Cytoskeletal Role in the Transition From Compensated to Decompensated Hypertrophy During Adult Canine Left Ventricular Pressure Overloading

Hirofumi Tagawa, Masaaki Koide, Hiroshi Sato, Michael R. Zile, Blase A. Carabello, George Cooper IV

Abstract—Increased microtubule density causes cardiocyte contractile dysfunction in right ventricular (RV) pressure-overload hypertrophy, and these linked phenotypic and contractile abnormalities persist and progress during the transition to failure. Although more severe in cells from failing than hypertrophied RVs, the mechanical defects are normalized in each case by microtubule depolymerization. To define the role of increased microtubule density in left ventricular (LV) pressure-overload hypertrophy and failure, in a given LV we examined ventricular mechanics, sarcomere mechanics, and free tubulin and microtubule levels in control dogs and in dogs with aortic stenosis both with LV hypertrophy alone and with initially compensated hypertrophy that had progressed to LV muscle failure. In comparing initial values with those at study 8 weeks later, dogs with hypertrophy alone had a very substantial increase in LV mass but preservation of a normal ejection fraction and mean systolic wall stress. Dogs with hypertrophy and associated failure had a substantial but lesser increase in LV mass and a reduction in ejection fraction, as well as a marked increase in mean systolic wall stress. Cardiocyte contractile function was equivalent, and unaffected by microtubule depolymerization, in cells from control LVs and those with compensated hypertrophy. In contrast, cardiocyte contractile function in cells from failing LVs was quite depressed but was normalized by microtubule depolymerization. Microtubules were increased only in failing LVs. These contractile and cytoskeletal changes, when assayed longitudinally in a given dog by biopsy, appeared in failing ventricles only when wall stress began to increase and function began to decrease. Thus, the microtubule-based cardiocyte contractile dysfunction characteristic of pressure-hypertrophied myocardium, originally described in the RV, obtains equally in the LV but is shown here to have a specific association with increased wall stress. (Circ Res. 1998;82:751-761.)

Key Words: heart failure ■ myocardial contraction ■ cytoskeleton ■ microtubule

A major long-term focus of this laboratory has been the attempt to establish a molecular basis for the contractile defects characteristic of myocardium hypertrophying in response to a pressure overload. We have now established in the feline right ventricle (RV) that the contractile defect originally observed at the tissue level1 is intrinsic to the isolated cardiac muscle cell,2 or cardiocyte, and that this defect is accounted for by an increased density of the microtubule component of the extramyofilament cytoskeleton.3,4 Furthermore, we have found that upregulation of α-tubulin and β-tubulin, the major microtubule proteins, on both the message and the protein levels persists not only during prolonged, functionally compensated RV pressure-overload hypertrophy5 but also after the transition to RV failure.6 Importantly, the contractile defects of cardiocytes isolated from RVs either with compensated hypertrophy alone or with associated decompensated failure were fully reversed after microtubule depolymerization. Thus, both the increased microtubule density and the associated contractile defects observed in compensated pressure-overload RV hypertrophy persist during, and thus potentially contribute to, the eventual development of right heart failure in this model when the pressure overload is severe.

In the present study, we turned our attention from feline RV hypertrophy in response to a fixed pressure overload to canine left ventricular (LV) hypertrophy in response to a progressive pressure overload. This was done for four reasons. First, since the ultimate goal of these studies is to gain insight into the causes of human heart failure, we wished to concentrate on the potential role of microtubules in the cardiac chamber wherein the great majority of clinical pathophysiology occurs. Second, we wished to generate an animal model in which the characteristics of pressure-overload induction were closely analogous to those seen clinically.
Third, using the LV of a large animal, wherein ventricular geometry and contractile function are readily characterized, allowed us to correlate ventricular and cellular mechanics in the same animal and thus establish the potential role of any microtubule-related cardiocyte contractile defects in ventricular contractile dysfunction. Fourth, this large animal model provided the opportunity to perform serial LV biopsies in defined settings of ventricular load and function, such that ventricular and cellular contractile properties and any underlying changes in tubulin synthesis and assembly could be related directly to progressive changes in LV mass, load, and wall stress.

**Materials and Methods**

**Experimental Model**

This model of canine LV hypertrophy in response to a progressive pressure overload was newly devised for the purposes of the present study; it was defined in some detail in a previous study from this laboratory. The two design criteria were as follows: (1) a progressive and readily controllable increase in afterload to a level comparable to that causing significant clinical pathophysiology in humans and (2) a resultant increase in LV mass comparable to that known to be associated with ventricular dysfunction in the setting of clinical LV pressure overload. Both of these criteria were met.

In brief, the 23 mongrel dogs of random sex aged 1 to 5 years that were used for the present study were characterized in terms of hemodynamics and LV mechanics before and at the time of aortic band placement. They were studied again at 2, 4, 6, and 8 weeks after banding; at each study except the last, the degree of aortic stenosis was increased. Echocardiography showed that the LV hypertrophic response after each increase in afterload was complete within the 2-week period before the marked augmentation of the degree of aortic stenosis. The band was a catheter-based externally controllable supracoronary ascending aorta constrictor, which was placed via a right thoracotomy and sectioned (Vibratome 3000, Technical Products, Inc) in Ca2+-free buffer containing 30 mmol/L 2,3-butanediol monoxime into 200- to 400-μm slices, which were treated in the same manner as the 2-mm LV cubes. In each case, cells were kept at 37°C for 1 hour and pH 7.4 in collagenase-free 2.5 mmol/L Ca2+ buffer before defining contractile function.

**Cardiocyte Microtubules in Left Ventricular Failure**

**Cardiocyte Mechanics**

The use of laser diffraction techniques for measuring sarcomere motion in isolated cardiocytes is well established; an outline of our method is as follows: An aliquot of isolated cells was added to 4 mL of the 2.5 mmol/L Ca2+ buffer in a well that was affixed to a glass slide. The cardiocytes came to rest on the bottom of this chamber, which was placed on the stage of an inverted microscope. The buffer was kept at 37±0.1°C by a thermostated heating stage. Only cardiocytes with the following characteristics were analyzed: single rod-shaped cells unattached to adjacent cells that contracted with each stimulus and were quiescent between stimuli. The cardiocytes were stimulated to contract between platinum wire electrodes by 0.25-Hz 100-μA DC pulses of alternating polarity. When after 10 to 15 contractions the extent of shortening was stable, 10 contractions were sampled and averaged to yield a final profile of sarcomere length and velocity versus time during contraction. Changes in sarcomere length were measured from movement of the first-order diffraction pattern cast by a stage laser light passing through the sarcomeres of a given cardiocyte onto diametrically opposed optical sensors situated above the microscope stage. Each sensor contained of a linear array of 256 photodiodes that was interrogated at a frequency of 1 kHz. The distance between the first-order diffraction patterns at every millisecond was then calculated by and stored in a computer.

After baseline sarcomere mechanics were evaluated, colchicine, which causes microtubule depolymerization, was added to the superfusate at a final concentration of 10−4 mol/L. Sarcomere mechanics were then assessed in each of a number of cardiocytes; each cell was studied sequentially at intervals of 10, 20, 30, 45, and 60 minutes after drug exposure. In a given sample, it was usually possible to define mechanical behavior using this protocol for two or three cardiocytes before the baseline sarcomere mechanics of a cardiocyte isolate began to change.

**Immunobots**

**Tubulin Protein**

For the immunoblot analysis, fresh 0.25-g LV specimens were homogenized in 5 mL of microtubule stabilizing buffer and were used to calculate LV mass, volume, pressure, ejection fraction, mean normalized systolic ejection rate (calculated as ejection fraction divided by ejection time), and mean systolic midwall stress (derived by averaging the stresses calculated frame by frame during systole). The basis for these measurements is given elsewhere.

**Experimental Model**

This model of canine LV hypertrophy in response to a progressive pressure overload was newly devised for the purposes of the present study; it was defined in some detail in a previous study from this laboratory. The two design criteria were as follows: (1) a progressive and readily controllable increase in afterload to a level comparable to that causing significant clinical pathophysiology in humans and (2) a resultant increase in LV mass comparable to that known to be associated with ventricular dysfunction in the setting of clinical LV pressure overload. Both of these criteria were met.

In brief, the 23 mongrel dogs of random sex aged 1 to 5 years that were used for the present study were characterized in terms of hemodynamics and LV mechanics before and at the time of aortic band placement. They were studied again at 2, 4, 6, and 8 weeks after banding; at each study except the last, the degree of aortic stenosis was increased. Echocardiography showed that the LV hypertrophic response after each increase in afterload was complete within the 2-week period before the marked augmentation of the degree of aortic stenosis. The band was a catheter-based externally controllable supracoronary ascending aorta constrictor, which was placed via a right thoracotomy and sectioned (Vibratome 3000, Technical Products, Inc) in Ca2+-free buffer containing 30 mmol/L 2,3-butanediol monoxime into 200- to 400-μm slices, which were treated in the same manner as the 2-mm LV cubes. In each case, cells were kept at 37°C for 1 hour and pH 7.4 in collagenase-free 2.5 mmol/L Ca2+ buffer before defining contractile function.
by guest on July 12, 2017 http://circres.ahajournals.org/ Downloaded from
glycerol in phosphate-buffered saline, and
They were then mounted with 1% triethylenediamine and 50%
fluorescein-conjugated secondary antibody (Vector Laboratories).

Indirect Immunofluorescence Micrographs
For visualization of the cardiocyte microtubule network, isolated
cardiocytes were sedimented onto laminin-coated coverslips at 1
min

<table>
<thead>
<tr>
<th>Characteristics of the Model</th>
<th>Hypertrophy (n=9)</th>
<th>Failure (n=14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV end-diastolic pressure, mm Hg</td>
<td>9.0±0.6</td>
<td>12.2±1.5</td>
</tr>
<tr>
<td>LV end-diastolic volume, mL/kg</td>
<td>2.75±0.18</td>
<td>2.64±0.14</td>
</tr>
<tr>
<td>Ejection fraction, %</td>
<td>73.0±2.1</td>
<td>77.3±3.1</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>21.9±0.6</td>
<td>21.1±0.6</td>
</tr>
<tr>
<td>9.7±0.9</td>
<td>16.4±1.1*‡</td>
<td></td>
</tr>
<tr>
<td>2.91±0.17</td>
<td>3.23±0.21‡</td>
<td></td>
</tr>
<tr>
<td>58.0±1.8‡</td>
<td>49.0±2.8‡</td>
<td></td>
</tr>
<tr>
<td>23.0±0.7</td>
<td>22.2±0.7</td>
<td></td>
</tr>
</tbody>
</table>

LV indicates left ventricular. After a one-sample Kolmogorov-Smirnov test showed that the data for each of these values were normally distributed, parametric statistical comparisons were made first via two-way ANOVA. If a difference was found, further comparisons were made via one-way ANOVA followed by the Bonferroni/Dunn procedure. A nonparametric Kruskal-Wallis one-way ANOVA by ranks confirmed the existence and rank order of significant differences among these values for the categories wherein a significant difference had been found by parametric analysis. Values are mean±SEM.

* P<.05 for a difference from the baseline value within either the hypertrophy group or the failure group; † P<.05 for a difference between baseline values for the hypertrophy vs failure groups; and ‡ P<.05 for a difference between final values for the hypertrophy vs failure groups.

Data Analysis
The mean±SEM values are shown for each group of data. Differences in selected measures were evaluated via either a paired or unpaired Student’s t test, as appropriate, with a significant difference said to exist at the level specified for each set of data. Where stated, group means were first compared by a one-way or two-way ANOVA, and if a difference was found, then each experimental mean was compared with that of the control group by the appropriate post hoc test as individually specified.

Results
Characteristics of the Experimental Model
The major features of the surgical model used in the present study are summarized in the Table and in Figures 1 and 2. Most notably, as treated specifically in our previous report that focused on the clinical relevance of the characteristics of this model,7 the present group of randomly selected mongrel dogs self-segregated into two distinct sets; this occurred despite the fact that there was no gender difference between the groups, and LV systolic pressure did not differ between the two groups (106±4 mm Hg for the group that retained normal LV function and 113±3 mm Hg for the group that developed dysfunction) before the imposition of an aortic pressure gradient. The first of these groups, hereafter designated the “hypertrophy” group, showed no change from baseline during progressive LV afterloading in terms of LV end-diastolic pressure, end-diastolic volume, or ejection fraction (Table); ie, LV contractile function remained well compensated throughout the development of extensive LV hypertrophy (Figure 1B). The second of these groups, hereafter designated the “failure” group, showed significant abnormalities in each of these measures (Table); ie, LV contractile function compensated during the development of significantly less extensive LV hypertrophy (Figure 1B).

However, at this relatively early stage, the dogs in the failure group exhibited myocardial failure rather than overt congestive heart failure, since they did not develop a decrease in resting cardiac output or an increase in arteriovenous O2 difference. These disparities between the two groups occurred despite the fact that LV afterload was equivalent in the two groups until the final 8-week measurement (Figure 1A). The contrasting functional consequences, for the LV myocardium of the two groups, of this differing response to progressive LV afterloading are summarized in Figure 2. In the hypertrophy group, a normal relationship as defined in this labo-
ratory between mean normalized systolic ejection rate and mean systolic stress obtained at the beginning, the midpoint, and the end of this study. In the failure group, this relationship was different from that of the hypertrophy group initially and showed progressive abnormalities thereafter, such that at the end of the study, there was a marked decrease in LV function and a marked increase in LV wall stress. In summary, the 14 dogs in the failure group, which were identified retrospectively on the basis of distinctly abnormal LV function at terminal study, were found to (1) differ at the outset of the study from the 9 dogs in the hypertrophy group in terms of LV mass and function, (2) display no further LV hypertrophy in response to further increases in afterload after the midpoint of the study, and (3) exhibit markedly decreased LV contractile function and markedly increased LV systolic wall stress at the end of the study.

Cardiocyte Mechanics: Effects of Colchicine

We have shown that microtubule depolymerization, either by low temperature or by colchicine, restores initially abnormal contractile performance of pressure-hypertrophied feline RV cardiocytes to normal after hypertrophy is complete, with or without the superimposition of right heart failure.3–6 This effect is based on the greatly increased density of the microtubule network in these cells. We sought to determine in the present study what role increased microtubule density might play in any contractile dysfunction found in pressure-hypertrophied cardiocytes isolated from canine LVs, with or without the superimposition of left heart failure.

Figure 1. Increases in left ventricular (LV) afterload and the resultant increments in LV mass are shown with progressive stenosis of the ascending aorta. The group of dogs denoted as “hypertrophy” maintained normal LV function throughout the course of the study; the group of dogs denoted as “failure” developed LV systolic dysfunction as the afterload became more severe. For both panels, the times at which the animals were studied and the aortic band was tightened are indicated on the abscissa; the vertical arrows indicate the times of LV biopsy and of final study. A, The aortic pressure gradient as measured by cardiac catheterization is indicated on the ordinate. B, The LV/body weight ratio as measured by ventriculography is indicated on the ordinate. Statistical comparisons, which considered all dogs from a given experimental group together, were by two-way ANOVA and a means comparison contrast, where n indicates number of dogs. *P<.01 for difference between the two groups of dogs at matched time points. †P<.01 for difference from the initial baseline value within a group.

Figure 2. The relationship between mean normalized systolic ejection rate and mean systolic stress for the same two groups of dogs whose data are given in Figure 1. The solid and parallel dashed lines define this relationship and its 95% confidence interval, calculated using a least squares linear regression analysis, for 40 β-blocked normal dogs studied in this laboratory. The arrows indicate for each group separately the progression through the indicated time points of this study. Statistical comparisons, which considered all dogs from a given experimental group together, were by two-way ANOVA and a means comparison contrast, where n indicates the number of dogs in each group and the number of dogs in the subset of each group submitted to left ventricular biopsy. †P<.01 for difference between the two groups of dogs at matched time points. †P<.01 for difference from the initial baseline value within a group.

Figure 3. The effect of colchicine on free and polymerized tubulin fractions of LV cardiocytes from the failure group at final study. Lanes 1 and 3 of this immunoblot represent free β-tubulin, and lanes 2 and 4 represent polymerized β-tubulin. The samples for lanes 1 and 2 were prepared from freshly isolated cells (time 0 in Figure 6), and the samples for lanes 3 and 4 were prepared from an aliquot of the same cells after a 60-minute exposure to 10−6 mol/L colchicine (time 60 in Figure 6).
Figure 4. Colchicine study in single cardiocytes from dogs in the hypertrophy and failure groups. Each panel shows sequential samples from a single left ventricular (LV) cardiocyte, where for each contraction sarcomere length versus time is given above and the rate of length change versus time is given below. The time in minutes after the addition of 10^{-6} mol/L colchicine is indicated. A, LV cardiocyte obtained by biopsy from a hypertrophy dog at 4 weeks after aortic banding. B, LV cardiocyte obtained from the same dog at terminal study. C, LV cardiocyte obtained by biopsy from a hypertrophy dog at 4 weeks after aortic banding. D, LV cardiocyte obtained from the same dog at final study.

Figure 3 shows that just as in pressure-hypertrophied feline RV cardiocytes, exposure of pressure-hypertrophied canine LV cardiocytes from the failure group to 10^{-6} mol/L colchicine for 1 hour causes essentially complete microtubule depolymerization. The same finding was obtained in canine LV cardiocytes from the control and hypertrophy groups (data not shown). Figures 4 to 6 show the effects of such microtubule depolymerization by colchicine on the mechanics of LV cardiocytes isolated at the midpoint of the hypertrophy process (designated as “biopsy” in Figures 1 and 2) and at terminal study (designated as “final” in Figures 1 and 2). Data from the hypertrophy and failure groups are presented separately; Figure 4 shows representative examples of actual data, and Figures 5 and 6 are presented as summary data. Each panel of Figure 4 shows sequential contractions of a single LV cardiocyte. The cardiocytes in panels A and B, respectively, are both from a single dog in the hypertrophy group, where the cardiocyte in panel A was isolated from the biopsy sample at 4 weeks, and the cardiocyte in panel B was isolated at final study. The cardiocytes in panels C and D, respectively, are both from a single dog in the failure group, where the cardiocyte in panel C was isolated from the biopsy sample at 4 weeks when ventricular function was normal, and the cardiocyte in panel D was isolated at final study when LV dysfunction was present. For each contraction, sarcomere length versus time is given above, and the rate of length change versus time is given below. The time in minutes after adding colchicine to a concentration of 10^{-6} mol/L is indicated.

Panels A and B of Figure 4 show that after the addition of 10^{-6} mol/L colchicine, sarcomere motion and its first derivative did not change appreciably during sequential sampling of LV cardiocytes from a dog that retained well-compensated LV function throughout the course of LV hypertrophy; ie, sarcomere motion was normal initially and was unaffected by microtubule depolymerization both in the biopsy specimen obtained at the midpoint of the hypertrophy process and in the specimen obtained at final study when the hypertrophy process was complete. Panel C shows that sarcomere motion of a cell from a hypertrophying LV that would eventually exhibit contractile dysfunction was normal at the time of biopsy and was unresponsive to colchicine, just as was the case for the cell in panel A. However, panel D shows markedly depressed contractile function for a cardiocyte removed from this same LV at final study. Importantly, although aftercontractions such as those seen here were found in about one third of the cardiocytes from the failure group, perhaps reflecting the abnormal Ca^{2+} metabolism that we have described in pressure-overload cardiac hypertrophy, this must be considered a distinctly second-order effect, since colchicine does not affect Ca^{2+} levels or kinetics in hypertrophied cardiocytes, and the initially depressed sarcomere shortening extent and shortening velocity of this cardiocyte from the failing pressure-overloaded LV returned fully to normal after exposure to colchicine.

Figure 5 provides summary data for the groups of cardiocytes exemplified in panels A and B of Figure 4 and compares these data with those for LV cardiocytes from control dogs. Panel A of Figure 5 shows the maximum extent of sarcomere shortening, defined as initial sarcomere length minus minimum sarcomere length, at the indicated times after the addition of colchicine to these three groups of LV cardiocytes. All cells were sampled sequentially at the indicated times after drug exposure. Panel B of Figure 5 shows the maximum velocity of sarcomere shortening, defined as the maximum positive rate of length change, for the same contractions summarized in panel A. As shown, with a single notable exception the contractile function of these three groups of LV cardiocytes was identical, and colchicine in no case had any appreciable effect on sarcomere mechanics. Thus, contractile function both of the LV and of its constituent cardiocytes was not depressed in this group of dogs and was unaffected by microtubule depolymerization. The notable and interesting exception is the augmented extent of sarcomere shortening seen in cardiocytes from the hypertrophy group at final study. For that study group, the extent of sarcomere shortening was significantly greater than that of either the control cells or hypertrophied cells obtained by biopsy at the midpoint of this growth process, and this was the case both before colchicine exposure (microtubules present) and after colchicine exposure (microtubules absent).
These data suggest that in the absence of increased wall stress and concomitant microtubule densification, pressure-overload LV hypertrophy invokes favorable compensatory modification(s) of the contractile apparatus, or of its regulation, in the hypertrophied cardiocyte.

Figure 6 provides summary data for the groups of cardiocytes exemplified in panels C and D of Figure 4 and compares these data with those from the same control LV cardiocytes shown in Figure 5. The format is the same as that for Figure 5. At biopsy, cardiocytes from these LVs, which are destined to go on to contractile failure after further afterload increases, exhibit normal sarcomere motion that is unaffected by microtubule depolymerization. But for LV cardiocytes from the failure group at final study, there was a profound depression of sarcomere mechanics in the basal state. However, the initial differences from the other two groups of LV cardiocytes were no longer statistically significant 45 minutes after the addition of 10^{-6} mol/L colchicine, and after 30 minutes there was a significant increase from the initial values for both the extent and velocity of sarcomere shortening. Thus, exposure of hypertrophied LV cardiocytes from the failure group at final study to colchicine essentially normalized what was initially quite abnormal contractile function, a response to microtubule depolymerization that is closely comparable to that which we observed in our earlier studies of hypertrophied cardiocytes from the pressure-overloaded feline RV.3–6

To exclude any potential nonspecific effects of colchicine on inotropic state as the basis for the amelioration of contractile dysfunction seen in the LV cardiocytes from the failure group, just as was done in our previous study of the...
pressure-overloaded feline RV, microtubules of eight LV cardiocytes from a single dog in the failure group at final study were depolymerized by exposure to 0°C for 1 hour followed by abrupt rewarming. Sarcomere mechanics were fully normalized via a 68% increase in the extent and a 98% increase in the velocity of sarcomere shortening. Again, just as in the prior study, when these cardiocytes were kept at 37°C for a further hour to allow microtubule repolymerization, there were moderate reductions in the extent (a 20% decrease) and the velocity (a 33% decrease) of sarcomere shortening during contraction. Thus, of particular interest, in the context of our hypothesis that increased microtubule density is initiated by cardiocyte stress loading, is the fact that when cardiocyte microtubules that had polymerized under a stress load in vivo repolymerized under zero load in vitro, the initial contractile abnormality was not fully recapitulated, despite the substantially increased concentration of αβ-tubulin heterodimers shown below.

**Cardiocyte Microtubules and Cytoarchitecture**

In Figure 7, an antibody that recognizes the isoform-common region of β-tubulin was used for immunofluorescence confocal micrographs of cardiocyte microtubules in LV cardiocytes of dogs from the control, hypertrophy, and failure groups. The micrographic density of the microtubule network is alike for LV cardiocytes from the control group (Figure 7A), the failure group at biopsy (when ventricular function is normal) (Figure 7B), and the hypertrophy group at final study (Figure 7C). Cardiocytes from the hypertrophy group at the time of biopsy had a similar microtubule density (data not shown). In comparison, however, a markedly increased microtubule density is apparent in the LV cardiocyte from the failure group at final study when LV dysfunction is present (Figure 7D).

Figure 8 shows that despite this marked change in the structure of the microtubule component of the extramyofilament cytoskeleton in LV cardiocytes from the failure group at final study, the cytoarchitecture of both the extramyofilament and the myofilament portions of the cardiocyte cytoskeleton is otherwise unaltered in these cells. That is, immunolocalization of desmin, the predominant protein of the intermediate filaments linking the Z lines to the sarcolemma, is unaltered (Figure 8A) despite the greatly increased microtubule density in the same cell (Figure 8B), and another cell from this same isolate shows normal immunolocalization of myosin (Figure 8C) within very well-ordered sarcomeres.

**Myocardial Free and Polymerized Tubulin**

The top panel of Figure 9 shows immunoblots of free (lanes 1 and 3), polymerized (lanes 2 and 4), and total (lanes 5 and 6) β-tubulin in samples of the same LVs of dogs from the hypertrophy and failure groups, both at biopsy and at final study. In each of these immunoblots, the samples from the same heart were run together, such that visual comparisons within that blot are valid; however, comparison of one immunoblot with another requires densitometric analysis using concurrently run β-tubulin standards, as was done in...
generating the data shown in the bottom panel of this figure. It is nonetheless clear that in the LV from the hypertrophy group, the biopsy and final samples are equivalent and show the 2:1 ratio of free to polymerized tubulin that we observe in the normal feline heart. The same is true for the LV from the failure group at the time of biopsy. However, in this LV from the failure group at final study, there is an obvious increase both in free and especially in polymerized β-tubulin, with a reversal of the ordinarily observed ratio of free to polymerized tubulin. Thus, for the hypertrophy group, the total tubulin was 15 ± 4 ng/mg total protein at biopsy and 13 ± 3 ng/mg total protein at final study, whereas for the failure group, these values were 18 ± 2 ng/mg total protein at biopsy and 34 ± 5 ng/mg total protein at final study. The bottom panel of Figure 9 provides summary data from these and additional blots from these two groups of dogs in terms of the final/biopsy ratios of free tubulin, polymerized tubulin, and total tubulin. In the hypertrophy group, this ratio remains near unity for all three fractions, such that there was no increase in any β-tubulin fraction during the progression of compensated LV hypertrophy, with the preservation of normal contractile function and systolic wall stress. In the failure group, in contrast, there was an increase in all three fractions, which became especially prominent for the polymerized microtubule fraction as these LVs progressed from compensated hypertrophy to decompensated failure, with attendant deterioration of contractile function and systolic wall stress.

Discussion

We had found in our earlier work that microtubules are selectively and persistently increased in RV myocardium hypertrophying in response to pressure overloading, that in cardiocytes from the pulmonary artery band model used therein this alteration is responsible both for the reduced extent and velocity of sarcomere shortening in externally unloaded cells and for the reduced extent of sarcomere and cellular shortening in externally loaded cells, that these contractile defects can be reproduced in normal cells when microtubule hyperpolymerization is induced by a chemical or physical as opposed to a pathophysiological stimulus, that these contractile defects are specific to the microtubule component of the cytoskeleton, and that the microtubule-dependent biophysical effect is one of imposing a primarily viscous intracellular load on the active sarcomeres characterized by frictional dissipation. As such, this single molecular defect could well be explicable not only of abnormal sarcomere and cellular motion during contraction but also of the linked contractile and energetic abnormalities that we have previously found to be characteristic of pressure-overload cardiac hypertrophy.

Two important issues, however, could not be addressed in this earlier work. First, although our findings have been confirmed in the rodent LV, the relevance of these data to the pressure-overloaded LV of a large animal with an adult-onset, progressive, and pathological LV afterload imposition similar to that found in human disease was undetermined. Second, the relationship of these cytoskeletal abnormalities to the mechanical environment of the cardiocyte in vivo was unknown, since characterization of RV mass, function, and wall stress, especially in a small animal, is infeasible. Further, since functional and physical ventricular and cellular sampling over time is similarly infeasible in the RV of a small animal, it was unknown whether increased microtubule density is a characteristic of pressure-overload hypertrophy per se or whether, instead, it has a specific relationship to progressive changes in the mechanical environment of the cardiocyte as myocardial stress loading increases. Given these issues, as well as the predominant importance of the LV in clinical pathophysiology, we devised a model of progressive pressure overload of the canine LV for the particular purposes of the present study.

An unexpected and interesting feature of this model is that although all dogs were selected randomly and were submitted to the same protocol, they self-segregated into two groups with respect to LV mass and function as the degree of aortic stenosis increased. These two groups of outbred mongrel dogs did not differ in terms of sex, age, weight, or any other grossly discernible characteristic. On retrospective examination, however, it became clear that the group of dogs destined to exhibit LV failure differed at baseline from the group of
dogs destined to exhibit compensatory LV hypertrophy, in that the failure group entered the study with significantly lower LV mass and mean normalized systolic ejection rate and significantly higher LV mean systolic stress (Figures 1B and 2). That is, although when considered together all of the dogs used in this study fell within the "normal" range for these variables as defined in this laboratory, their response to the stress of progressive LV loading evoked a basic heterogeneity of LV properties, especially that of hypertrophic growth capacity, that apparently stemmed from intrinsic, and perhaps genetically based differences among these animals.

The intriguing similarity between this heterogeneous canine response to pathological LV afterloading and that observed clinically in humans is treated fully in our description of this animal model. But to return to the two issues that formed the basis for the present study, considerable insight was gained in each case. First, the same microtubule-based cardiocyte contractile defect found in the pressure-overloaded feline RV, where with fixed afterloading wall stress is probably continuously elevated from the outset, was also found in the pressure-overloaded canine LV. However, the present study allows us to assign considerably more specificity to this finding. In this canine model we were able to sample LV mechanical and geometrical properties, on the organ and on the cellular levels, throughout the development of LV hypertrophy that was of a degree relevant to human disease and that was also in response to a pattern of progressive afterloading that is also relevant to human disease. In the context of considering compensatory hypertrophy to be that in which myocardial mass increases so as to maintain normal ventricular wall stress, we found that those animals whose capacity for LV growth was such that normal LV wall stress was sustained despite a very high aortic pressure gradient (Figure 1A) retained normal ventricular (Figure 2) and cellular (Figures 4 and 5) contractile function. Furthermore, they exhibited increases neither in tubulin protein (Figure 9) nor in the density of the cytoskeletal microtubule network (Figure 7). Indeed, a unique and unanticipated finding was that cellular contractile function, in terms of the extent of sarcomere shortening, was normal in LV biopsy specimens obtained at the midpoint of the hypertrophic growth response in this group but was significantly increased at final study (Figures 4B and 5A), despite the fact that LV mass had doubled. This finding raises the provocative possibility that as-yet-unknown compensatory mechanisms may have a significant and heretofore unrecognized role in the maintenance of normal contractile function on the organ level, ie, functionally compensatory hypertrophy.

The findings for the group of dogs that developed LV failure were quite different from those seen in the group of dogs that maintained compensated LV hypertrophy and quite similar to those seen in cats with RV pressure-overload hypertrophy with associated right heart failure. The hallmark that distinguished this group of failure dogs from the compensated hypertrophy dogs was a conspicuous breakdown of the LV growth response to progressive afterloading. That is, through the time of biopsy, these dogs showed progressive increases in LV mass in response to progressive increases in LV afterload, albeit to a lesser extent than for the hypertrophy group (Figure 1B) and with the early development of abnormalities in LV stress and function (Figure 2). Furthermore, normal contractile function was maintained at this time in cardiocytes from these LVs (Figures 4C and 6), and no abnormalities of cytoskeletal tubulin or microtubules were apparent (Figures 7B and 9). However, after the time of biopsy, there was no further compensatory growth response of the LV to further afterloading in these dogs (Figure 1B). As a direct consequence, there was a striking increase in LV mean systolic stress measured both after β-blockade (Figure 2) and before β-blockade (data not shown). Of greatest functional consequence, however, there was also a striking deterioration of LV contractile function (Figure 2), which was mimicked by, and may well have had its basis in, a parallel striking deterioration of sarcomere contractile mechanics in cardiocytes from these same LVs (Figures 4D and 6). These observations, and their contrast with those made in the group of dogs with compensated hypertrophy, are lent particular cogency by the fact that in many cases the same dogs were sampled longitudinally in both groups and that the ventricular, cellular, and biochemical data were all gathered from the same animals.

Just as in the feline RV, wherein severe pressure overloading caused RV failure, these dogs from the LV failure group demonstrated a profound but colchicine-reversible depression of sarcomere mechanics (Figures 4D and 6) that was associated with remarkable increases both in free and polymerized tubulin (Figure 9) and in the density of the cellular microtubule network (Figure 7D). But whereas in pressure-hypertrophied but nonfailing feline RV myocardium there were persistent and concordant increases in both the free and polymerized tubulin fractions, such
that the ordinary 2:1 ratio of these fractions was maintained, the increases in these two pools were not concordant either in pressure-hypertrophied and failing feline RV myocardium in our previous work\(^6\) or in pressure-hypertrophied and failing canine LV myocardium in our present work. Instead, as shown in Figure 10 for the group of dogs with LV failure, there was a major increase in the percentage of the total tubulin pool found in the polymerized fraction. Indeed, given the central importance of tubulin in cellular contractile dysfunction, a search for the basis for this apparent increase in microtubule stability in hypertrophied failing myocardium is a major focus of our present research.

Conclusions

Increased microtubule density and its functional consequences are properties of both RV and LV pressure-overload hypertrophy; this cytoskeletal alteration is not, as has been posited elsewhere,\(^{20}\) either species or chamber specific. But data from the present model, where ventricular mechanics can be defined and where longitudinal sampling is possible, show clearly that this is not a ubiquitous feature of pressure-overload cardiac hypertrophy. Instead, it has a specific association with increased ventricular wall stress, which in turn appears during progressive LV pressure overloading when, and in those animals wherein, the hypertrophic response to load is exhausted. Thus, this cardiac cytoskeletal response to a hemodynamic overload is dependent on the type of ventricular loading, being absent in eccentric volume overload and present in concentric pressure overload,\(^3,4\) and it is also dependent on the age of the subject in which the load is applied, the extent of the resultant hypertrophy, the duration of the hypertrophy, and whether the hypertrophic response is sufficient to normalize ventricular wall stress. Microtubule hyperpolymerization, therefore, should not be viewed as the only mechanism causing the development of contractile dysfunction in hemodynamically overloaded, hypertrophied, and failing myocardium. Indeed, confusion about this point could lead to interpreting several recent studies noted below as negating the results of our present and previous work in this area.

For example, as we have reported before,\(^6\) the abnormalities in cellular systolic function that may occur in chronic LV pressure-overload hypertrophy with normal or subnormal wall stress in the juvenile mammal are not accompanied by an increase in the microtubule portion of the cytoskeleton and are not corrected by altering the microtubule polymerization state. This situation is consistent with, rather than in opposition to, recent findings\(^21\) in chronic progressive pressure overload of the kitten LV, where, although ventricular mechanics were not characterized, the LV mass and pressure data would strongly suggest a relatively low LV systolic wall stress and where microtubules were not found to play a role in the cardiocyte contractile dysfunction. In contrast, as noted here, with substantial, fixed pressure overloading of the adult RV, wall stress is probably elevated from the outset, thus explicating our findings of persistent cytoskeletal and contractile abnormalities for RV pressure overloading within the context provided by the present adult LV data, which show the crucial role of increased wall stress.

Again, although our findings have been confirmed by others in both rodent\(^{18,19}\) and human\(^2\) LV, there are two further studies\(^{23,24}\) of the pressure-overloaded rodent LV in which this is not the case. Of note, however, LV wall stress was not defined in these latter two studies, and the finding in one of these studies\(^25\) of no increase in microtubules in the pressure-overloaded guinea pig LV has since been challenged by the finding of a very substantial increase in cardiocyte microtubules in the identical animal model.\(^19\) In the context of the present data set and that of another\(^25\) showing only a transient increase in cardiocyte microtubule density after fixed LV pressure overloading that disappeared during the compensatory hypertrophic growth process wherein, presumably, initially increased LV wall stress was renormalized, these apparently disparate findings may well be reconciled in terms of the specific linkage of these cytoskeletal and contractile abnormalities to increased wall stress.

Thus, in summary, when hypertrophic cardiac growth in response to pathological afterloading does not renormalize myocardial stress, this inadequate quantitative response is compounded by a qualitative defect that further jeopardizes the ability of the heart to compensate functionally. But as we have discussed fully elsewhere,\(^5\) one would expect multiple myocardial abnormalities in the setting of advanced heart failure. We have found, for example, that the decreased contractile state caused by the chronic volume overloading attendant on mitral regurgitation is not accompanied by an increase in microtubule density, is not corrected by altering microtubule polymerization, and is instead probably caused by abnormalities in other myocardial proteins and processes. Furthermore, in ischemia-, virus-, or tachycardia-induced cardiomyopathies, changes in microtubules would not be expected to be an important mechanism for contractile dysfunction. Finally, when pressure-overload hypertrophy or any other cardiomyopathic process reaches an advanced and irreversible stage, there are almost certainly multiple changes that are responsible for the myocardial dysfunction that is present. At such an advanced stage, it would be quite unlikely indeed either that changes in only one protein would cause all of the myocardial dysfunction that is present or that normalizing the changes in that single protein would restore normal myocardial function. Therefore, the most remarkable feature of the present data set is the full reversibility of the cardiocyte contractile defects after microtubule depolymerization at this early stage of pressure-overload-induced LV contractile dysfunction.

On a basic level, we are now extending these findings to studies of the control of microtubule density\(^28\) and the control of tubulin synthesis in the pressure-hypertrophied cardiocyte, since insight into these closely interrelated problems will be essential if we are to understand the mechanisms responsible for the increased microtubule density and the associated contractile defects in pressure-overload cardiac hypertrophy. On a more applied level, these findings must now be extended to the in vitro and in vivo tissue levels and to studies of clinical disease in humans, since our long-standing goal of learning the causes of the transition from initially compensatory pressure-overload cardiac hypertrophy to decompensated...
cardiac failure constitutes the relevant clinical rationale for this work.

Acknowledgments

This study was supported by grants HL-37196 and HL-48788 from the National Heart, Lung, and Blood Institute and by research funds from the Department of Veterans Affairs. The authors thank Heather Downs, Sebette Hamill, and Mary Barnes for technical assistance.

References

Cytoskeletal Role in the Transition From Compensated to Decompensated Hypertrophy During Adult Canine Left Ventricular Pressure Overloading

Hirofumi Tagawa, Masaaki Koide, Hiroshi Sato, Michael R. Zile, Blase A. Carabello and George Cooper IV

_Circ Res._ 1998;82:751-761
doi: 10.1161/01.RES.82.7.751

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1998 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/82/7/751