Left Ventricular Hypertrophy in Ascending Aortic Stenosis Mice

Anoikis and the Progression to Early Failure

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Background—To determine potential mechanisms of the transition from hypertrophy to very early failure, we examined apoptosis in a model of ascending aortic stenosis (AS) in male FVB/n mice.

Methods and Results—Compared with age-matched controls, 4-week and 7-week AS animals (n = 12 to 16 per group) had increased ratios of left ventricular weight to body weight (4.7 ± 0.7 versus 3.1 ± 0.2 and 5.7 ± 0.4 versus 2.7 ± 0.1 mg/g, respectively, P < 0.05) with similar body weights. Myocyte width was also increased in 4-week and 7-week AS mice compared with controls (19.0 ± 0.8 and 25.2 ± 1.8 versus 14.1 ± 0.5 μm, respectively, P < 0.01). By 7 weeks, AS myocytes displayed branching with distinct differences in intercalated disk size and staining for β1-integrin on both cell surface and adjacent extracellular matrix. In vivo left ventricular systolic developed pressure per gram as well as endocardial fractional shortening were similar in 4-week AS and controls but depressed in 7-week AS mice. Myocyte apoptosis estimated by in situ nick end-labeling (TUNEL) was extremely rare in 4-week AS and control mice; however, a low prevalence of TUNEL-positive myocytes and DNA laddering were detected in 7-week AS mice. The specificity of TUNEL labeling was confirmed by in situ ligation of hairpin oligonucleotides.

Conclusions—Our findings indicate that myocyte apoptosis develops during the transition from hypertrophy to early failure in mice with chronic biomechanical stress and support the hypothesis that the disruption of normal myocyte anchorage to adjacent extracellular matrix and cells, a process called anoikis, may signal apoptosis.

Key Words: myocytes • hypertrophy • heart failure • integrins • apoptosis

Although apoptotic myocytes have been observed in end-stage human failing hearts, the potential role of programmed cell death during the transition from adaptive hypertrophy to early failure is not known. We investigated a mouse model of ascending aortic stenosis (AS) with fidelity to those clinical features that characterize the transition from adaptive hypertrophy to very early failure, including an initial phase of compensation followed by the development of left ventricular (LV) systolic dysfunction. At the stage of very early failure, we observed that LV myocytes exhibit aberrant myocyte branching and β1-integrin deposition in situ in association with a very low prevalence of myocyte apoptosis estimated by terminal dUTP nick end-labeling (TUNEL) and ligation of hairpin oligonucleotide probes. These observations raise the hypothesis that anoikis, the process of abnormal cell–cell and cell–extracellular matrix (ECM) contact that initiates the cell death program, occurs early and may contribute to the progression to very early heart failure.

Methods

Animal Preparation and Hemodynamic Measurements

Ascending AS was performed in male FVB/n mice (body weight 12 to 15 g, Charles River Breeding Laboratories, Wilmington, Mass) as described by our laboratory.1 Age-matched animals underwent the same procedure without placement of the aortic ligature to serve as controls. In vivo LV pressure measurements were performed before euthanasia (n = 12 to 16 per group) by methods previously described in AS rats2–3 and mice.4 AS animals and age-matched sham-operated controls were euthanized at 4 and 7 weeks after surgery (n = 25 to 30 per group). This stage of 7-week AS, which was selected as the transition to failure, precedes the development of severe decompensation that develops by 13-week AS, which is characterized by...
severely increased mortality (>75%) and liver and lung congestion with pleural effusion.

2D Targeted M-Mode Echocardiography

After measurement of body weight, animals were lightly anesthetized with intraperitoneal ketamine 50 mg/kg and xylazine 2.5 mg/kg and studied in the conscious condition on a warming pad. 2D guided M-mode echocardiography in the mouse was performed with a 12-MHz transducer (Hewlett Packard) as described by our laboratory. In addition to standard analysis of LV wall thickness, cavity size, and endocardial fractional shortening, we also used midwall fractional shortening as an index to estimate LV systolic function, because endocardial edge chamber dynamics may overestimate function. In addition, the relationship between LV volume measured by echocardiography and postmortem volume was determined in mice (n=6) that underwent echocardiography as described above immediately followed by diastolic arrest of the heart via aortocoronary potassium chloride perfusion in situ. The paraformaldehyde-fixed LV was sectioned into ten 0.5-mm sections from apex to base, the sections were stained with hematoxylin-eosin, and the volumes of all sections were calculated by use of an ellipsoid model and summed for determination of LV volume by Simpson’s rule. These data demonstrate a strong correlation between echocardiographic and postmortem measurements of LV volume: volume = 0.97 × calculated echocardiographic volume – 19.8 μL, r = 0.94, P = 0.001.

Analysis of β-integrin Protein Levels

Additional hearts (n=6 per group) underwent collagenase perfusion for isolated LV myocyte dissociation to achieve a highly myocyte-enriched suspension (98% myocytes and <2% fragments of endothelial and fibroblast cells) as described by our laboratory. After homogenization, proteins were quantified with a BCA assay (Biochrominichinic Assay, Pierce Chemical). Proteins were separated by SDS-PAGE on 12% gradient gels and transferred onto nitrocellulose membranes by standard procedures. Membranes were blocked in 5% nonfat dry milk solution (w/v) in PBS with 0.1% Tween-20) followed by incubation with the polyclonal primary antibody followed by incubation with the polyclonal rabbit IgG conjugated with horseradish peroxidase (Amersham) and the chemiluminescent detection system (Amerham). Observed band densities were quantified with an AlphaImage densitometric software. The β-integrin antibody was prepared with affinity-purified β-integrin as an immunogen as previously described.

Confocal Microscopic Analyses

Hearts (n=6 to 7 per group) were removed and rinsed for 1 minute in 0.1 mol/L PBS with 50 mmol/L KCl, pH 7.2, and subsequently fixed overnight at 4°C in 4% paraformaldehyde prepared in PBS. Vibratome sections (100 μm) from similar areas of the LV free wall and the septum were stained in a 1:20 dilution of rhodamine phalloidin (Molecular Probes) and imaged with a BioRad MRC 1000 confocal microscopic laser microscope. A minimum of 5 optical sections were collected from the free wall of the LV of each animal with a Nikon ×60 NA 1.4 lens. Myocyte widths (60 to 120 myocytes per heart) were measured perpendicular to the long axis of the sarcomeres from unbranched areas of the myocytes near an intercalated disk with the length/profile function in the BioRad MRC1000 COMOS program. Myocyte length was obtained by measuring the distance between the intercalated disks.

In addition, in situ TUNEL was analyzed by confocal microscopy. Vibratome sections (100 μm) from 3 different hearts from each group were cut from similar regions of the hearts and triple-labeled with (1) rhodamine phalloidin, which is specific for the f-actin in the cytoplasmic domain, as previously described; (2) TOPRO-3, which is a DNA-specific stain used for localizing nuclei; and (3) a Boehringer Mannheim TUNEL kit with FITC as the reactive fluorophore, according to the manufacturer’s instructions. Positive controls for TUNEL staining consisted of treating sections with 2% DNase 1 for 1 hour at 37°C before proceeding with the TUNEL reaction staining protocol. Negative controls for the TUNEL reaction consisted of treating sections with the TUNEL reaction mixture without terminal transferase. Sections were subsequently examined with a BioRad MRC 1000 confocal microscope with an argon/krypton laser capable of imaging the 3 fluorophores in distinct channels. Sequential images were obtained with the ×10 objective from 5 random fields per section in each of the 3 channels without adjustment of the microscope stage position. Images were subsequently overlaid in the BioRad Confocal Assistant program, and nuclei stained with both the TUNEL reaction and TOPRO-3 were considered positive for the apoptosis reaction. Co-localization of the TUNEL-positive nuclei with rhodamine phalloidin–stained myocytes was used to confirm that apoptosis was occurring in myocytes.

Vibratome sections (100 μm) collected adjacent to those used for the TUNEL staining were also stained with a primary antibody against heart β-integrin, which recognizes both the extracellular and intracellular domains, as previously described. Sections were incubated at 4°C overnight in the primary antibody, rinsed in PBS, and stained at 4°C overnight with Alexa 488 (Molecular Probes) conjugated to the secondary antibody. Sections were then rinsed in PBS and mounted in a 1:3 mixture of glycerin and PBS with 10 mg/mL DABCO (Sigma). In addition, isolated fixed LV myocytes from the 7-week AS mice and age-matched normal mice were stained with this polyclonal antibody by methods published by our laboratory. To determine the specificity of the β-integrin staining in the extracellular space in the tissue sections, sections were stained with a polyclonal antibody made against specific peptides of the cytoplasmic domain of the β-integrin (a gift from Dr Clayton Buck, Wistar Institute). Controls consisted of incubation in nonimmune serum rather than the primary antibody followed by incubation with conjugated antibody as described above.

In Situ Ligation Assay

In additional hearts (n=2 per group), the TUNEL assay as well as in situ ligation using hairpin oligonucleotide probes was performed to identify double-strand DNA breaks, which are highly specific for apoptotic DNA fragmentation. In situ ligation using hairpin oligonucleotide probes was performed as described in detail by Didenko et al., with some modifications. Briefly, paraformaldehyde-fixed LV sections were deparaffinized with xylene and rehydrated in graded alcohol concentrations. After a washing in water, sections were incubated with 25 μg/mL proteinase K (Intergen) in PBS for 15 minutes. Sections were then rinsed with water. A mixture of 50 mmol/L Tris/HCl (pH 7.8), 10 mmol/L MgCl2, 10 mmol/L dithiothreitol, 1 mmol/L ATP, 15% polyethylene glycol (8000 MW, Sigma) with hairpin oligonucleotide probes at 35 μg/mL, and T4DNA ligase at 250 U/mL was added. Sections were placed in a humidified box for 16 hours, then washed in water 3 times for 20 minutes each. Fluorescein-isothiocyanate conjugate (Vector Laboratories) was added at 4 μg/mL in 50 mmol/L sodium chloride (pH 8.2) for 45 minutes. Sections were washed 3 times for 10 minutes in the same buffer and then rinsed in water before the addition of the terminal deoxynucleotidyl transferase mixture for TUNEL staining. For TUNEL staining to detect free 3’ DNA hydroxyl groups with terminal transferase, the published procedure was used, modified to accommodate the use of Texas Red as label rather than biotin. A mixture of Tris/HCl, 1 mmol/L, Tris/HCl (pH 7.2), 140 mmol/L sodium cacodylate, 1 mmol/L CoCl2, 0.1 mmol/L dithiothreitol, 8 μmol/L Texas Red dUTP (Molecular Probes), and 800 U/mL terminal transferase was added for 1 hour at 37°C in a humidified incubator. After 2 washings in water for 20 minutes, the sections were counterstained with the DNA-binding dye DAPI (1 μg/mL), mounted in Vectashield, and examined by fluorescence microscopy.

DNA Laddering

Genomic DNA was isolated from LV tissue (n=3 per group) with a DNA isolation kit (Gentra). One microgram of extracted DNA was labeled by enzymatic assay with 3’ terminal deoxynucleotidyl transferase (TdT) as described by Didenko et al., The DNA was labeled in a buffer containing 2 mmol/L CoCl2, 0.5 mmol/L DTT, 100 mmol/L potassium cacodylate, 166 mmol/L [32P]dCTP (3000 Ci/mmol), 664
nmol/L dCTP, and 20 U TdT. The samples were incubated for 60 minutes at 37°C. One-tenth of the total reaction volume was loaded to a phosphor-sensitive screen. As a standard positive control for the presence of depressed systolic performance in 7-week AS mice, LV systolic developed pressure per gram was depressed compared with age-matched controls. In the 7-week AS mice, absolute LV systolic pressure was lower than in the 4-week AS mice despite a greater magnitude of hypertrophy. In the 7-week AS mice, LV systolic developed pressure per gram was depressed compared with the control group. In the absence of LV chamber dilatation (see below), the ratio between systolic developed pressure and mass is directly proportional to systolic wall stress and is a measure of force development per unit of force-developing myocardium.15 These data indicate the presence of depressed systolic performance in 7-week AS mice. In vivo LV end-diastolic pressure was higher in both AS groups than in age-matched controls. Heart rate was similar between the groups. For comparison, LV hemodynamics were also measured in a small cohort of surviving 13-week AS mice with advanced failure, in whom mortality is high (>75% mortality), that exhibited lethargic cage behavior, tachypnea, foot-pad edema, and pleural effusions. At end-stage failure, there is further depression of LV systolic developed pressure and elevation of LV end-diastolic pressure.

**Echocardiographic Assessment**

In vivo 2D targeted M-mode echocardiograms were obtained in 4-week and 7-week AS mice (Figure 1). As reported in

**TABLE 1. Characteristics of Ascending AS Mice**

<table>
<thead>
<tr>
<th>Measure</th>
<th>Controls, 4 wk</th>
<th>AS, 4 wk</th>
<th>Controls, 7 wk</th>
<th>AS, 7 wk</th>
<th>Controls, 13 wk</th>
<th>AS, 13 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>25.4±1.9</td>
<td>26.0±1.7</td>
<td>28.6±1.0</td>
<td>28.2±1.3</td>
<td>29.9±1.6</td>
<td>30.4±1.0</td>
</tr>
<tr>
<td>LV weight, mg</td>
<td>78±5</td>
<td>122±11*</td>
<td>79±6</td>
<td>164±12*</td>
<td>90±1</td>
<td>140±4*</td>
</tr>
<tr>
<td>LV/body weight, mg/g</td>
<td>3.1±0.2</td>
<td>4.7±0.7*</td>
<td>2.7±0.1</td>
<td>5.7±0.4*</td>
<td>2.9±0.1</td>
<td>4.7±0.1*</td>
</tr>
<tr>
<td>RV weight, mg</td>
<td>18±2</td>
<td>20±2</td>
<td>19±3</td>
<td>21±2</td>
<td>20±1</td>
<td>30±1*</td>
</tr>
<tr>
<td>RV/body weight, mg/g</td>
<td>0.70±0.06</td>
<td>0.72±0.10</td>
<td>0.68±0.08</td>
<td>0.68±0.07</td>
<td>0.70±0.01</td>
<td>1.10±0.02*</td>
</tr>
<tr>
<td>LV systolic pressure, mm Hg</td>
<td>63±13</td>
<td>110±20*</td>
<td>59±7</td>
<td>97±12*</td>
<td>68±3</td>
<td>87±3*</td>
</tr>
<tr>
<td>LV diastolic pressure, mm Hg</td>
<td>3.6±0.7</td>
<td>6.0±0.9*</td>
<td>3.7±0.8</td>
<td>7.7±1.2*</td>
<td>3.7±3</td>
<td>11.1±0.8*</td>
</tr>
<tr>
<td>LV developed pressure, mm Hg/g</td>
<td>812±199</td>
<td>856±166</td>
<td>694±91</td>
<td>575±119*</td>
<td>775±33</td>
<td>582±21*</td>
</tr>
<tr>
<td>Peak +dP/dt, + mm Hg/s</td>
<td>7717±357</td>
<td>11 687±732*</td>
<td>7654±675</td>
<td>10 233±835*</td>
<td>6135±187</td>
<td>10 618±498*</td>
</tr>
<tr>
<td>Peak −dP/dt, − mm Hg/s</td>
<td>4650±322</td>
<td>9021±230*</td>
<td>5038±580</td>
<td>7483±681*</td>
<td>5300±372</td>
<td>9600±577*</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>267±38</td>
<td>279±38</td>
<td>241±36</td>
<td>256±41</td>
<td>305±11</td>
<td>296±6</td>
</tr>
</tbody>
</table>

Control indicates age-matched sham-operated normal mice; AS, ascending AS mice studied at 4, 7, and 13 weeks after operation; RV, right ventricular; and LV, left ventricular. n=11 to 16 per group. All data are expressed as mean±SEM.

*P<0.05 vs age-matched control group by ANOVA and Student’s t test.

**Statistical Analysis**

All values are expressed as mean±SEM. Statistical analysis of differences observed between the AS groups and the control groups was done by ANOVA comparison or by ANOVA for repeated measures where appropriate. An unpaired Student’s t test was used for comparison among the groups at the same age after operation. Statistical significance was accepted at the level of P<0.05.

**Results**

**LV Hypertrophy and Hemodynamics**

As shown in Table 1, LV weight was increased by 56% in 4-week AS mice and by 108% in 7-week AS mice compared with age-matched controls. At these stages of hypertrophy, there were no differences in body weight or RV weight compared with controls. In the 4-week AS mice, absolute LV systolic pressure was elevated and LV systolic developed pressure per gram was similar compared with the age-matched control group. In the 7-week AS mice, absolute LV systolic pressure was lower than in the 4-week AS mice despite a greater magnitude of hypertrophy. In the 7-week AS mice, LV systolic developed pressure per gram was depressed compared with the control group. In the absence of LV chamber dilatation (see below), the ratio between systolic developed pressure and mass is directly proportional to systolic wall stress and is a measure of force development per unit of force-developing myocardium.15 These data indicate the presence of depressed systolic performance in 7-week AS mice. In vivo LV end-diastolic pressure was higher in both AS groups than in age-matched controls. Heart rate was similar between the groups. For comparison, LV hemodynamics were also measured in a small cohort of surviving 13-week AS mice with advanced failure, in whom mortality is high (>75% mortality), that exhibited lethargic cage behavior, tachypnea, foot-pad edema, and pleural effusions. At end-stage failure, there is further depression of LV systolic developed pressure and elevation of LV end-diastolic pressure.

**Figure 1.** Representative images of in vivo 2D targeted M-mode echocardiograms of LV chamber in age-matched control mouse 7 weeks after operation (left), 4-week ascending AS (AS 4 WK) mouse (middle), and 7-week ascending AS (AS 7 WK) mouse (right). Both 4-week and 7-week AS mice show phenotypic hallmark of concentric pressure overload hypertrophy: LV anterior and posterior walls (AW and PW) are thickened, but LV diastolic chamber dimension is not increased. LV endocardial and midwall fractional shortening are preserved in 4-week AS mouse; in contrast, these ejection indices of LV systolic function are depressed by 7 weeks of AS.
Confocal Analysis of Morphology and β⁺-Integrin Localization

The confocal microscopic analysis of AS and control hearts showed marked differences in wall thickness (Figure 2), as well as myocyte width (Figure 3 and Table 3). Both AS groups also showed slight increases in myocyte length compared with controls. In the 7-week AS animals, an increase in myocyte width (an index of concentric hypertrophy) was increased compared with age-matched control groups. In 4-week AS mice, both endocardial fractional shortening and midwall fractional shortening were preserved compared with age-matched controls. In 4-week AS mice, the antibody staining for the β⁺-integrin showed an increased staining in the ECM surrounding the myocytes as well as on the cell surface (Figure 3, B through D). As illustrated in Figure 3D, this staining pattern was prominent in all 7-week AS hearts at the stage of early failure. These images represent a 10-μm stacked image that provides the localization of antigens at the cell surface in relation to the surrounding extracellular environment. Staining at the Z line is also evident in these stacked confocal images and is consistent with previous reports from our laboratory describing the localization of the β⁺-integrin on the cell surface. Because this previous analysis of β⁺-integrin staining was performed on isolated cells, isolated LV myocytes from 7-week AS mice were also stained with the same antibody. As illustrated in Figure 3E, this analysis in isolated cells revealed a pattern of localization of the antibody only at the Z bands, as previously reported. No staining on the cell surface or the ECM was evident in tissue sections when the preimmune antibody was used, indicating that the pattern was not due to nonspecific binding in the extracellular compartment (Figure 3A). In addition, further staining with an antibody made against peptides from the cytoplasmic domain of β⁺-integrin (Figure 3F) showed only intracellular staining. Taken together, these confocal images from LV tissue showed aberrant staining of both the cell surface and adjacent matrix in AS mice, whereas the isolated myocytes showed the localization of β⁺-integrin only on the cell surface.

We next determined whether the abnormal pattern of β⁺-integrin staining in situ was related predominantly to an increased expression of β⁺-integrin versus change in its localization in AS hearts. β⁺-Integrin protein levels were measured by Western blotting in protein from LV myocytes from control and AS hearts. As shown in Figure 4, LV β⁺-integrin protein levels were similar in 4-week and 7-week AS hearts compared with controls.

Confocal Analysis of Apoptosis

In situ TUNEL in vibratome sections of LVs from each group was performed (Figure 5). Five random fields per vibratome  

**Table 2. In Vivo Echocardiographic Measures of LV Function**

<table>
<thead>
<tr>
<th></th>
<th>Controls, 4 wk</th>
<th>AS, 4 wk</th>
<th>Controls, 7 wk</th>
<th>AS, 7 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Posterior wall thickness, mm</td>
<td>0.54±0.07</td>
<td>0.97±0.10*</td>
<td>0.63±0.05</td>
<td>1.11±0.12*</td>
</tr>
<tr>
<td>Anterior wall thickness, mm</td>
<td>0.51±0.5</td>
<td>0.95±0.10*</td>
<td>0.57±0.05</td>
<td>1.08±12*</td>
</tr>
<tr>
<td>Relative wall thickness, %</td>
<td>0.27±0.05</td>
<td>0.60±0.10*</td>
<td>0.32±0.03</td>
<td>0.65±0.10*</td>
</tr>
<tr>
<td>LV diastolic dimension, mm</td>
<td>4.01±0.22</td>
<td>3.25±0.29*</td>
<td>3.88±0.17</td>
<td>3.50±0.47</td>
</tr>
<tr>
<td>LV systolic dimension, mm</td>
<td>1.74±0.25</td>
<td>1.33±0.14*</td>
<td>1.73±0.37</td>
<td>1.80±0.20</td>
</tr>
<tr>
<td>Endocardial fractional shortening, %</td>
<td>56±5</td>
<td>58±6</td>
<td>55±7</td>
<td>48±5*</td>
</tr>
<tr>
<td>Midwall fractional shortening, %</td>
<td>33±5</td>
<td>30±3</td>
<td>33±5</td>
<td>24±4*</td>
</tr>
</tbody>
</table>

Abbreviations as in Table 1.

*P<0.05 vs age-matched control group by ANOVA and Student’s t test.
section from 3 different hearts from each group were obtained with the ×10 objective in the confocal microscope. Each field displayed ~3000 myocyte nuclei. Myocyte TUNEL loading was extremely rare in the LVs of both age-matched control and 4-week AS mice, with a range of 0 to 2 positive nuclei per field. In comparison, TUNEL-positive myocyte nuclei were observed in all 7-week AS mice, with a range of 0 to 17 nuclei per field. In corroboration, DNA laddering was observed in 7-week AS mice and the positive control tissue (involuting thymus after dexamethasone injection) but not in 4-week AS mice or age-matched controls (data not shown).

Because TUNEL labeling may identify DNA fragmentation from nonapoptotic processes, experiments were performed in additional hearts from each group to determine whether myocyte TUNEL labeling colocalized with in situ ligation of hairpin oligonucleotide probes, which is considered to be highly specific and sensitive for detection of apoptotic double-strand DNA breaks.14–16 Consistent with the quantitative TUNEL labeling analysis described above, TUNEL labeling was absent or extremely rare in control and 4-week AS hearts but was more common in 7-week AS hearts. In all instances, in situ ligation was present and colocalized with TUNEL-positive myocyte nuclei, as illustrated in Figure 6.

Discussion

The present study demonstrates that the ascending AS mouse provides a model to study both initial compensated LV hypertrophy and very early systolic dysfunction. Our observations show that aberrant myocyte branching and abnormal β1-integrin deposition appear in situ at this stage of early failure in association with a very low incidence of myocyte

<table>
<thead>
<tr>
<th>TABLE 3. LV Myocyte Morphometry</th>
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<tbody>
<tr>
<td>LV Myocyte Length, μm</td>
</tr>
<tr>
<td>------------------------</td>
</tr>
<tr>
<td>Controls</td>
</tr>
<tr>
<td>AS, 4 wk</td>
</tr>
<tr>
<td>AS, 7 wk</td>
</tr>
</tbody>
</table>

Control indicates 7-week sham-operated normal mice; AS, ascending AS mice studied at 4 and 7 weeks after operation. n=6 to 7 per group. LV indicates LV free wall; septum, the interventricular septum.

*P<0.05 vs sham-operated control group; †P<0.01 vs 4-week AS by ANOVA and Student’s t test.
apoptosis as reported by positive TUNEL reaction, in situ ligation, and DNA laddering. These data raise the hypothesis that the dynamic changes in cell-cell and cell-ECM anchorage inherent in multicellular growth may be a signal that contributes to the development of apoptosis in chronic pressure overload hypertrophy.

**Apoptosis and Early Heart Failure**

Programmed cell death is critical for the regulation of embryological development, immune cell growth, and controlled dismantling of cells with defective transit through the cell cycle.\(^{21-23}\) The activation of the highly regulated caspase-protease cascade results in proteolytic damage to the nuclear lamina, cytoskeleton, and internucleosomal cleavage of DNA.\(^{24,25}\) The in situ TUNEL technique\(^{13,24,25}\) has been used to identify DNA fragmentation attributed to apoptosis in studies of end-stage human and experimental heart failure. The prevalence of TUNEL-positive cells has varied widely, ranging from values as high as 35 to as low as 0.2 to 0.4 positive nuclei per 100 myocytes.\(^{26-29}\)

Using confocal microscopy, we observed that positive in situ TUNEL was virtually absent in LV vibratome sections of both controls and 4-week AS animals. Consistent with this finding in early adaptive hypertrophy, Teiger et al.\(^{17}\) reported a very transient wave of TUNEL-positive myocytes immediately after aortic banding in rats but observed no increase in apoptosis in early hypertrophy 30 days after pressure overload. In contrast, in the 7-week AS mice with early heart failure, we observed in situ TUNEL of LV myocytes as well as DNA laddering by DNA gel electrophoresis consistent with DNA internucleosomal cleavage. Although the incidence of TUNEL-positive myocyte nuclei was very low, the frequency of TUNEL-positive nuclei observed in 7-week AS mice was similar to that described in a recent report of transgenic mice with \(\text{Gs}_\alpha\) overexpression that were studied during the transition to early failure.\(^{29}\) Regions of positive labeling were not associated with infiltration of inflammatory cells.

**Limitations**

A limitation of the TUNEL analysis is that nick end-labeling of 3' DNA hydroxyl groups may not discriminate highly regulated apoptotic DNA cleavage from nonspecific DNA fragmentation due to necrosis, in vitro autolysis, RNA synthesis, or DNA repair.\(^{14-16,30,31}\) Recent experiments by...
Didenko et al\textsuperscript{14–16} that examined different forms of DNA damage have demonstrated that in situ ligation of DNA with hairpin oligonucleotides that are complementary to the ends of double-strand DNA breaks is highly sensitive and specific in the identification of apoptotic nuclei and discrimination between other forms of nonspecific DNA fragmentation. Therefore, we performed additional experiments that demonstrated that positive labeling for in situ ligation colocalized with TUNEL-positive myocyte nuclei labeling in all instances. Taken together, these data provide support that myocyte apoptosis is not a ubiquitous outcome of pressure overload and is extremely rare in early adaptive hypertrophy. However, myocyte apoptosis is detected at the stage of early failure. The onset of a very low frequency of repetitive dropout of myocytes via apoptosis has the potential to contribute to the loss of force-developing myocytes and promote the gradual progression of systolic dysfunction. In pathological hypertrophy, multiple signals may increase the risk of apoptosis, including tissue hypoxia, free-radical injury, local production of growth-stimulating peptides such as angiotensin II, and perturbed calcium regulation.\textsuperscript{32,33} However, anoikis has received little attention as a potential signal of apoptosis in in vivo chronic hypertrophy.

**Anoikis and Hypertrophy**

In the present study, the combined morphological evidence of increased myocyte branching, changes in the intercalated disk, changes in myocyte size, and altered $\beta_1$-integrin deposition were prominent at the stage of early failure in this model. Previous studies in isolated myocytes have also suggested that changes in myocyte cell shape appear long before overt failure.\textsuperscript{34} The data presented here suggest that chronic biomechanical stress results in altered cell-cell and cell-ECM interactions as well as an increase in cardiac myocyte size. Studies of apoptosis using a variety of cell types have demonstrated that release of cells from normal cell-cell and/or cell-ECM contacts are events that are sufficient to trigger apoptosis, a process called anoikis.\textsuperscript{35–37} Fibronectin and integrins, specifically $\alpha_5\beta_1$, have been shown to be important components in triggering apoptosis,\textsuperscript{36,37} and altered expression may be present in cardiac hypertrophy.\textsuperscript{10,38,39} The release of myocytes from normal anchorage-dependent binding to collagen and/or fibronectin via the $\alpha_5\beta_1$-integrin to an anchorage-independent status could be an important signal in triggering apoptosis during chronic hypertrophic growth.

Previous reports, as well as the staining pattern of isolated hypertrophied myocytes in this study, suggest that $\beta_1$-integrins are confined to the Z bands of the myocyte; however, these studies did not use confocal microscopy of tissue, so staining of the ECM would not have been evident. This study provides new observations that support the concept that abnormal myocyte-ECM anchorage develops during chronic hypertrophy. The data obtained from staining with antibodies against $\beta_1$-integrin indicated an increase in the presence of immunoreactive material both on the cell surface and in the ECM surrounding the myocytes. The staining in the ECM appeared to increase from 4- to 7-week AS compared with controls. Measurement of $\beta_1$-integrin protein levels by Western blots indicated that there was no significant change in the amount of $\beta_1$-integrin from isolated myocytes from AS and control hearts. However, these data do not account for $\beta_1$-integrin immunoreactive material in the ECM. Control experiments using preimmune antisera and antisera specific to only the cytoplasmic domain of $\beta_1$ showed no staining in the ECM. The characterization of specific integrins as well as the immunoreactive material that appears to be shed into the ECM is currently under way.
Shedding of the integrins into the ECM is consistent with cell growth.40,41 Because integrin attachment to the ECM represents fixed anchorage points,42 it is logical to assume that these points of attachment must repeatedly change as myocyte growth and changes in morphology occur. This would be expected to be more prominent in the later stages of hypertrophy (7-week AS), when the myocyte shows branching as well as increases in size. Thus, we propose that this shedding of the integrin-stable material is a significant feature of hypertrophic growth; however, the precise mechanism by which this event happens is not clear. One likely mechanism is the shedding of β1-integrin due to the presence of cell surface proteases, such as the matrix metalloproteases or the ADAMS proteins (A Disintegrin and Metalloprotease).40,41,43,44 Both classes of proteases are associated with integrins45–47 and could be important components in the interactions between the ECM and cell surface that contribute to the dynamic balance between cell growth and apoptosis.

In summary, these observations in AS mice support the hypothesis that myocyte apoptosis develops during the transition from hypertrophy to early failure in response to chronic biomechanical stress. Future experiments are necessary to investigate integrin–ADAMS protein regulation in hypertrophy and to conclusively demonstrate that changes in myocyte anchorage to the ECM contribute to apoptosis signaling in adult myocytes.

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