End-Stage Cardiac Failure in Humans Is Coupled With the Induction of Proliferating Cell Nuclear Antigen and Nuclear Mitotic Division in Ventricular Myocytes

Federico Quaini, Elena Cigola, Costanza Lagrasta, Gloria Saccani, Eugenio Quaini, Cristiano Rossi, Giorgio Olivetti, Piero Anversa

Abstract  Proliferating cell nuclear antigen (PCNA) is a late growth-regulated gene that is expressed at the G1-S boundary of the cell cycle and is required for DNA synthesis and cell proliferation. Since quantitative results suggest that myocyte hyperplasia occurs in the decompenated human heart, we postulated that induction of the PCNA gene may be present in the failing heart in humans. PCNA protein was detected in myocardial samples obtained from the left and right ventricles of patients with congestive heart failure. Endomycocardial biopsies collected from donor subjects were used as control tissue. The percentage of positively stained myocyte nuclei in the ventricles was established by using PCNA monoclonal antibody and the immunoperoxidase technique. The localization of PCNA in myocytes was confirmed by a sarcomeric actin antibody staining. PCNA labeling was present in left ventricular myocytes of 29 of the 32 hearts examined. In the right ventricle, 24 of the 29 samples showed positive staining. In a subset of 25 patients, the percentage of PCNA-labeled myocyte nuclei was measured and found to constitute 49±22% of left ventricular myocytes. A similar analysis for the right ventricle, conducted in 21 patients, showed that 49±19% of the myocyte nuclei exhibited PCNA protein. In addition, mitotic figures in myocytes were documented. A quantitative analysis of this cellular process revealed that 11 myocyte nuclei per 1 million cells exhibited mitotic images in chronic heart failure. Immediately after myocardial infarction, two cells per million showed mitotic division, and this phenomenon was restricted to the region adjacent to the necrotic tissue. No PCNA labeling or nuclear mitotic images were detected in the ventricular myocardium of control subjects. Thus, the observation that diffuse PCNA labeling and myocyte mitotic division are present in hearts with end-stage failure strongly suggests that adult ventricular myocytes are not terminally differentiated cells and that myocyte cellular hyperplasia may constitute a growth reserve mechanism of the diseased heart. (Circ Res. 1994;75:1050-1063.)

Key Words: late growth-related genes • myocyte nuclear mitotic division • myocyte hyperplasia • end-stage cardiac failure • human heart

There are several gene products that are required for cell proliferation.1 Proliferating cell nuclear antigen (PCNA), a cofactor of DNA polymerase delta,2,3 is a nuclear protein necessary for DNA synthesis and for cell cycle progression.4 When cells are in G1, PCNA mRNA levels are low but rapidly increase in the presence of growth factors, stimulating cells to divide.3,5 In continually dividing cells, PCNA mRNA and the protein itself vary little in amount throughout the cell cycle.5 The crucial role of PCNA for cell growth has been documented by inhibition of cell proliferation by an antisense oligodeoxynucleotide to PCNA mRNA.6,7 Under these conditions, cell growth is abolished. Thus, the induction of PCNA protein in myocytes may be considered an important indicator that these cells may enter the cell cycle and undergo DNA synthesis and nuclear mitotic division. This contention is supported by the observation that PCNA protein has been detected in ventricular myocytes of the mammalian rat heart during embryonic and early postnatal life,6 during which myocyte cellular hyperplasia predominates.10 In contrast, PCNA protein has not been demonstrated in normal adult ventricular myocytes, in spite of the fact that PCNA mRNA is present.4 However, in pathological states characterized by severe ventricular dysfunction and failure, the expression of PCNA mRNA is markedly enhanced in association with the appearance of PCNA protein in the stressed myocytes.11,12 DNA synthesis, myocyte mitotic division, and myocyte cellular hyperplasia have been documented as well.13-14 These experimental observations constituted the basis for the present investigation, in which the hypothesis was advanced that activation of late growth-regulated genes may occur in the failing human heart. Specifically, the induction of PCNA protein in myocytes was examined in samples of ventricular myocardium from patients undergoing cardiac transplantation. Although the identification of PCNA protein in these cells may provide supporting evidence that adult ventricular myocytes are not terminally differentiated cells, it cannot be determined from this phenomenon whether the activation of the DNA synthetic machinery actually results in myocyte nuclear mitotic division. Therefore, a careful analysis of the occurrence of mitosis in myocytes.

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was performed and compared with the magnitude of this cellular process in the fetal human heart. An identical evaluation was conducted at the level of the nonmyocyte compartment of the myocardium. This type of determination is without precedent in humans and animal models in both normal conditions and pathological states of the heart. Moreover, mitotic images in myocytes of the adult human heart have never been identified.

Materials and Methods
Cardiac Function and Ventricular Anatomy
The hemodynamic and gross anatomic properties of the hearts included in the present study were established between 1 and 6 months before cardiac transplantation. Specifically, measurements of systolic and diastolic ventricular dimensions and fractional shortening were performed by two-dimensional echocardiography. Stroke volume, cardiac output, cardiac index, ejection fraction, right ventricular end-diastolic pressure, and pulmonary wedge pressure were determined by cardiac catheterization.

Tissue Fixation and Sampling
Thirty-two hearts were sampled immediately after cardiacectomy from patients undergoing cardiac transplantation at the De Gasperi Division of Cardiac Surgery in Milan. Specimens were obtained from the left and right ventricular myocardium and fixed in 10% buffered formalin. These tissue fragments were taken halfway between the base and the apex in the subendocardial-midmyocardial region of the anterior wall of each ventricle. Technical limitations did not permit fixation of all samples for an identical time period. Twenty-five of the 32 left ventricular specimens and 22 of the 29 right ventricular specimens were uniformly fixed for 30 minutes and used for the quantitative analysis of PCNA distribution in myocytes. The remaining cases were used for qualitative detection of PCNA in the myocardium but were excluded from the quantitative determinations. To ensure rapid and adequate fixation, the initial specimens were cut into small tissue blocks, 2 to 3 mm in size, which were embedded in paraffin for light microscopy. Five to 25 tissue samples were obtained in each case in each ventricle.

Four additional left ventricular samples were collected at autopsy from four patients who died within 5 days after acute myocardial infarction and intractable congestive heart failure. These patients were included since tissue fragments became available within 2 hours after death. However, they were used only for qualitative detection of PCNA protein in the tissue and for a careful search of mitotic figures in the myocardium.

Endomyocardial biopsies performed before cardiopulmonary arrest in five donor subjects were used as control tissue for PCNA localization. By this approach, only a limited number of tissue fragments were collected, and sampling was restricted to the right ventricle. In spite of these limitations, this myocardium was considered to represent the most appropriate control for baseline PCNA immunohistochemistry. Finally, three fetal hearts were obtained within 5 to 10 hours from abortion and treated in a similar manner for the detection of PCNA protein in ventricular cells and analysis of the number of mitotic divisions in the myocardium.

PCNA Localization
Immunohistochemical staining was performed by using a modification of the immunoperoxidase method of Hsu et al. The monoclonal mouse antibody anti-PCNA (PC10, Dako A/S) was used for this study. The specificity of this antibody has been established previously. Five-micrometer-thick sections were rehydrated in phosphate-buffered saline (PBS) and then exposed to 3% hydrogen peroxide for 5 minutes to block endogenous peroxidase activity. Subsequently, the sections were treated with normal serum to quench nonspecific protein binding. The antibody, diluted 1:100 with PBS, 1% bovine serum albumin, and 0.1% sodium azide, was applied for 10 minutes at room temperature. Sections were then subjected to two-step incubations of 10 minutes each with biotinylated antisemur followed by peroxidase-labeled streptavidin (commercial kit, Dako LSAB). Finally, the sections were counterstained with Mayer’s hematoxylin and mounted with glycero gelatin. Negative controls in which PBS replaced the primary antiserum were performed with each batch of stains. Lymph node sections were used as positive controls.

After the qualitative detection of PCNA labeling in the myocardium, the percentage of positively stained myocyte nuclei was determined in both ventricles at a final magnification of x400. This analysis was achieved by examining an average of 600 myocyte nuclei in each ventricle in each case.

The distinction between myocyte nuclei and nonmyocyte nuclei could not be performed in the fetal myocardium. Nuclei are closely adjacent to each other and are surrounded by a small amount of cytoplasm, which makes the recognition of the cell of origin often uncertain. Therefore, the extent of PCNA labeling in these cases was measured in relation to all cell populations. This determination included the analysis of 4500 to 5000 nuclei per heart.

α-Sarcomeric Actin Staining
To demonstrate further that PCNA protein labeling occurred in adult ventricular myocyte nuclei, sections from a limited number of samples were stained first for PCNA by using alkaline phosphatase-labeled streptavidin (commercial kit, Dako LSAB/R) and fast red chromogen (Biogenex). Subsequently, sections were incubated for 10 minutes with monoclonal anti-rabbit sarcomeric actin antibody (Dako-sarcomeric actin, Alpha-Sr-1) diluted 1:40, followed by peroxidase-labeled streptavidin. As a chromogen, 3-amino-9-ethyl carbazole was used. By this approach, PCNA-labeled nuclei appeared reddish, and α-sarcomeric actin staining of the cytoplasm appeared brownish.

Quantitative Analysis of the Incidence of Mitosis in Myocardial Cells
The frequency of mitosis in myocytes, interstitial cells, and cells that could not be clearly identified was established in 7 of the 25 left ventricular specimens obtained from patients undergoing cardiac transplantation as well as in the 4 autopsy specimens collected from subjects who died from acute myocardial infarction. The three fetal hearts sampled at the time of abortion were similarly analyzed and used for comparison. Mitotic figures were expected to be numerous in the fast-growing fetal myocardium. It is necessary to emphasize that mitotic figures in myocytes and nonmyocyte cell populations in the adult human heart have never been identified, indicating that a quantitative analysis of this phenomenon would require an extremely large amount of sampling. Such an approach was used in the present study.

A second relevant issue was to establish the histological criteria for the recognition of the cell of origin of mitotic figures. A very stringent rule was followed: Mitotic images were considered to pertain to myocytes only if myofibrils with sarcomere striation could be identified in the cytoplasm. Under no other conditions were mitotic figures attributed to myocytes. Mitotic figures in cells located in the interstitial space or in the endothelial lining of the wall of coronary vessels were assigned to nonmyocytes. Since tissue specimens were not fixed by vascular perfusion, the relation between the myocyte and nonmyocyte compartments of the myocardium was, at times, not clearly defined, making the recognition of cells uncertain. This identification was further complicated by the absence of myofibrils in dividing cells, which by location and appearance may resemble myocytes. Under these circum-
TABLE 1. Clinical Characteristics

<table>
<thead>
<tr>
<th>No. of Patients</th>
<th>Age, y</th>
<th>Length of History, mo</th>
<th>Interval From Heart Failure to Transplant, mo</th>
</tr>
</thead>
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<tr>
<td>Idiopathic dilated cardiomyopathy</td>
<td>14</td>
<td>42±9</td>
<td>26-58</td>
</tr>
<tr>
<td>Ischemic cardiomyopathy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transplantation</td>
<td>13</td>
<td>54±5</td>
<td>45-63</td>
</tr>
<tr>
<td>Autopsy</td>
<td>4</td>
<td>58±16</td>
<td>39-80</td>
</tr>
<tr>
<td>Valvular heart diseases</td>
<td>5</td>
<td>46±11</td>
<td>35-62</td>
</tr>
<tr>
<td>Total</td>
<td>36</td>
<td>47±10</td>
<td>26-80</td>
</tr>
</tbody>
</table>

Statistical Analysis

Data were collected blindly, and the code was broken at the end of the study. Results are presented as mean±SD. Statistical significance for comparison between two measurements was determined by the unpaired two-tailed Student’s t test. Statistical significance for comparison among groups was determined by ANOVA and Bonferroni’s method. Values of P<.05 were considered to be significant.

Results

Patient Population

The present study was performed by using myocardial samples from 32 patients who underwent cardiac transplantation and from 4 patients who died shortly after acute myocardial infarction and intractable congestive heart failure. Tissue specimens were obtained from the latter 4 patients within 2 hours after death. Right ventricular endomyocardial biopsies, collected from 5 donor subjects before cardioplegic arrest and removal of the heart, were used as control myocardium. The entire population included only white individuals.

The myocardial samples obtained from the explanted hearts were collected during a period of 10 months, from April 1992 to February 1993, at the De Gasperi Division of Cardiac Surgery in Milan. No preoperative criteria were established in the selection of the cases. Only two additional hearts became available during this period, but they were excluded since one was a transplanted heart with late graft failure and the second was not properly treated for PCNA protein detection. This approach was followed in order to include all patients with congestive heart failure regardless of the etiology of the process, sex, age, and the therapeutic treatment.

Five samples of normal myocardium were collected from four males who died of cranial trauma and one female who died of spontaneous cerebral hemorrhage. Hearts were eligible for homograft transplant so that only small biopsy specimens from the right ventricle could be obtained. The age of these patients varied from 18 to 53 years (average, 35 years).

Anatomic and Functional Characteristics

Table 1 shows that of the 36 patients involved, there were 14 with idiopathic dilated cardiomyopathy, 17 with ischemic heart disease, and 5 with acquired valvular defects. Age, sex, duration of the disease process, and the time interval between the occurrence of heart failure (New York Heart Association [NYHA] class 3)
and cardiac transplantation are also listed in Table 1. With the exception of the 4 patients for whom samples were collected at autopsy and for whom no data were available, 26 patients were in NYHA functional class 4, and 6 patients were in NYHA functional class 3. At the time of surgery, 24 patients were under intravenous inotropic therapy, and 2 patients needed a ventricular assistance device.

Although the left ventricle was the most affected site in 12 of the 14 patients with idiopathic dilated cardiomyopathy, the right ventricle was the primary site of localization in 2 of the patients. Moreover, for one of these 2 patients, left ventricular function was maintained, whereas in the other, a depressed left-side performance was documented. Left ventricular assistance was required in 2 patients for 2 and 46 days before transplantation.

With one exception, the 17 patients in the ischemic cardiomyopathic group had a history of myocardial infarction. Coronary angiography showed three-vessel disease in 6 patients, two-vessel disease in 2 patients, one-vessel disease in 3 patients, and essentially normal coronary arteries in another patient. In one instance, coronary angiography was not performed, but a large aneurysmatic dilation of the left ventricle was present. In the patient with normal coronary arteries, the primary event was an anterior myocardial infarction, which occurred during pregnancy, ≈13 years before transplantation. In the 4 patients for whom samples were collected at autopsy, extensive acute myocardial infarction was present. In addition, 3 patients showed marked biventricular hypertrophy, which was associated with aneurysms of the ascending and abdominal aorta in one patient and severe and diffuse atherosclerosis of the coronary arteries in the others.

In the valvular heart disease group, two of the five patients had involvement of the mitral and aortic valves, two had involvement of the mitral valve only, and the last had a defect restricted to the aortic valve. This subdivision corresponds to the initial diagnosis. All five individuals had valve replacement during the course of the disease.

Table 2 presents measurements of cardiac hemodynamics and anatomic properties collected 1 to 6 months before cardiac transplantation. These data show a marked reduction in the ejection fraction coupled with a significant increase in left ventricular diastolic and systolic diameters. These changes produced a severe decrease in fractional shortening, which was found to be comparable in the three groups of patients. In addition, the ratio of wall thickness to chamber radius was reduced, and left ventricular end-diastolic volume was nearly doubled in all cases. Finally, the cardiac index was depressed, and pulmonary wedge pressure was elevated. Right ventricular end-diastolic pressure was lower in the group with ischemic cardiomyopathy.

**PCNA Labeling of the Ventricular Myocardium**

The distribution of PCNA protein in the myocardium was detected by the immunoperoxidase technique. Sections from lymph nodes were used as controls for the immunohistochemical reaction. The myocardial samples obtained from the right ventricle of control hearts showed no PCNA labeling in myocyte and nonmyocyte nuclei. In contrast, labeling was found in myocyte nuclei and other cell types of the left and right ventricles of patients affected by end-stage cardiac failure. Similarly, PCNA protein was detected in myocytes located in the proximity of the infarcted tissue of the four hearts collected at autopsy. Extensive search in the left ventricular tissue remote from the infarction failed to reveal positively stained myocyte nuclei. In addition, no PCNA staining was observed in the right ventricle of these hearts. PCNA labeling was also observed in the three fetal hearts examined.
Fig 1. Detection of proliferating cell nuclear antigen (PCNA) by the immunoperoxidase technique in the left ventricular myocardium of a patient with end-stage idiopathic dilated cardiomyopathy. Photomicrographs show numerous myocyte nuclei exhibiting positive staining (arrows). Unlabeled myocyte nuclei are also present (arrowheads). The number of unlabeled myocyte nuclei is higher in panel b than in panel a. Hematoxylin staining was light. Original magnification ×600.

Fig 2. Photomicrographs showing double staining of proliferating cell nuclear antigen (PCNA) and α-sarcomeric actin in sections of myocardium obtained from the left ventricle of a patient with ischemic cardiomyopathy. PCNA protein was detected by alkaline phosphatase streptavidin labeling, revealed by fast red chromogen, whereas α-sarcomeric actin was detected by immunoperoxidase, revealed by 3-amino-9-ethyl-carbazole. a, Numerous labeled nuclei within positively stained myocyte cytoplasm are shown (arrows). Smooth muscle cell nuclei are also labeled (arrowheads). b, Positively stained myocyte nucleus (arrow) is illustrated in combination with an unstained nucleus (arrowhead). All myocytes are labeled by α-sarcomeric actin (original magnifications ×700 [a] and ×1100 [b]).

Quantitative Analysis of PCNA Labeling in Myocytes

Table 3 shows the number of left and right ventricular samples obtained in each group of patients and the number of patients for which myocyte and nonmyocyte nuclei were positively labeled for PCNA protein. It should be apparent that PCNA in myocytes was always found in combination with labeling of interstitial cells in the left and right ventricles. However, nonmyocyte cells exhibited PCNA protein in the absence of positive results in ventricular myocytes. As stated above, no labeling of myocyte and nonmyocyte nuclei was seen in biopsy specimens obtained from the right ventricle of normal hearts.

In the left ventricle, positively stained myocyte nuclei for PCNA protein were found for all patients with ischemic cardiomyopathy and valvular heart disease. For the patients with idiopathic dilated cardiomyopathy, nuclei were negative for PCNA protein in 3 of the 14 patients. The first of these 3 patients exhibited a predominantly right ventricular localization of the myocardy. In this regard, the cardiac index was within normal levels (2570 mL·min⁻¹·m⁻²), and the ejection fraction was 51%. For the second patient, neither myocyte nor nonmyocyte nuclei were labeled, suggesting a potential technical artifact in the preparation. We
have no explanation for the lack of PCNA labeling in myocytes from the third patient, since interstitial cells showed positive PCNA immunoreactivity.

Table 3 also demonstrates that PCNA labeling of myocytes in the right ventricle was not apparent in two patients with idiopathic dilated cardiomyopathy or in three patients affected by ischemic cardiomyopathy. For the two patients in the group with idiopathic dilated cardiomyopathy, labeling was absent in all cell populations of both ventricles in the first patient, implying a technical limitation; in the second, nonmyocyte nuclei were occasionally labeled, so the absence of PCNA in myocyte nuclei cannot be explained. The prevailing effect of ischemia on the left ventricular myocardium may be the cause of the negative results in the three patients in the group with ischemic cardiomyopathy.

The data listed in Table 4 are restricted to the number of patients in which comparable fixation and staining conditions were obtained for quantitative analysis of the fraction of labeled and nonlabeled myocyte nuclei in the myocardium. Approximately 600 myocyte nuclei were examined in each ventricle in each patient. With this type of sampling, an average of 49% of nuclei were seen to be positively stained for PCNA in the left and right ventricles. Modest variations in this parameter were noted among the three groups of patients, but none of the changes was statistically significant.

In the three fetal hearts, a total of 14 219 nuclei were counted, and 4765 were found to be positively labeled by PCNA. This yielded an average 33.51±1.60% magnitude of labeling. Individual values were 35.3%, 31.4%, and 33.8%. It should be emphasized that tissue samples in these fetal hearts were obtained between 5 and 10 hours after abortion.

**Mitotic Figures**

A detailed analysis of tissue sections obtained for the quantitative measurements of PCNA labeling in the myocardium revealed mitotic images in myocytes in 7 (4 with ischemic cardiomyopathy, 2 with idiopathic dilated cardiomyopathy, and 1 with valvular heart disease) of the 25 patients. Mitotic figures in nonmyocyte cells were also observed. Similarly, mitotic images were detected in hearts collected at autopsy. Since these hearts showed positive PCNA labeling of muscle cell nuclei in the region adjacent to the infarcted myocardium, this zone of tissue was extensively sampled. Multiple specimens of myocardium remote from the necrotic area of the left ventricle were also examined. Fig 3 illustrates mitotic images in myocyte nuclei found in these autopsy cases. These cells were all located in the proximity of the infarction, whereas mitosis was never seen in the myocardium, distant from the necrotic zone.

Mitotic figures were also found in myocardial samples obtained from patients undergoing cardiac transplantation. Such an image, in which metaphase chromosomes in the center of a transversely sectioned myocyte profile were noted, is shown in Fig 4. There is no difficulty in recognizing the cell of origin in this case, which belonged to a patient affected by dilated cardiomyopathy.

An approach identical to that described above for the localization of PCNA protein was followed here for the identification and confirmation of mitotic figures in myocytes. Fig 5 demonstrates a mitotic image at four different focal planes in a myocyte stained for α-sarcomeromic actin. The myocardial sample was obtained from a patient with ischemic cardiomyopathy.

Difficulties existed in the evaluation of the number of mitoses in myocytes because the frequency was low and their recognition was complex. In some occasions, it was impossible to establish whether a mitotic image pertained to a myocyte or nonmyocyte cell. For example, Fig 6 illustrates two mitotic figures that appear to be located in myocytes, although this could not be proven with certainty. It is important to note that PCNA was not consistently present in dividing nuclei.
Incidence of Mitotic Figures in the Fetal Human Heart

Table 5 shows the quantitative analysis performed to evaluate the total number of nuclei present in the three fetal hearts obtained for this investigation. This information, combined with the evaluation of the number of mitotic images in myocytes, nonmyocytes, and nonidentifiable cells, allowed the computation of the total number of mitotic figures in the heart for each of these three classes of cells. Table 5 lists the area of fetal myocardium sampled and the number of nuclei counted in each case. By examining a total of 8.29 mm² of tissue and counting 28,280 nuclei, it was found that the nuclear density per square millimeter of fetal myocardium averaged 3431 nuclei. Since mean nuclear length, obtained from the collection of 3240 individual measurements, was found to be 8.00 μm, the number of nuclei per cubic millimeter of fetal tissue was 452,994. This parameter multiplied by the volume of the heart yielded a value of 987×10⁶ nuclei, which corresponds to the total number of nuclei in the fetal heart.

Since in the 28,280 nuclei counted there were 5 mitotic figures in myocytes, 5 in nonmyocytes, and 10 in nonidentifiable cells, the numbers of mitotic images per 10⁶ nuclei and in the total fetal heart were computed...
and are illustrated in Fig 7. Although the number of mitoses in the nonmyocyte population per $10^6$ nuclei was slightly higher than in myocytes, the 21% difference was not statistically significant. When the entire heart was considered, it could be seen that $173 \times 10^6$ myocytes and $222 \times 10^6$ nonmyocytes were undergoing nuclear

### Table 5. Incidence of Mitotic Figures in Fetal Human Heart

<table>
<thead>
<tr>
<th>Patient</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, wk</td>
<td>22</td>
<td>26</td>
<td>20</td>
<td>22.7±2.5</td>
</tr>
<tr>
<td>Heart weight, g</td>
<td>1.9</td>
<td>2.6</td>
<td>2.4</td>
<td>2.30±0.29</td>
</tr>
<tr>
<td>Heart volume, mm$^3\times10^3$</td>
<td>1.79</td>
<td>2.45</td>
<td>2.26</td>
<td>2.17±0.28</td>
</tr>
<tr>
<td>Area of myocardium sampled, mm$^2$</td>
<td>3.11</td>
<td>2.76</td>
<td>2.41</td>
<td>2.76±0.28</td>
</tr>
<tr>
<td>Number of nuclei counted</td>
<td>9592</td>
<td>10 092</td>
<td>8596</td>
<td>9427±622</td>
</tr>
<tr>
<td>Number of nuclei/mm$^2$ of myocardium</td>
<td>3084</td>
<td>3657</td>
<td>3552</td>
<td>3431±249</td>
</tr>
<tr>
<td>Average nuclear length, μm</td>
<td>6.93</td>
<td>7.3</td>
<td>8.6</td>
<td>8.00±0.72</td>
</tr>
<tr>
<td>Number of nuclei/mm$^3$ of myocardium</td>
<td>445 057</td>
<td>500 893</td>
<td>413 031</td>
<td>452 994±36 306</td>
</tr>
<tr>
<td>Total number of nuclei in the heart $\times10^6$</td>
<td>798</td>
<td>1229</td>
<td>935</td>
<td>987±180</td>
</tr>
<tr>
<td>Number of mitotic figures in the sampled myocardium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myocytes</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1.67±0.47</td>
</tr>
<tr>
<td>Nonmyocytes</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>2.00±0.82</td>
</tr>
<tr>
<td>Nonidentifiable cells</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>3.33±0.94</td>
</tr>
<tr>
<td>All cells</td>
<td>5</td>
<td>9</td>
<td>7</td>
<td>7.00±1.63</td>
</tr>
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</table>
mitotic division at this stage of fetal development. This 28% difference was also not statistically significant.

**Incidence of Mitotic Figures in the Failing Adult Human Heart**

Table 6 shows an analysis similar to that described above for the fetal heart for 7 of the 25 patients undergoing cardiac transplantation in whom mitotic images in the left ventricle were found. However, the distinction between myocyte and nonmyocyte nuclei was possible in the well-differentiated adult heart, so the parameters of myocyte nuclear density and nonmyocyte nuclear density in the myocardium were obtained. By counting 27 452 myocyte nuclei and 78 167 nonmyocyte nuclei in 106 mm² of myocardium, the numerical density of myocyte nuclei and nonmyocyte nuclei per square millimeter of tissue was measured. Myocytes were found to possess a nuclear numerical density that was 66% ($P<.0001$) lower than that of nonmyocytes. These values were the bases for the subsequent calculations listed in Table 6.

To identify mitotic images in myocytes, an average of 425 mm² of myocardium had to be examined in each case. This amount of sampling was 28-fold greater than that used for the estimation of the nuclear density of myocytes and nonmyocytes listed in Table 6. In addition, it is important to recognize that the frequency of mitotic figures in tissue sections for any cell type is dependent on its nuclear density in the myocardium. Therefore, the product of the number of nuclei per unit area of myocardium and the area of myocardium sampled for the detection of mitotic figures yielded the aggregate number of nuclei examined for each cell population. By this approach, the percentage of myocyte nuclei and nonmyocyte nuclei showing mitotic images was obtained and is listed last in Table 6.

Fig 8 illustrates the number of mitotic figures in each cell group per $10^6$ nuclei. This analysis showed that the frequency of mitotic images in myocyte nuclei was 1.71-fold greater than that in nonmyocyte nuclei, and this difference was statistically significant ($P<.05$). In addition, mitoses in myocytes were 3.20-fold more numerous than in nonidentifiable cells, and this value was also statistically significant ($P<.001$). Finally, it should be recognized that when all cell populations are considered, the examination of 1 000 000 nuclei in the myocardium will yield two or three mitotic images in myocyte nuclei.

As discussed in the preceding section, parameters identical to those described above could not be obtained in the fetal myocardium. Therefore, a direct comparison between the fetal and failing heart was not possible.

### Table 6. Incidence of Myocyte Nuclear Mitotic Division in the Failing Human Heart

<table>
<thead>
<tr>
<th>Patients</th>
<th>Ischemic</th>
<th>Ischemic</th>
<th>Ischemic</th>
<th>Ischemic</th>
<th>Dilated</th>
<th>Dilated</th>
<th>Valvular</th>
<th>Mean±SD</th>
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<td>Age, y</td>
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<td>46</td>
<td>45</td>
<td>55</td>
<td>38</td>
<td>29</td>
<td>35</td>
<td>43±9</td>
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<tr>
<td>Area of myocardium sampled, mm²</td>
<td>15</td>
<td>17</td>
<td>15</td>
<td>14</td>
<td>18</td>
<td>14</td>
<td>15</td>
<td>15±1</td>
</tr>
<tr>
<td>Number of nuclei counted</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myocytes</td>
<td>5192</td>
<td>5204</td>
<td>4308</td>
<td>2154</td>
<td>4163</td>
<td>1404</td>
<td>5027</td>
<td>3922±1422</td>
</tr>
<tr>
<td>Nonmyocytes</td>
<td>10 908</td>
<td>13 549</td>
<td>12 427</td>
<td>13 902</td>
<td>7626</td>
<td>8667</td>
<td>11 088</td>
<td>11 167±2191</td>
</tr>
<tr>
<td>All cells</td>
<td>16 100</td>
<td>18 753</td>
<td>16 735</td>
<td>16 056</td>
<td>11 789</td>
<td>10 071</td>
<td>16 115</td>
<td>15 088±2809</td>
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<tr>
<td>Number of nuclei/mm² of myocardium</td>
<td></td>
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<tr>
<td>Myocytes</td>
<td>347</td>
<td>313</td>
<td>295</td>
<td>156</td>
<td>237</td>
<td>104</td>
<td>336</td>
<td>255±87</td>
</tr>
<tr>
<td>Nonmyocytes</td>
<td>729</td>
<td>816</td>
<td>851</td>
<td>1007</td>
<td>434</td>
<td>642</td>
<td>741</td>
<td>746±166</td>
</tr>
<tr>
<td>All cells</td>
<td>1076</td>
<td>1129</td>
<td>1146</td>
<td>1163</td>
<td>671</td>
<td>746</td>
<td>1077</td>
<td>1001±189</td>
</tr>
<tr>
<td>Area of myocardium sampled for mitosis, mm²</td>
<td>220</td>
<td>405</td>
<td>480</td>
<td>360</td>
<td>550</td>
<td>510</td>
<td>448</td>
<td>425±102</td>
</tr>
<tr>
<td>Number of mitotic figures in the sampled myocardium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myocytes</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1.00±0.00</td>
</tr>
<tr>
<td>Nonmyocytes</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>2.00±0.76</td>
</tr>
<tr>
<td>Nonidentifiable cells</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1.43±0.49</td>
</tr>
<tr>
<td>All cells</td>
<td>3</td>
<td>6</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>4.43±1.05</td>
</tr>
<tr>
<td>Percentage of mitotic figures</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myocytes</td>
<td>0.00131</td>
<td>0.00079</td>
<td>0.00071</td>
<td>0.00177</td>
<td>0.00077</td>
<td>0.00188</td>
<td>0.00066</td>
<td>0.00113±0.00049</td>
</tr>
<tr>
<td>Nonmyocytes</td>
<td>0.00062</td>
<td>0.00090</td>
<td>0.00049</td>
<td>0.00055</td>
<td>0.00084</td>
<td>0.00092</td>
<td>0.00030</td>
<td>0.00066±0.00022</td>
</tr>
<tr>
<td>Nonidentifiable cells</td>
<td>0.00042</td>
<td>0.00043</td>
<td>0.00036</td>
<td>0.00024</td>
<td>0.00054</td>
<td>0.00026</td>
<td>0.00021</td>
<td>0.00035±0.00011</td>
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<tr>
<td>All cells</td>
<td>0.00127</td>
<td>0.00131</td>
<td>0.00091</td>
<td>0.00135</td>
<td>0.00135</td>
<td>0.00131</td>
<td>0.00062</td>
<td>0.00110±0.00026</td>
</tr>
</tbody>
</table>

Ischemic indicates ischemic heart disease; dilated, idiopathic dilated cardiomyopathy; and valvular, acquired valvular defects.
However, it has been previously shown that the aggregate number of myocyte nuclei present in the left ventricle of individuals varying in age from 29 to 55 years is $5.2 \times 10^9$.\textsuperscript{22} This would imply that the total number of myocyte nuclei undergoing mitosis in the group of seven patients listed in Table 6 would be nearly equal in the fetal heart, the total number of left ventricular myocyte nuclei in mitosis would be 86,500. Thus, the fetal left ventricle appears to exhibit 47% more mitotic images than the adult failing left ventricle.

**Incidence of Mitotic Figures Immediately After Myocardial Infarction**

Table 7 shows the area of myocardium examined and the number of nuclei counted to estimate the myocyte and nonmyocyte numerical density in the tissue of four patients who died of congestive heart failure shortly after coronary artery occlusion. These data, in combination with the numbers of mitotic figures in myocytes and nonmyocytes encountered in a larger area of myocardium, were used to compute the percentage of myocyte and nonmyocyte nuclei undergoing mitosis. Since mitotic images were not observed in the myocardium remote from the infarct, this analysis was restricted to the surviving tissue contained within the first 5 to 10 mm lateral to the necrotic region.

Fig 9 illustrates that for every $10^6$ myocyte nuclei, two muscle cell nuclei exhibited mitotic figures. The frequency of mitosis in nonmyocyte nuclei was of the same magnitude and was also limited to the peri-infarcted zone of viable myocardium. Thus, in the period immediately following infarction, mitosis of myocytes and nonmyocytes was significantly less frequent than in

![Bar graph showing the number of mitotic figures in the different cell populations of the myocardium in seven patients with end-stage heart failure. Results are presented as mean±SD. *P<.05 vs value for myocytes.](http://circres.ahajournals.org/)
chronic heart failure and did not involve the portion of the ventricle distant from the damaged tissue.

Discussion

The results of the present study indicate that end-stage cardiac failure is characterized by the induction of PCNA protein in myocytes biventricularly. This response involved >40% of the myocyte population and was not disease dependent, being comparable in idiopathic dilated cardiomyopathy, chronic coronary artery disease, and valvular heart disease. PCNA labeling was also present in the other cell populations of the myocardium, including smooth muscle cells, endothelial cells, and fibroblasts. In addition, mitotic images in myocyte and nonmyocyte nuclei were found in the myocardium of both ventricles. Importantly, a quantitative analysis of the frequency of mitosis in myocyte nuclei of the failing left ventricle documented that this cellular process was only 32% lower than that measured in the fetal heart. Similarly, myocyte nuclear mitotic divisions were seen in the viable tissue after acute myocardial infarction. However, in the latter condition, mitosis in myocytes was restricted to the region bordering the infarct, and its magnitude was significantly less than that detected in chronic heart failure. Finally, the incidence of mitosis in myocyte nuclei was equal to or greater than that in the other cell populations of the ventricular myocardium. In contrast, no PCNA protein or mitotic images were detected in myocytes and connective tissue cells from normal hearts. Thus, the abnormalities in hemodynamic load generated by a prolonged impairment in ventricular pump function appear to be coupled with the activation of a late growth-related gene, which is essential for DNA synthesis and cell cycle progression. This phenomenon, in combination with the presence of myocyte nuclear mitotic division, strongly supports the notion that ventricular myocytes are not terminally differentiated cells.

Growth-Regulated Genes and the Mammalian Heart

PCNA and histone-H2a are late growth-regulated genes that are expressed at the G1-S phase boundary.23,24 These genes are responsible for the progression of the cell cycle and nuclear mitotic division.23,24 In the myocardium, increased PCNA transcription has been linked to myocyte proliferation, but this association has been claimed to be restricted to prenatal and early postnatal life.9 With attenuation of physiological growth and the development of the adult circulatory system,25 myocyte cellular hypertrophy becomes the major growth mechanism of the heart,26 and PCNA protein is no longer detected in cardiac myocytes.9 On the other hand, experimentally, in the presence of ventricular failure induced by acute myocardial infarction11,13 or conditions of global ischemia,12 PCNA mRNA is upregulated in the surviving myocytes, and the distribution of PCNA protein correlates closely with DNA synthesis in these viable cells.12,13 Myocyte nuclear mitotic division12-14 and myocyte cellular hyperplasia14 have been documented as well.

It should be recognized that the 49% PCNA labeling of left and right ventricular myocytes found here in terminal heart failure cannot be equated with a similar magnitude of myocyte cellular hyperplasia. This observation simply indicates that numerous myocytes are in the process of entering the cell cycle and synthesizing DNA.1 Limitations due to sample size and difficulties in assessing DNA synthesis in human hearts did not allow morphometric estimations of myocyte number and/or DNA replication in the available specimens. In the absence of this information, it is impossible to quantify from the fraction of PCNA-labeled cells the actual degree of myocyte proliferation in the myocardium.

The absence of PCNA protein in tissue samples from normal hearts found here in a small group of patients is consistent with recent findings indicating that 89% of normal individuals (63 of 71) do not exhibit PCNA in the myocardium.27 In the remaining 11% of normal individuals (8 of 71), only 1% to 2% of myocytes were labeled, and this small percentage is significantly lower than the values obtained in the present study in subjects affected by end-stage heart failure. Importantly, cardiac disease processes with no signs of ventricular dysfunction have been found to be characterized by nearly 10% of PCNA-stained myocytes,27 which represents one fifth of the magnitude of labeling documented in this investigation. Thus, the induction of the PCNA gene appears to be coupled with pathological states of the heart and particularly with the abnormalities in ventricular loading generated by intractable congestive heart failure. Whether aging may participate in the activation of this system cannot be excluded, since myocyte mitotic division and cellular hyperplasia have been shown to occur experimentally in the senescent myocardium.19,28 Moreover, myocyte nuclear and possibly cellular hyperplasia has been documented in elderly patients affected by severe cardiac decompensation.29

The percentage of myocyte and nonmyocyte nuclei showing PCNA labeling in the fetal heart was similar to that detected in myocardial samples from patients undergoing cardiac transplantation. However, the fraction of nuclei exhibiting mitosis in the fetal heart was severalfold greater than in the adult myocardium. This difference is difficult to explain, although the possibility may be advanced that the lengths of the S, G2, and M phases of the cell cycle are not comparable in the fetal and adult ventricular tissue, influencing the relative number of PCNA-labeled cells. The lower mitosis—to-
PCNA-labeled nuclei ratio in the adult heart most likely reflects a markedly longer myocyte cell cycle. Alternatively, the detection of PCNA-stained nuclei in the fetal tissue that could not be obtained immediately after ablation may have decreased the actual number of labeled cells. Last and most important, it has repeatedly been shown that highly proliferating malignant tumor cells exhibit one mitotic division per 1000 cells in the presence of 30% PCNA-positive cells. This clearly indicates the complexity of inferring the extent of cell division from PCNA labeling in any tissue when no information is available regarding the duration of the S phase of the cell cycle and the time required for mitosis to occur. Finally, it should be recognized that the high level of expression of PCNA in myocytes may also represent an abortive reaction that only occasionally is followed by mitosis and the formation of new cells.

Myocyte Nuclear Mitotic Division and the Mammalian Heart

Several reports have suggested that ventricular myocytes in the adult human heart may reenter the cell cycle and synthesize DNA. However, the perennial issue of whether DNA replication leads exclusively to the formation of ploidy or to nuclear mitotic division and cell proliferation has been a matter of controversy. Although morphometric studies have demonstrated unequivocally that myocyte nuclear hyperplasia occurs in the failing hypertrophied heart and that the proportion of mononucleated and binucleated cells does not change significantly in the myocardium, mitotic figures in myocytes have never been observed, and on this basis, the validity of these results is frequently challenged. The observations in the present study not only document for the first time that mitotic images can be found in adult myocyte nuclei but also provide a quantitative estimate of this phenomenon. This information is without precedent. The occurrence and frequency of mitosis in the nonmyocyte cell population have also been measured. Again, no comparable data have been obtained previously experimentally or in the human heart during fetal development and in pathological conditions.

The mitotic index for myocyte nuclei measured in the present study was higher in the fetal heart, intermediate in the failing myocardium, and lower in the surviving tissue bordering the acute myocardial infarction. However, the difference between the fetal and the failing human heart was relatively small when the actual number of mitotic figures per ventricle was taken into account. In this regard, <0.5 billion nuclei were present in each ventricle of the fetal heart, and this computation included interstitial cells as well. The adult left ventricle contains an average of 5.2 billion myocyte nuclei, which have been claimed to represent only one third of the entire number of cells in the ventricle. It should be apparent that a mitotic index for myocytes similar to that observed in the fetal tissue would be incompatible with the size of the heart reported in normal and pathological states. The observation that myocyte nuclear mitotic division after acute myocardial infarction was restricted to the region adjacent to the necrotic tissue is consistent with experimental results in the rat heart. Importantly, with the evolution of myocardial infarction, DNA synthesis and mitotic division do occur in the remote tissue as well. Thus, infarction and ventricular failure activate the DNA replicatory machinery of all remaining viable cells.

Although it is a common assumption that the extent of proliferation in interstitial cells is significantly greater than in myocytes, results in the present study indicate that the mitotic index was higher in myocyte than in nonmyocyte nuclei of the failing heart. However, this information does not take into account the total number of endothelial cells and fibroblasts in the myocardium that exceed the aggregate number of myocytes. In addition, the duration of the cell cycle may differ among these three cell populations. Finally, the activation of connective tissue cell proliferation may occur at different times during the evolution of the cardiomyopathic heart of ischemic and nonischemic origin. The present data simply indicate that a large sampling is required not only for the detection of mitosis in myocytes but also for the identification of this process in endothelial cells and fibroblasts.

Difficulties exist in the evaluation of myocyte cellular hyperplasia by various methodologies. So far, quantitative measurements of myocyte nuclei in the ventricle, combined with the evaluation of the distribution of nuclei in myocytes, appear to be the only approach that can demonstrate myocyte cellular hyperplasia unequivocally. However, the concomitant presence of myocyte loss and myocardial damage complicates this type of analysis. Autoradiographic detection of thymidine-labeled tissue has also been used to establish whether DNA synthesis and cellular hyperplasia occur in myocytes. On the other hand, it is impossible by this technique to distinguish whether DNA synthesis in nuclei is due to nuclear hyperplasia, ploidy formation, or DNA repair. Since myocardial damage and diffuse and segmental cell loss are common findings in the failing human heart, the approach followed here, in which the presence of a nuclear protein essential for the initiation of DNA synthesis and cell cycle progression was demonstrated in combination with mitotic division, may offer the advantage of documenting an important molecular event involved in cell proliferation.

Myocyte Cellular Hyperplasia and the Mammalian Heart

The possibility that myocyte cellular hyperplasia may contribute to the hypertrophic growth of the myocardium in pathological states of the heart remains controversial. It is a general belief that once myocyte proliferation ceases shortly after birth in the mammalian heart, physiological and induced postnatal myocardial growth occur principally through hypertrophy of myocytes. In contrast, a hyperplastic component persists in interstitial fibroblasts and capillary endothelial cells, which, in combination with cellular hypertrophy, participate in the expansion of the overloaded myocardium. These conclusions have been based on a limited number of studies performed in animal models of pressure-overload hypertrophy in which little or no thymidine labeling has been found in the stressed myocardium. De novo DNA synthesis was seen to be restricted mostly to the nonmyocyte populations of the ventricle, suggesting the absence of myocyte cellular hyperplasia during the development and progression of cardiac hypertrophy.
Although the observations above support the concept of the inability of cardiac muscle cells to divide, it has been demonstrated recently that myocyte cellular hyperplasia can occur in the left and right ventricular myocardium in association with a prolonged and sustained elevation in workload on the heart\textsuperscript{14,44-47} or with aging and senescence.\textsuperscript{19,28,29,47} In addition, DNA synthesis in myocyte nuclei has been found immediately after myocardial infarction\textsuperscript{13,48} and after coronary artery constriction.\textsuperscript{12,14} These experimental findings appear to confirm quantitative results obtained in the human heart that strongly indicate the occurrence of myocyte proliferation with severe degrees of cardiac hypertrophy.\textsuperscript{29,31-34} It should be emphasized, however, that documentation consistent with myocyte regeneration were obtained only in the presence of profound alterations in ventricular pump function and congestive heart failure in both humans\textsuperscript{29,31-34} and animals.\textsuperscript{11-14,19,46-48} Thus, activation of the replicatory machinery of adult human cardiac myocytes may occur exclusively in response to the marked abnormalities in myocardial loading encountered in the failing heart. In contrast, cellular hypertrophy may be the dominant growth mechanism of the stressed myocardium in its compensated stage.

**Limitations of the Study and Conclusions**

There are several limitations in the present investigation that must be acknowledged. The observations of PCNA protein immunostaining in myocytes and nuclear mitotic division cannot be interpreted as unequivocal indicators of myocyte cellular hyperplasia. As stated previously, the lack of information concerning the duration of the cell cycle and the time necessary for mitosis to occur does not permit a correlation between the degree of PCNA labeling and the fraction of myocytes entering the S phase and subsequently dividing. Moreover, PCNA may lead to DNA synthesis, but this process may be coupled with polypliodization or DNA repair.\textsuperscript{19,28,40,44} In this regard, a large fraction of nuclei in the hypertrophied human heart have been found to be polypliod.\textsuperscript{45,46} Since the destiny of mitotic cells is not known, the possibility cannot be excluded that mitosis of differentiated cardiac muscle cells is accompanied by nuclear fragmentation and cell death. Similarly, nuclear mitotic division may result in multinucleation of cells without cell division.\textsuperscript{14,19,28,44,48} It should be emphasized, however, that the number of myocyte nuclei per cell tends to decrease in cardiac failure experimentally.\textsuperscript{14,19} Myocyte nuclear mitotic division\textsuperscript{22,23,38} results consistently in myocyte cellular hyperplasia.\textsuperscript{14,19} On the other hand, with the exception of the fetal heart, the morphometric approach used examined enough myocardium to obtain one myocyte nuclear mitotic image in each case. This type of analysis may reflect an underestimation of the real magnitude of this phenomenon in the heart or an overestimation. Nevertheless, additional work for a more precise characterization of the phenomenon of mitosis in the human heart does not appear justified. Variability in the duration of the disease, medical history, diet, smoking habits, and living conditions may contribute significantly to the large standard deviation of the mean values obtained here. Since, by definition, cells terminally differentiated cannot reenter the cell cycle and undergo nuclear mitotic division, the present findings provide the first unequivocal demonstration that adult ventricular myocytes in humans do not belong to this class of cells.

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End-stage cardiac failure in humans is coupled with the induction of proliferating cell nuclear antigen and nuclear mitotic division in ventricular myocytes.

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