.2 ± 1.9 [control] TUNEL-positive myocytes/10,000 myocytes; n=3 each group).

Discussion

Unlike the early embryonic lethality of the traditional β1KO mice, our cardiac myocyte-specific β1KO mice survived to...
adulthood likely because some β1 integrin protein was still present. Residual protein was present because of the incomplete nature of Cre-mediated excision effected by MLC2v/Cre or αMHC-Cre, and is in agreement with previous published work that used these two Cre mice to effect excision of the connexin 43 and gp130 genes. It is likely that further reduction of β1 integrin protein in the cardiac myocyte below a yet-unidentified “critical level” would result in abnormal cardiac development and embryonic lethality. This is currently being tested by mating our β1 integrin–floxed mice with animals that may express Cre in a more efficient manner currently being tested by mating our MHC-Cre mice to our Lox/Lox mice. This is cur-

these findings demonstrate that β1 integrin is an important mechanotransducer in the cardiac myocyte as in other cell types. Appropriate linkage of ECM and cytoskeleton through β1 integrins is essential for preservation of myocyte function. When β1 integrin is reduced at the cell surface, this linkage is disrupted.

In older adult mice, we noted increased amounts of myocardial fibrosis and also found that the mice developed a dilated cardiomyopathy by 6 months of age. There was no consistent pattern of fibrosis within the ventricular wall, although some mice were found that displayed it in a subepicardial manner. Myocytes in the β1KO mice displayed membrane abnormalities as detected by positive EBD staining. We hypothesize that the ECM-cytoskeletal linkage, which in part occurs through integrins, is essential for maintenance of the cardiac myocyte, which undergoes continual hemodynamic demands in the beating myocardium. When the integrin is lost from the membrane, this important equilibrium is altered, myocyte integrity is compromised in a progressive manner, and myocyte necrosis occurs, leading ultimately to fibrotic replacement in the areas of the ventricle with myocyte loss. Cell adhesion has been suggested to be required for cell survival. More specifically, loss of adhesion to the ECM has been linked to cell apoptosis, a process termed anoikis. It is for this reason that we tested for accelerated apoptosis in our KO mice, but no increase over control values was detected at the time assessed. It is likely that the cardiomyopathy resulted not only from replacement...
fibrosis of myocytes, but also from altered mechanotransduction of the β1 integrin–deficient myocytes. Deficiency of β1 integrin would lead to alterations in numerous αβ1 integrin heterodimer pairs, including α1β1. It is interesting to note that α1 integrin–null mice have increased expression of several matrix metalloproteinases (MMPs). 25 MMPs are linked to the remodeling that occurs during the development of cardiomyopathies, and thus it is possible that alterations in these molecules may also be present in our knockout mice. This is currently being investigated.

**Parallels can be drawn between these integrin KO mice and changes that occur in muscular dystrophy. Like integrins, the dystrophin-glycoprotein complex also links the ECM and intracellular cytoskeleton. With loss of components of this complex, alteration in membrane permeability is seen and cardiomyopathy can result. 17,26 Although integrin mutations have been linked to skeletal muscle dystrophies in animal models and in humans, they have not been previously linked to cardiomyopathy. 27–31 Further, it is interesting to note that in muscles from mdx mice and patients with Duchenne and Becker dystrophies, increased expression of α7 integrin transcript and protein were detected. Thus, integrins may compensate for absence of components of the dystrophin-dystroglycan complex. This has been recently shown in a mouse model of muscular dystrophy in which overexpression of α7 integrin improved many of the clinical features of the disease. 32 Further, α7 integrin–null mice develop progressive skeletal muscular dystrophy, although cardiac function has not been studied in this model. In contrast to integrin upregulation in the absence of dystrophin-dystroglycan complex proteins, no compensatory change in dystrophin, dystroglycan, or α-sarcoglycan was detected in the α7 integrin–null background. 31 Thus, this model gives further credence to the importance of integrins as an indispensable linkage between muscle fiber (and perhaps cardiac myocytes) and the ECM.**
This linkage appears independent of the dystrophin-dystroglycan complex–mediated interaction of the cytoskeleton with the muscle basement membrane. Thus, there are clearly distinct functions of these two types of transmembrane proteins. This is emphasized by recent data that found that dystroglycan was necessary for initial binding of laminin to the cell surface, whereas β1 integrin was subsequently required for laminin matrix assembly after the binding.33 We would suggest that integrin mutations could ultimately be detected in a subset of cardiomyopathy patients.

The strategy we used to target exons 2 and 3 of the β1 integrin gene reduces all isoforms of this protein. In the heart, the dominant postnatal isoform of β1 integrin is β1D.6 Previous work has shown that β1D cannot substitute for β1A integrin in all organs; doing so leads to fetal lethality with multiple developmental defects.34 Conversely, full ablation of β1A or β1D as the prime cause of the resultant phenotype. Studies are currently underway to test the ability of cardiac-specific expression of β1A or β1D, to alter the β1KO phenotype.

In summary, we have produced a murine model with cardiac-specific β1 integrin deficiency resulting in abnormal cardiac function and cardiomyopathy. This animal model will be useful for determination of integrin function in the myocardium and other tissues.

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