Ventricular Expression of a MLC-2v-ras Fusion Gene Induces Cardiac Hypertrophy and Selective Diastolic Dysfunction in Transgenic Mice*

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Cardiac muscle hypertrophy is one of the most important compensatory responses of the heart (1, 2). Long standing hypertension, myocardial injury, or increased cardiac work elicit a hypertrophic response, which permits the myocardium to adapt appropriately by increasing cardiac muscle mass. This increase in mass is due to an enlargement of individual muscle cells and is not accompanied by a proliferative response. Although this process is initially compensatory, there can be a pathological transition in which the myocardium becomes dysfunctional. In this regard, the onset of hypertrophy is a well accepted prognostic indicator for subsequent cardiac dysfunction and morbid events (3). Although the signaling pathways that mediate the development of hypertrophy and the transition to overt heart failure have been the subject of numerous descriptive studies, proof of their involvement has been lacking due to the absence of in vivo animal model systems that, by altering intracellular signal transduction in the myocyte itself, faithfully reproduce key features of human cardiac hypertrophy.

To approach this problem, we have recently characterized mouse model systems that allow use of genetically based approaches to study this physiological response in the in vivo context. Recent studies have established that the mouse is a valid model system to study cardiac muscle hypertrophy and dysfunction in vivo (4–7), which conserves many features of larger mammalian model systems. By fusing constitutively active mutants of candidate signaling proteins to cardiac muscle promoters that can target expression to specific cardiac chambers in transgenic mice, the generation of genetically based mouse models of cardiac muscle hypertrophy and failure appears to be entirely feasible (8, 9), as has been shown for cardiac myocyte proliferation (8, 10). The availability of miniaturized technology, microsurgical approaches, and techniques to quantitatively assess in vivo murine cardiac physiology, offers the further possibility of discriminating among various forms of hypertrophy and categorizing their effects on function (9). In addition to the in vivo physiological techniques used here, other investigators have demonstrated the feasibility of applying other physiological assays to the mouse heart, including the isolated heart preparation (11) and the analysis of isolated myocytes (12).

Utilizing a well characterized cultured cardiac myocyte model system (13–17), three independent signaling pathways (ras (18), Gq (13), and gp130 (19)),1 have recently been implicated in the activation of key features of the hypertrophic response in vitro (19). The question remains as to whether the activation of any of these signaling molecules is sufficient to generate a hypertrophic response in vivo, and whether it would induce concomitant cardiac dysfunction and/or specific subsets of cardiac hypertrophy and cardiomyopathy that have been well defined in clinical human disease. To examine whether ras-dependent pathways are sufficient to activate a hypertrophic response in the in vivo context, in the current study the transgenic approach was used to target the expression of oncogenic ras into the mouse cardiac ventricular chamber under the control of the MLC-2v promoter.

1 K. Wollert and K. R. Chien, submitted for publication.
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EXPERIMENTAL PROCEDURES

Transient Transfection Studies—pMLCSVOA utilizes the promoter-less luciferase vector pSVOALΔS (20) as a backbone, which contains an SV40 polyadenylation signal upstream of luciferase to prevent read-through transcription from cryptic promoters; the luciferase structural gene was excised as a HindIII-SacI fragment. The 250-bp MLC-2v promoter fragment was excised from pMLCΔS (21) as a HindIII-EcoRI fragment and subcloned into pBluescript SK+ (Stratagene); the promoter and polylinking site was re-excisected as a HindIII-SacI fragment and inserted into the backbone vector above. The luciferase polyadenylation signal/site was duplicated as a synthetic dinucleotide and subcloned between the SacI and SacII sites to yield pMLCSVOA.

Transient transfection studies in neonatal rat ventricular myocytes were performed by calcium phosphate precipitation as described (22). 8 μg of plasmid were transfected with 2 μg of ANF luciferase reporter plasmid and 1 μg of cytomegalovirus promoter-β-galactosidase plasmid (to correct for differences in transfection efficiency). Cells were harvested 72 h after transfection and analyzed for luciferase activity. Fold induction is expressed as the ratio of the luciferase value (corrected for β-galactosidase) for the active plasmid construct to that for the backbone vector pMLCSVOA. Each experiment was performed in triplicate, and the results of three experiments were pooled.

Reverse Transcriptase-Polymerase Chain Reaction—Total RNA was prepared from various flash-frozen tissues from representative line F21 (23), where 3-day-old pups were anesthetized with pentobarbital (50 mg/kg administered intraperitoneally). Hearts were arrested in diastole with 1 M KCN and fixed at constant pressure (aortic root, 60 mm Hg, left atrium, 5 mm Hg). For histologic studies, fixation was performed using the SuperPrep protocol (Life Technologies, Inc.), with random primers. After RNase H digestion, PCR was carried out using a set of transgene-specific primers (sense, 5'-CTCTTGTGATCTCCTGACCC-3' (5'-untranslated MLC-2v with upstream polylinking sequence); antisense, 5'-CTCTTGCCCGCGT-ATCCAG-3' (bases 166–186 of human coding sequence, 57% homology to mouse), fragment size 243 bp). PCR was carried out as follows: 1 cycle of 94 °C for 4 min., 27 cycles of 94 °C for 1 min/58 °C for 1 min/72 °C for 2 min., and 1 cycle of 72 °C for 10 min. At 27 cycles, band intensity is increasing exponentially (data not shown); thus, relatively high band intensity reflects relative amounts of substrate cDNA/mRNA. One half of the PCR reaction was analyzed on a 2% agarose gel, and transferred to nylon membranes (Magna NT, Micron Separations, Inc.) by capillary blotting. After prehybridization (QuickHyb, Stratagene), membranes were hybridized with a 32P-labeled probe (specific for the 5' end of the transgene) generated by random priming (PrimeII, Stratagene), washed to a final stringency of 0.1 × SSC, 1% SDS, 55 °C, and exposed to film. Densitometry was performed on an Ultrascan LKB laser densitometer, and normalized for cDNA concentration to a separate PCR (same RT reaction) using primers for the ubiquitin mRNA for β-tubulin (not shown).

Histological Analysis—Animals were sacrificed using an overdose of pentobarbital (50 mg/kg administered intraperitoneally). Hearts were arrested in diastole with 1 k KCl and fixed at constant pressure (aortic root, 60 mm Hg, left atrium, 5 mm Hg). For histologic studies, fixation was in 10% formalin; after paraffin embedding, serial 1 μm sections were obtained. For cell size determination, fixation was in 4% paraformaldehyde, 1% glutaraldehyde; tissue was prepared, and ventricular midwall myocyte cross-sectional area was determined as described (24). An average of 238 cells/heart were measured.

Left Ventricular Hemodynamics—Animals were anesthetized with a single dose mixture of ketamine (100 mg/kg intraperitoneally) and xylazine (5 mg/kg intraperitoneally). After bilateral vagotomy, LV pressure was measured in the anesthetized, open-chest animal with a 2-French high fidelity micromanometer catheter, inserted through the LA, and advanced into the LV (23). Continuous aortic pressure, LV systolic and diastolic pressures, and LV dp/dt were recorded on an eight-channel chart recorder and in digitized form for beat averaging (codas, Dataq Instruments, Akron, OH). Ten sequential beats were averaged for each measurement. A sampling rate of 1000 Hz for LV pressure was required for accurate measurement of dp/dt, which in turn was necessary for calculation of VR; VR was calculated by fitting the decay of LV pressure (from dp/dtmax) to a monoexponential equation assuming a zero asymptote (i.e. that the ventricle would ultimately relax to zero transmural pressure). The equation simplifies to P = P0 exp (-UT), where P0 is pressure at time zero (dp/dtmax) and T is: T = VR

2 The abbreviations used are: bp, base pair(s); PCR, polymerase chain reaction; ANF, atrial natriuretic factor; RT, reverse transcriptase; LV, left ventricle; RV, right ventricle; HCM, hypertrophic cardiomyopathy.
which 5 had integration of the transgene detectable by screening tail DNA by PCR using a 5' primer specific for the MLC-2v promoter and a 3' primer in the Ha-ras cDNA. PCR screening of the founders was confirmed by genomic Southern blotting, which also was utilized to identify mice homozygous for the MLC-ras transgene in subsequent progeny; homozygosity was confirmed in several animals by outcrosses with wild type breeders. Three founders transmitted the transgene. One line (F21) was chosen for analysis, and a second line (F48) showed an identical phenotype to F21, while that of a third line (F26) displayed a qualitatively similar, but less prominent phenotype. As assessed by RT-PCR analysis (Fig. 2, A and B), the expression of the MLC-ras transgene in line F21 was predominantly restricted to the cardiac ventricles; the RV level from a +/- animal was approximately 85% of that seen in the LV, while the atria displayed <5% of the ventricular level. No detectable expression was present in uterus, lung, or spleen; the level in skeletal muscle was slightly greater than that in the atrium (all corrected for β-tubulin cDNA); liver, kidney, and brain had lower but detectable levels (all corrected for β-tubulin cDNA). Expression in the LV of a +/- animal was 25% of that in the +/- LV.

To monitor for the presence of a hypertrophic phenotype, we utilized three independent criteria: an increase in chamber mass (normalized to body weight), an enlargement in myocardial cell size, and the activation of a marker of the embryonic gene program (the reactivation of ANF gene expression in the ventricular chamber). These represent conserved features of the hypertrophic response found in large mammalian species (including humans), as well as in the hypertrophied mouse ventricular chamber following pressure overload in vivo (4–7). Mice (line F21) heterozygous (+/-) for the MLC-ras transgene showed a modest yet statistically significant 7.8% increase in LV mass (3.75 ± 0.07 mg/g body weight in +/- (n = 49), versus 3.48 ± 0.13 mg/g in +/- (n = 27)), while mice homozygous for the MLC-ras transgene (+/+) exhibited a marked 57.5% increase (5.48 ± 0.14 mg/g (n = 4)) (Fig. 3). The magnitude and pattern of increase in chamber mass was similar in line F48, although the increase in LV mass in line F26 +/- was approximately 30% (data not shown). There were no differences in body weight among any of the experimental groups, indicating that the increase in LV/body weight ratio primarily reflected an increase in the mass of the left sided cardiac chambers. This increase in LV mass was substantially greater than that seen in wild type mice exposed to in vivo pressure overload (average 41% increase in whole heart weight; Refs. 4 and 5) and is consistent with the activation of a hypertrophic response in the left ventricular chamber. In contrast to the LV, only a trend toward an increase in RV mass, which did not reach statistical significance, was observed in the homozygous MLC-ras mice, indicating that the effect of ras was selective for LV chamber. Interestingly, left atrial mass was also significantly increased (175% versus wild type, 0.37 ± 0.05 mg/g versus 0.13 ± 0.01), while right atrial mass was not different between groups. Since the MLC-ras transgene was not significantly expressed in the atrial compartment, the increase in LA mass is most likely not secondary to a direct effect of ras on the atrial hypertrophic response. However, this finding raised the possibility that the increase in LA mass was secondary to chronic exposure of the

**Fig. 2. Tissue expression of the transgene.** A, chamber-restricted expression of the transgene. RT-PCR products amplified from transgene-specific primers were transferred to a nylon membrane and probed with a transgene-specific DNA probe. LV samples are shown from a homozygote (+/+) animal, a heterozygote (+/-), and a wild type (-/-); RV and atrial (A) samples are shown from the homozygote. B, tissue-restricted expression. Using the same RT-PCR as in panel A, comparison is made between LV and other tissues from a homozygote. Sk, skeletal muscle; Ut, uterus; Lu, lung; Ki, kidney; Li, liver; Sp, spleen; Br, brain. The exquisite detectability of cDNA by Southern blotting of RT-PCR products explains the apparent difference in tissue selectivity between these transgenic mice and those assayed for the presence of the reporter protein luciferase (26) or β-galactosidase (R. Ross and K. Chien, unpublished observations); these lines have demonstrated that at the protein level, gene expression directed by the MLC2v promoter is higher in the ventricular compartment by at least 2 orders of magnitude (26).

**Fig. 3. Chamber wet weights.** Data are shown from 3 litters; n = 4 for each group. RA, right atrium; RV, right ventricle; LA, left atrium; LV, left ventricle. Data shown are mean ± S.E. and were analyzed with single factor analysis of variance with post hoc tests based on t test with Bonferroni correction for three comparisons (*, p < 0.05 versus both other groups; †, p < 0.05 versus wild type; ‡, p < 0.001 versus both other groups).
Histology. A, transverse sections of hearts fixed at constant pressure (11) at the papillary muscle level, showing gross left and right ventricular hypertrophy in a +/+ animal (right), compared to a −/− littermate (left). Hematoxylin/eosin stain was used. B, transverse sections of hearts from +/+ and −/− animals, fixed and stained as in A, were projected at constant magnification and traced by two observers blinded to genotype, and the area occupied by ventricular myocardium was measured by planimetry. Data shown as mean ± S.E. and analyzed by t test (*, p = 0.05). C, section demonstrating focus of myocyte disarray (right) and a comparable area of a nearby section (10 μm away) from the same heart (as a control). Both sections are from the interventricular septum of the +/+ mouse in Fig. 4A, above. Hematoxylin/eosin stain, 100 × magnification, is shown.

Evidence for tumorigenesis was seen, and there was no significant perivascular, interstitial, or subendocardial fibrosis. However, as seen in Fig. 4C, focal areas of myocyte disarray were evident in 2 of 8 +/+ hearts studied (one sectioned completely from base to apex, the others only sectioned at 100-μm intervals from midwall to base); this histologic picture is virtually indistinguishable from that found in human hypertrophic cardiomyopathy.

To determine if the left ventricular hypertrophy is associated with activation of embryonic genes (a marker of the hypertrophic response; Refs. 29 and 30), RNA from −/−, +/−, and +/+ animals was analyzed for ANF expression by Northern blotting (Fig. 5). As in other models of hypertrophy, there was a profound increase in ANF mRNA in the +/+ group, with no significant increase in +/− animals compared to −/−.

To assess cardiac function, in vivo LV pressures and the maximal first derivative of LV pressure (dP/dtmax) were obtained in the open-chest mouse. Peak LV systolic pressure was not different between +/+ and −/− littermates (Fig. 6A), and no significant differences were observed in LV end-diastolic pressure (Fig. 6B) or LV dP/dtmax (an index of contractile state, Fig. 6C); therefore, the hypertrophy observed in the +/+ MLCK-ras mice was not simply due to impaired cardiac contractile function. To assess the effect of hypertrophy on relaxation, the time constant of isovolumic LV pressure decay (τ) was determined (Fig. 6D) (24). τ is the most frequently used index of relaxation time constant of isovolumic LV pressure decay (τ) was determined (Fig. 6D) (24). τ is the most frequently used index of relaxation.
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**DISCUSSION**

In summary, the present study provides direct evidence that ras is sufficient to activate a hypertrophic response in cardiac muscle in the *in vivo* context. Activation of the hypertrophic response has been monitored by several independent criteria, including an increase in LV mass/body weight ratio, increased myocardial cell size, and increased expression of an embryonic genetic marker of the hypertrophic response, ANF. The increase in these structural, morphological, and genetic markers of hypertrophy achieved by targeting oncogenic ras expression to the ventricular muscle cells are qualitatively similar to those seen in hypertrophy due to pressure overload in murine myocardium, but they occurred in the absence of concomitant valvular disease, hypertension, or other systemic effects. The hypertrophic response was more marked, however, in the +/+ mice than in mice with pressure overload, except when the overload was very severe (100 mm Hg trans-stenotic gradient during thoracic aortic constriction; Ref. 7). Analysis of *in vivo* cardiac physiology in these mice has demonstrated an effect of ras expression to produce diastolic dysfunction, as assessed by altered LV relaxation and a selective increase in left atrial (versus right atrial) mass (consistent with a chronic increase in LV diastolic pressure). In contrast, basal and β-adrnergic-stimulated systolic contractile function remained normal. These effects of ras may be direct, or some of them may be mediated, at least in part, by autocrine/paracrine factors or by cross-talk with other signaling pathways. There also appears to be a threshold effect, such that a certain amount of activated ras is necessary for the hypertrophic response, since animals heterozygous for the transgene had very little hypertrophy.

These mice exhibited a functional phenotype that in most animals is qualitatively similar to that seen in compensated human hypertensive heart disease. The selective diastolic dysfunction, in association with hypertrophy resulting from a primary cardiac muscle disorder (without accompanying hypertension or valvular disease), bears functional similarity to that seen in human hypertrophic cardiomyopathy (HCM); likewise, the myocyte disarray seen in at least some +/+ mice is characteristic of HCM, although it has been described less frequently in other forms of human hypertrophy. The findings in this murine model suggest a role for ras-dependent pathways in the genesis of cardiac hypertrophy, as well as in the transition between compensatory hypertrophy and the onset of cardiac muscle dysfunction and the development of myocyte disarray. These mice should be valuable in genetically based approaches to identify further downstream signaling pathways that mediate this form of cardiac muscle dysfunction, using molecular physiological analysis to characterize the functional phenotype. While this transgenic mouse does not model the human familial HCM genotype per se, nevertheless, it may provide useful insights into the molecular mechanisms that lead to cardiac ventricular hypertrophy and the onset of diastolic dysfunction, which are important entities in the clinical disease process. While familial HCM in humans can be caused by mutations in sarcomeric protein genes, the mechanisms by which these mutations lead to the activation of the hypertrophic phenotype is unclear. Our current studies suggest the possibility that ras-dependent pathways might play a role, either directly or indirectly, in this pathological process. As a genetically based model of cardiac muscle disease, these mice should now permit dissection of the interaction of ras with retinoids and other signaling pathways, through genetic crosses with other transgenic strains, as well as physiological and pharmacological manipulations which induce or impair the development of hypertrophy. Furthermore, they identify ras and subsequent downstream signaling pathways as potential targets for interrupting the pathological process of hypertrophy in the *in vivo* context. In fact, based on these observations, we have recently demonstrated that agents that can block the oncogenic effects of ras, such as retinoids, can serve as suppres-

**Fig. 5.** Northern blot of LV total RNA, using rat ANF probe. −/−, wild type; +/−, heterozygous for transgene; +/+ homozygous for transgene. Each lane represents RNA from one animal; similarity of RNA loading is shown by methylene blue staining of the 28 S ribosomal RNA band.

**Fig. 6.** Hemodynamic measurements performed in the open-chest animal. Responses are shown for the basal and peak dose of isoproterenol. A, peak LV systolic pressure; B, LV end-diastolic pressure; C, maximum dP/dt; D, τ (13). Data shown are mean ± S.E.; basal values for +/+ are significantly different between homozygotes and wild type mice (*, p < 0.05, t test).
sors of myocardial cell hypertrophy in an in vitro model system (33), supporting the potential therapeutic importance of these observations.

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