Cell Death in Acromegalic Cardiomyopathy

Andrea Frustaci, MD; Cristina Chimenti, MD; Manabu Setoguchi, MD; Sabrina Guerra, BS; Salvatore Corsello, MD; Filippo Crea, MD; Annarosa Leri, MD; Jan Kajstura, PhD; Piero Anversa, MD; Attilio Maseri, MD

Background—Prolonged untreated acromegaly leads to a nonspecific myopathy characterized by ventricular dysfunction and failure. However, the mechanisms responsible for the alterations of cardiac pump function remain to be defined. Because cell death is implicated in most cardiac disease processes, the possibility has been raised that myocyte apoptosis may occur in the acromegalic heart, contributing to the deterioration of ventricular hemodynamics.

Methods and Results—Ten acromegalic patients with diastolic dysfunction and 4 also with systolic dysfunction were subjected to electrocardiography, Holter monitoring, 2-dimensional echocardiography, cardiac catheterization, and biventricular and coronary angiography before surgical removal of a growth hormone–secreting pituitary adenoma. Endomyocardial biopsies were obtained and analyzed quantitatively in terms of tissue scarring and myocyte and nonmyocyte apoptosis. Myocardial samples from papillary muscles of patients who underwent valve replacement for mitral stenosis were used for comparison. The presence of apoptosis in myocytes and interstitial cells was determined by confocal microscopy with the use of 2 histochemical methods, consisting of terminal deoxynucleotidyl transferase (TdT) assay and Taq probe in situ ligation. Acromegaly was characterized by a 495-fold and 305-fold increase in apoptosis of myocytes and nonmyocytes, respectively. The magnitude of myocyte apoptosis correlated with the extent of impairment in ejection fraction and the duration of the disease. A similar correlation was found with the magnitude of collagen accumulation, indicative of previous myocyte necrosis. Myocyte death was independent from the hormonal levels of growth hormone and insulin-like growth factor-1. Apoptosis of interstitial cells did not correlate with ejection fraction.

Conclusions—Myocyte cell death, apoptotic and necrotic in nature, may be critical for the development of ventricular dysfunction and its progression to cardiac failure with acromegaly. (Circulation. 1999;99:1426-1434.)

Key Words: cells ■ heart failure ■ growth substances ■ cardiomyopathy

Growth hormone (GH) excess leads to cardiovascular alterations whose underlying cause remains controversial. Hypertension, glucose intolerance, or chronic exposure to GH may alone be implicated in the abnormalities of ventricular hemodynamics.1,2 Heart failure, involving both the left and right ventricles, may occur late in acromegaly,3 but the events implicated in the progressive deterioration of pump performance have not been identified. Myocardial hypertrophy and cavitary dilation are consistent findings.4 Focal areas of replacement fibrosis and diffuse interstitial fibrosis, in combination with muscle fiber disarray, lymphocyte infiltrates, and small vessel disease have been described,5,4 but the amount of healthy myocardium largely predominates, raising questions on the underlying basis responsible for the impairment in the functional capacity of the acromegalic heart. Recent observations have indicated that ongoing myocyte cell death, apoptotic in nature, affects the decompensated human heart.5,6 Programmed cell death is not associated with collagen deposition,7 which typically follows cell necrosis.8 Additionally, myocyte apoptosis is scattered across the wall and, rarely, involves groups of 2 to 3 cells. This form of cell death can be triggered by humoral factors9 and physical forces10,11 and has a significant impact on myocardial mechanics by reducing the force-generating capacity of the muscle.10 The ability of developing comparable degrees of resting tension with changes in sarcomere length is also altered; the Frank-Starling relation is severely modified by myocyte apoptosis of <0.4%.10,12 These physiological deficiencies are accompanied by restructuring of the wall with side-to-side slippage of myocytes, mural thinning, and cavitary dilation.13 Therefore, the possibility may be advanced that programmed myocyte cell death occurs in the acromegalic heart, playing a key role in the deterioration of function with time. This hypothesis was tested with the use of cardiac biopsies from the left and right ventricular myocardium of patients with acromegaly.

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From the Departments of Cardiology and Endocrinology, Sacred Heart Catholic University, Rome, Italy (A.F., C.C., S.C., F.C., A.M.); and the Department of Medicine, New York Medical College, Valhalla, NY (M.S., S.G., A.L., J.K., P.A.).
Reprint requests to Piero Anversa, MD, Department of Medicine, Vosburgh Pavilion, Room 302, New York Medical College, Valhalla, NY 10595.
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Methods

Patients
Ten patients, 5 men and 5 women, with active acromegaly and cardiac dysfunction, varying from New York Heart Association class II to IV, were studied. All patients underwent noninvasive cardiac examinations consisting of ECG, 2-dimensional echocardiography with Doppler analysis, and Holter monitoring. Invasive studies consisting of cardiac catheterization, coronary angiography, and ventricular endomyocardial biopsy also were performed. Echocardiography included the measurement of left ventricular end-systolic and end-diastolic diameters and the evaluation of the thickness of the septum and the posterior aspect of the left ventricular wall. Systolic function was assessed by the analysis of left ventricular ejection fraction (EF), whereas diastolic function was determined by the E/A ratio. Left ventricular mass was derived with the use of the echocardiographic method described by Devereux and Reichek \(^{14}\) and, subsequently, normalized by body surface area to obtain a left ventricular mass index. These parameters were collected before surgical removal of a GH-secreting pituitary adenoma. Samples from papillary muscles of 10 patients of comparable age and sex who underwent valve replacement for mitral stenosis were used for comparison. Although these were not healthy individuals, their papillary muscles were not overloaded. However, some unloading caused by the stenosis was present. Despite this limitation, a more appropriate control myocardium could not be obtained. This study was approved by the institutional review committee, and patients gave informed consent. Procedures followed were in accordance with institutional guidelines.

Hormone Levels
Plasma levels of GH were measured in duplicate by an immunoradiometric assay kit (bGH-IRMA-CT, Radim). Similarly, insulin-like growth factor-1 (IGF-1) concentration was assessed by radioimmunoassay (Medgenix Diagnostics). Levels of GH and IGF-1 were expressed as mean values obtained by averaging the results from 4 daily blood samples taken at 8:00 AM and noon and at 5:00 and 11:00 PM.

Light Microscopy
In acromegalic patients, 3 to 4 endomyocardial biopsies, \(\sim 3 \text{ mm}^2\) each, were collected from the septal apical region of the right and left ventricle. Specimens of papillary muscles from patients with mitral stenosis were used for comparison. Tissue was fixed in 10% formalin, paraffin-embedded, and stained with hematoxylin-eosin or trichrome. Myocardial fibrosis was measured with the use of a computer-assisted image analyzer with KS-500 software (Zeiss). A total of 24 serial sections were examined in each patient.

Confocal Microscopy
Sections were incubated in a solution containing 5 U of terminal deoxynucleotidyl transferase (TdT) (Boehringer Mannheim), 2.5 mmol/L CoCl\(_2\), 0.2 mol/L potassium cacodylate, 25 mmol/L Tris-HCl, 0.25% BSA, and 0.5 mmol/L biotinylated 2'-deoxyuridine-5'-triphosphate (biotin-16-dUTP). After exposure to 5 \(\mu\)g/mL of FITC-labeled extravidin, samples were stained with \(\alpha\)-sarcomeric actin antibody (clone 5C5, Sigma Chemical Co) and then with TRITC-labeled anti-mouse IgG. Nuclei were visualized with the use of propidium iodide–labeled nuclei included in a \(\alpha\)-sarcomeric actin-positive and \(\alpha\)-sarcomeric actin-negative cells. In acromegaly, these values were 211±45 mm\(^2\) of tissue for myocytes and 599±94 mm\(^2\) of myocardium for nonmyocytes. In subjects with mitral stenosis, these values were 277±45 mm\(^2\) for myocytes and 587±85 for nonmyocytes. These parameters were used to compute the number of TdT-stained apoptotic nuclei per 10\(^6\) cells.\(^{6}\)

Inturnucleosomal DNA cleavage was confirmed by in situ ligation\(^{12}\) because the TdT assay may overestimate the extent of cell death as a marker of apoptosis.\(^{13}\) Specifically, double-strand DNA fragments for in situ ligation to \(3'\) overhangs were prepared with primers 5'-GTGCGCTCAGCCGATCCGGCAAGCTCTACCT-3' and 5'-GGCTGTTGTCGCCGGCTTTCGACCCTGC-3'. Complementary to pBluescript-bSD1 plasmid. The reaction included 50 mmol/L Tris-HCl, pH 8.3, 10 mmol/L KCl, 1.5 mmol/L MgCl\(_2\), 16.6 \(\mu\)mol/L digoxigenin-11-dUTP (Boehringer Mannheim), 16.6 \(\mu\)mol/L TTP, 50 \(\mu\)mol/L of dATP, dCTP, and dTTP, 100 pmol of each primer, and 10 pg of plasmid. Inv polymerase (2.5 U) was added to the reaction mixture after heating to 80°C. Polymerase chain reaction was performed with 35 cycles of 20 seconds at 95°C, 20 seconds at 60°C, and 120 seconds at 74°C, the final cycle having an extension time of 4 minutes. Gel electrophoresis was performed to document a single polymerase chain reaction product. Amplified product was purified with the Qiagen kit. Digoxigenin-labeled fragments were ligated to DNA in tissue sections with the use of T4 ligase. Sections were treated with protease K (50 \(\mu\)g/mL PBS) for 30 minutes at 37°C, and a mixture of 50 mmol/L Tris-HCl, pH 7.8, 10 mmol/L MgCl\(_2\), 10 mmol/L DTT, 1 mmol/L ATP, 25 \(\mu\)g/mL BSA, 15% polyethylene glycol (8000 MW), 1 \(\mu\)g/mL probe, and 25 U/mL DNA T4 ligase was applied for 4 hours. Sections were washed with water at 70°C and incubated with anti-digoxigenin mouse monoclonal antibody (Boehringer Mannheim) followed by exposure to FITC-labeled goat anti-mouse IgG. Myocyte cytoplasm and nuclei were labeled as described in the TdT assay. Sections exposed to DNase I were used as positive control, whereas omission of T4 ligase was used as negative control. This methodology has previously been used in our laboratory.\(^{11}\) Essentially the same amount of myocardium indicated for TdT was examined by confocal microscopy in both groups of patients. The average numbers of myocyte and nonmyocyte nuclei analyzed by the TdT polymerase probe in acromegalic subjects were 3025±972 and 8922±1813, respectively. In cases with mitral stenosis, 13 561±14 229 and 30 032±30 070 myocyte and nonmyocyte nuclei were evaluated. The numerical density of myocyte and nonmyocyte nuclei was then determined. In acromegaly, values were 204±47 mm\(^2\) of tissue for myocytes and 616±92 mm\(^2\) of myocardium for nonmyocytes. Corresponding values with mitral stenosis were 285±59 mm\(^2\) and 568±65 mm\(^2\). On this basis, the number of TdT-positive apoptotic nuclei per 10\(^6\) cells was computed.

Statistical Analysis
All tissue samples were coded, and the code was broken at the end of the study. Results are presented as mean±SD. Statistical significance between 2 measurements was determined by the 2-tailed unpaired Student's t test; probability values <0.05 were considered significant. Linear regression analysis was performed to correlate duration of the disease and EF with the magnitude of apoptosis in myocytes and interstitial cells.\(^{36}\)

Results
Patients
Table 1 lists the sex, age, duration of disease, and hormonal profile of the 10 patients included in the current study. They consisted of 5 men, 53±14 years old, and 5 women, 57±4 years old, who had active acromegaly. Time from diagnosis varied from 2 to 15 years and did not differ between the 2 groups: men, 6.0±5.6 years; women, 4.8±3.3 years (not significant). In men, the levels of GH and IGF-1 were 12±5 ng/mL and 649±144 ng/mL, respectively. Values in women were 26±17 ng/mL and 693±141 ng/mL. Acromegaly was

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Acromegaly and Apoptosis

confirmed by the inability of an oral glucose load to decrease the plasma concentration of GH. The normal upper limit for GH is 5 ng/mL and for IGF-1, 300 ng/mL. With the exception of 1 male patient, who received lanreotide and cabergoline treatment, the other 9 individuals were not under medical therapy at the time of cardiac biopsies.

Cardiac Characteristics
The ECG showed signs of ventricular hypertrophy in all cases. Rhythm defects were not observed in 9 patients, but 1 individual, patient 2 in Table 1, had left bundle-branch block. Holter monitoring in this subject revealed frequent ventricular ectopic beats with some doublets and triplets. Phases of nonsustained ventricular tachycardia were also detected. This patient was considered to be in Lown class IVb. Anatomic and functional parameters are listed in Table 2. In subjects with mitral stenosis, the thickness of the septum and posterior aspect of the left ventricular wall was 9.3±0.71 mm and 9.0±0.58 mm, respectively. These values increased 33% (P<0.001, 12.4±1.1 mm) and 32% (P<0.001, 11.9±1.5 mm) in acromegalic subjects. However, comparable changes in ventricular thickness were noted in acromegalic men (12±1.9 mm) and women (11.8±1.0 mm). A similar adaptation was also noted in septal thickness (men, 12.1±1.2 mm; women, 12.6±1.1 mm). Left ventricular end-diastolic diameter was within normal range in 9 patients, indicating that wall thickening exceeded the alteration in chamber radius. However, a marked increase in cavitary volume was noted in the subject with rhythm disturbances. Thus in 9 cases, mural thickening was the prevailing response of the heart, resulting in an increase in wall thickness-to-chamber radius ratio. Left ventricular mass index in subjects with mitral stenosis was 92±6 g/m². With acromegaly, this parameter was within normal range in 1 man (114 g/m²) but increased in other 4 men (213±85 g/m²) and 5 women (160±13 g/m²). No statistical difference was noted between sexes. The average value in the entire population of 10 patients was 177±61 g/m².

EF was within normal limits (>50%) in 6 (56%±7%) but was reduced in 4 (36%±10%) acromegalic individuals (P<0.01). The average value of this parameter in the 10 patients was 48%±13%. Moreover, ventricular contractility, assessed by ventriculography, was compromised in these 4 subjects. Doppler evaluation of mitral flow documented diastolic dysfunction in all patients because the E/A ratio was consistently <1 (Table 2). Subjects with mitral stenosis had an E/A ratio of 1.3±0.3, which decreased 51% (P<0.001, 0.64±0.09) with acromegaly. Left ventricular end-diastolic pressure was elevated in all cases, varying from a minimum of 13 mm Hg to a maximum of 25 mm Hg, averaging 15±4 mm Hg. Control value is <12 mm Hg. Left ventricular systolic pressure was not increased in acromegalic patients. Similarly, coronary angiography did not detect defects in the arterial tree.

Myocardial Structure
The amount of interstitial collagen in combination with small foci of replacement fibrosis occupied 1.0%±0.5% of the

<p>| TABLE 1. Characteristics of Acromegalic Patients at Myocardial Biopsy |
|------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|------------------|</p>
<table>
<thead>
<tr>
<th>Patients</th>
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<th>Age, y</th>
<th>Duration of Disease, y</th>
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<th>IGF-1, ng/mL</th>
<th>PRL</th>
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</tr>
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</table>

GH indicates growth hormone; IGF-1, insulin-like growth factor-1; PRL, prolactin; N, normal; ↑, hyperfunction; and ↓, hypofunction.

*Lanreotide plus cabergoline.

<p>| TABLE 2. Anatomic and Functional Characteristics of Acromegalic Patients |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Patient</th>
<th>LV Mass/BSA, g/m²</th>
<th>E/A</th>
<th>LVEDD, mm</th>
<th>LVEDP, mm Hg</th>
<th>EF, %</th>
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LV indicates left ventricle; BSA, body surface area; E/A, ratio between early and late diastolic ventricular filling; LVEDD, left ventricular end-diastolic diameter; LVEDP, left ventricular end-diastolic pressure; and EF, ejection fraction.
myocardium of subjects with mitral stenosis and 7.9%±4.7% of the tissue in acromegalic patients. This 8-fold increase in the extent of connective tissue was statistically significant (P<0.001). Comparable values were obtained in acromegalic men (7.7%±5.8%) and women (8.1%±4.1%). The degree of myocardial fibrosis was inversely correlated with the impairment in EF (r=−0.92; P<0.0003) and positively correlated with the duration of the disease (r=0.91; P<0.0003). Myocytes appeared to be enlarged, and nuclei were prominent and irregular in shape.

**Myocyte and Interstitial Cell Apoptosis**

Apoptosis was detected by 2 independent histochemical methods, consisting of the TdT assay and in situ ligation. Tissue sections were examined by confocal microscopy. Figure 1, A through C, illustrates a myocyte undergoing apoptosis; nuclear damage with half-moon appearance is depicted by the red fluorescence of propidium iodide staining. The presence of DNA strand breaks is documented separately by the green fluorescence of the TdT assay. The myofibrillar component of the cytoplasm is shown by the red fluorescence of α-sarcomeric actin antibody labeling. Chromatin margination and loss in DNA, or nuclear pyknosis, are demonstrated in Figure 2, A through D and E through F. Similar nuclear modifications in interstitial cells are depicted in Figures 3 and 4.

Apoptosis was confirmed by a second method consisting of Taq polymerase in situ ligation. This technique identifies double-strand cleavage of the DNA with single-base 3’ overhangs that occur exclusively with apoptosis. Figure 5 illustrates 2 myocytes undergoing apoptosis in 2 patients with acromegaly. Nuclear morphology, which was largely preserved, is shown by the red fluorescence of propidium iodide staining (Figure 5, A and D). Double-strand DNA cleavage with single-base 3’ overhangs is depicted by green fluorescence in panels B and E. The combination of these 2 nuclear stainings with α-sarcomeric actin antibody labeling of the myocyte cytoplasm is represented in panels C and F. The specificity of in situ ligation to recognize DNA damage was established by exposing tissue sections to DNase I; diffuse staining of nuclei was observed (positive control). Conversely, the omission of T4 ligase resulted in the lack of labeling of nuclei (negative control).

Low levels of myocyte apoptosis were present in samples from patients with mitral stenosis. TdT assay yielded an average value of 6.2±13 per 10^6 and Taq 5.4±11 per 10^6. Corresponding values in interstitial cells were 16±27 per 10^6 and 14±28 per 10^6. With acromegaly, the TdT reaction showed values of 2810±2725 per 10^6 in myocytes and 4453±3665 per 10^6 in interstitial cells, whereas in situ ligation demonstrated 2671±2469 per 10^6 in myocytes and 4273±3952 per 10^6 in interstitial cells. On the basis of TdT, acromegaly was characterized by a 453-fold (P<0.005) and 278-fold (P<0.002) increase in the number of myocytes and nonmyocytes dying by apoptosis, respectively. With Taq, apoptosis in acromegaly increased 495-fold (P<0.004) and 305-fold (P<0.002) in myocytes and interstitial cells. None of the small differences between TdT and in situ ligation data were statistically significant. The extent of apoptosis in acromegalic patients was obtained by examining 8 right ventricular and 2 left ventricular myocardial biopsies. When ejection fraction was compared with the magnitude of apoptosis, it was apparent that severe impairment in cardiac pump function was accompanied by higher levels of myocyte death (Figure 6A). Additionally, the duration of acromegaly correlated with the degree of apoptosis (Figure 6B). This was not always the case for interstitial cells. Apoptosis in nonmyocytes was independent from the ventricular hemodynamics, for example, EF (not shown), but it correlated with the time of the disease (Figure 6C). These correlations were done with the use of the data collected with the Taq probe because of its specificity. However, similar results were obtained when TdT values were used.

**Figure 1. Acromegaly: TdT assay and confocal microscopy of the left ventricle. A, By red fluorescence of propidium iodide, half-moon image of the nucleus is shown (arrow); B, by green fluorescence, DNA strand breaks by the TdT assay (arrow). Combination of these 2 nuclear stainings with red fluorescence of α-sarcomeric actin is shown in C. Magnification ×1500. Bar corresponds to 10 μm.**
Discussion

Cell Death in Acromegalic Heart

Findings in this investigation document that the increase in cardiac mass with acromegaly was characterized by structural abnormalities involving tissue fibrosis and ongoing myocyte and interstitial apoptotic cell death. Replacement fibrosis is the consequence of focal myocyte necrosis, whereas interstitial fibrosis may be independent from scattered cell necrosis and may result from the reaction of connective tissue cells to pathological loads. This is supported by the lack of evidence of acute myocyte necrosis, although difficulty exists in detecting this form of damage in the absence of specific markers. Apoptosis occurs without collagen accumulation in the ventricular wall, suggesting that the effects of this type of cell death do not involve changes in the volume composition of the myocardium. This is a well-established phenomenon in all organs. In 9 of the 10 cases analyzed here, there was thickening of the wall without a significant increase in cavitary diameter, indicating that concentric ventricular hypertrophy developed with acromegaly. Although defects in the compliance properties and shortening velocity occur
with concentric hypertrophy, tissue scarring may alter muscle mechanical behavior. Similarly, myocyte apoptosis may impair the ability of the myocardium to develop force, decreasing pump function and EF. In the patient who had chamber dilation, relative wall thinning, and a 22% EF, myocyte death and collagen quantity were very high. In spite of these findings, it is not possible to establish whether chronic apoptotic cell death is the predominant event responsible for the deterioration of ventricular performance with acromegaly. However, it is likely that myocyte death, apoptotic and necrotic in nature, participates in the onset of ventricular dysfunction and its progression to cardiac failure. Cell death may account for the slow and partial recovery of contractility even after a successful removal of a GH-secreting pituitary adenoma.

The 0.3% level of myocyte apoptosis detected here may question its role in ventricular dysfunction of acromegalic patients. In most cell types, this process is completed between 20 minutes and 2 hours, suggesting that the magnitude of cell death with time may be highly significant. In this regard,
apoptosis in myocytes is responsible for mural slippage of cells and sudden changes in ventricular dimension.\textsuperscript{10,12} Ongoing cell loss negatively influences the anatomy and the hemodynamics of the heart; prevention of myocyte death in the surviving myocardium after infarction attenuates ventricular dilation, myocardial loading, and reactive hypertrophy.\textsuperscript{23} Comparable results have been obtained in hypertensive animals during the transition from compensated to decompensated myocardial hypertrophy.\textsuperscript{9} Lack of correlation between interstitial cell apoptosis and ventricular dysfunction is difficult to explain. Fibroblasts respond to apoptotic stimuli operative in myocytes, such as mechanical stretch,\textsuperscript{10} and this may be linked to the release of angiotensin II.\textsuperscript{11} However, fibroblasts are less susceptible to undergo apoptosis.\textsuperscript{10} Cell death in nonmyocytes may not represent a primary event of the cardiac myopathy with acromegaly, but this does not diminish the profound impact of collagen accumulation on myocardial performance.

**IGF-1 and Cell Death in Acromegalic Heart**

Several in vitro\textsuperscript{24} and in vivo\textsuperscript{23} studies have demonstrated that IGF-1 interferes with apoptosis and necrosis in various cell systems. Moreover, IGF-1 enhances cardiac hypertrophy and limits ventricular remodeling, improving myocardial function.
after infarction. Similar results have been obtained in patients with dilated cardiomyopathy. In both cases, the favorable changes produced by IGF-1 may be the consequence of attenuation of myocyte death in the heart. IGF-1 administration reduces cell necrosis and apoptosis in ischemia-reperfusion injury, and overexpression of IGF-1 in transgenic mice prevents the activation of necrotic and apoptotic myocyte death in the surviving myocardium after infarction. On the basis of these observations, the detection of myocyte death in the surviving myocardium after infarction may have resulted in a downregulation of IGF-1 receptors on cardiac muscle and nonmuscle cells.

**Study Limitations**

There are limitations that must be acknowledged. Control myocardium from healthy human beings was not available. Papillary muscles from patients with mitral stenosis are inevitably exposed to some unloading that could have influenced the collected results. Although 2 histochemical methods were used to evaluate apoptosis, this phenomenon could not be confirmed by DNA agarose gel electrophoresis. TdT reaction and Taq in situ ligation provided essentially identical results, documenting that the TdT assay with a fluorescent probe is a reliable technique for the detection of apoptosis. Ongoing myocyte necrosis could not be evaluated; this form of cell death may be relevant in the progression of the cardiac disease. Finally, biopsies are small in size and were collected at only 1 time point. All these variables have to be considered in the interpretation of the results obtained here.

**Acknowledgments**

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