

EVIDENCE THAT HUMAN CARDIAC MYOCYTES DIVIDE AFTER MYOCARDIAL INFARCTION

ANTONIO P. BELTRAMI, M.D., KONRAD URBANEK, M.D., JAN KAJSTURA, PH.D., SHAO-MIN YAN, M.D., NICOLETTA FINATO, M.D., ROSSANA BUSSANI, M.D., BERNARDO NADAL-GINARD, M.D., PH.D., FURIO SILVESTRI, M.D., ANNAROSA LERI, M.D., C. ALBERTO BELTRAMI, M.D., AND PIERO ANVERSA, M.D.

ABSTRACT

Background The scarring of the heart that results from myocardial infarction has been interpreted as evidence that the heart is composed of myocytes that are unable to divide. However, recent observations have provided evidence of proliferation of myocytes in the adult heart. Therefore, we studied the extent of mitosis among myocytes after myocardial infarction in humans.

Methods Samples from the border of the infarct and from areas of the myocardium distant from the infarct were obtained from 13 patients who had died 4 to 12 days after infarction. Ten normal hearts were used as controls. Myocytes that had entered the cell cycle in preparation for cell division were measured by labeling of the nuclear antigen Ki-67, which is associated with cell division. The fraction of myocyte nuclei that were undergoing mitosis was determined, and the mitotic index (the ratio of the number of nuclei undergoing mitosis to the number not undergoing mitosis) was calculated. The presence of mitotic spindles, contractile rings, karyokinesis, and cytokinesis was also recorded.

Results In the infarcted hearts, Ki-67 expression was detected in 4 percent of myocyte nuclei in the regions adjacent to the infarcts and in 1 percent of those in regions distant from the infarcts. The reentry of myocytes into the cell cycle resulted in mitotic indexes of 0.08 percent and 0.03 percent, respectively, in the zones adjacent to and distant from the infarcts. Events characteristic of cell division — the formation of the mitotic spindles, the formation of contractile rings, karyokinesis, and cytokinesis — were identified; these features demonstrated that there was myocyte proliferation after myocardial infarction.

Conclusions Our results challenge the dogma that the adult heart is a postmitotic organ and raise the possibility that the regeneration of myocytes may contribute to the increase in muscle mass of the myocardium. (N Engl J Med 2001;344:1750-7.)

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MYOCYTE replication occurs in the failing human heart,¹ and this form of cell growth tends to compensate for the exhaustion of myocyte hypertrophy.² In chronic heart failure, myocytes at most double in size, and when this limit has been reached no further enlargement of the heart occurs.^{2,3} For decades, it has been doubted whether the heart can grow by multi-

plication of myocytes.⁴ The demonstration that the adult human brain contains a population of cells that are able to regenerate neurons⁵ has not prompted a comprehensive reexamination of the notion that the heart is a postmitotic organ,⁶ even though cardiac endothelial cells, smooth-muscle cells, and fibroblasts are known to proliferate.^{3,4} Recently, a myocyte mitotic index (the ratio of the number of nuclei undergoing mitosis to the number not undergoing mitosis) of 0.015 percent was measured in explanted hearts from patients in the terminal stages of cardiac decompensation.¹ The importance of these results was questioned on the assumption that this level of myocyte replication has no clinical significance.⁴ In the absence of supporting evidence, it has been claimed that rates of myocyte proliferation ranging from 0.05 to 0.1 percent would be required for meaningful therapeutic reconstitution of damaged myocardium.⁴

Although a mitotic index of 0.015 percent, if sustained, could result in the formation of 100 g of myocardium in less than three months,¹ the fraction of mitotic myocytes in patients with late cardiac failure may reflect the ultimate growth reserve of this cell population. The mechanical overload in a disease that lasts several years may progressively exhaust the replicative capacity of myocytes. Cells cannot divide indefinitely. In contrast, after extensive myocardial infarction, abrupt increases in the need for growth may cause more myocytes to reenter the cell cycle than during chronic heart failure. To test this hypothesis, we determined the percentages of cycling myocytes and the mitotic indexes in patients who had died within a short time after extensive myocardial infarction. Cycling myocytes were identified by the expression of Ki-67 in nuclei. This nuclear protein is associated only with cell division.^{7,8} Although the function of Ki-67 is not clear, it appears to promote cell proliferation by interfering with the binding of p53 to DNA.⁹ To avoid errors in the identification of myocytes, we assessed mitotic divisions of myocyte nuclei by confocal microscopy of immunolabeled cell cytoplasm.^{1,3} The measures of cell regeneration were determined separately

From the Department of Medicine, New York Medical College, Valhalla (A.P.B., K.U., J.K., B.N.-G., A.L., P.A.); the Department of Pathology, University of Udine, Udine, Italy (S.-M.Y., N.F., C.A.B.); and the Department of Pathology, University of Trieste, Trieste, Italy (R.B., F.S.). Address reprint requests to Dr. Anversa at the Department of Medicine, Vossburgh Pavilion, Rm. 302, New York Medical College, Valhalla, NY 10595, or at piero_anversa@nymc.edu.

in areas bordering on and distant from the infarcts. To investigate characteristics of cell division *in vivo*, we searched for the formation of the mitotic spindle by microtubules¹⁰ and of the contractile ring by actin accumulation,¹¹ as well as karyokinesis and cytokinesis.

METHODS

Patients

Thirteen hearts were obtained from patients who had died 4 to 12 days after myocardial infarction. There were seven men and six women, with a mean (\pm SD) age of 64 ± 15 years. As previously described,¹² we obtained 10 control hearts at autopsy from five men and five women, with a mean age of 61 ± 20 years, who had died from causes other than cardiovascular disease. Infarct size was determined by measuring the area of grossly detectable necrotic myocardium within the left ventricular free wall; this was expressed as a fraction of the total area.¹³ The rest of the left ventricular free wall was divided into three equal parts, which were defined as the border zone, the intermediate region, and the distant myocardium. Since the average size of the infarct was 35 percent, the average size of each area of viable tissue was nearly 20 percent of the left ventricular free wall. Samples were obtained only from the border zone and the distant myocardium. Tissue specimens from comparable areas of noninfarcted control hearts were examined.

Confocal Microscopy and Immunocytochemical Analysis

The specimens were fixed in 10 percent phosphate-buffered formalin and embedded in paraffin. The histologic sections were stained with propidium iodide (10 μ g per milliliter) and antibody to α -sarcomeric actin (clone 5C5, Sigma), diluted 1:20 in phosphate-buffered saline. For identification of mitotic spindles, the samples were exposed to a mouse monoclonal antitubulin antibody (Zymed). Fluorescein isothiocyanate-conjugated antimouse IgG was used as a secondary antibody. For the detection of Ki-67, the samples were exposed for one hour at 37°C to a mouse monoclonal antibody against Ki-67 (clone MIB-1, Diagnostic Biosystems), diluted 1:40 in phosphate-buffered saline.⁷ Fluorescein isothiocyanate-conjugated antimouse IgG was again used as a secondary antibody. Subsequently, specimens were processed for confocal microscopy^{1,3} and examined with a confocal microscope (MRC-1000, Bio-Rad).

Statistical Analysis

The numbers of myocyte nuclei labeled by Ki-67 were determined by evaluating approximately 3000 nuclei in the border zone and 9000 to 11,000 nuclei in the distant myocardium of each infarcted heart. Approximately 100,000 to 125,000 nuclei were evaluated in each control heart. The numbers of myocyte nuclei undergoing mitosis were determined by evaluating an average of 80,000 nuclei in the border zone and 104,000 nuclei in the distant myocardium of each infarcted heart. The values in corresponding anatomical areas of the control hearts were 100,000 and 110,000. Sampling for mitoses was larger than sampling for Ki-67 because of the lower frequency of mitoses in the myocardium. A total of 1165 Ki-67-positive myocyte nuclei was counted in infarcted hearts. This yielded an overall sampling error of 2.9 percent (the sampling error equals the square root of n divided by n , where n equals the total count). The number of myocyte nuclei undergoing mitosis was 590, reflecting a 4.1 percent sampling error.¹⁴ These values are less than the biologic variability among humans, which is at least 20 percent.¹⁴ Since the numbers of Ki-67-labeled nuclei and mitotic images were similar in these two control regions analogous to border and distant myocardium in infarcted heart, separate measurements were combined to generate a single value. The results are presented as means \pm SD. The significance of the differences was determined with the use of Student's *t*-test for comparisons of two values and the Bonferroni method for multiple comparisons.¹⁵

RESULTS

Patients

The hearts of the 13 patients with myocardial infarction were obtained 7 to 17 hours after death. Coronary atherosclerosis was severe and affected the left and right coronary arteries in all cases. Myocardial infarction consistently involved the anterior and inferior aspects of the left ventricle and was associated with cardiac rupture in three subjects. The size of the infarct ranged from 26 to 44 percent, averaging 35 ± 7 percent. In three cases, an old fibrotic infarct was noted, and foci of replacement fibrosis and areas of interstitial fibrosis were identified in all three of these hearts. None of the patients had a history of systemic hypertension or diabetes. Two of the 13 patients had been treated with thrombolytic agents. The average weight of the hearts with myocardial infarction was 497 ± 129 g, and that of the control hearts was 361 ± 51 g ($P=0.005$). The control hearts were from subjects of similar age who did not have primary heart disease or major risk factors for coronary artery disease, including hypertension, diabetes, obesity, and severe atherosclerosis. Autopsy and histologic examination of all organs ruled out the presence of diffuse metastatic malignant neoplasms and chronic inflammation. Six patients died from acute trauma, one from gastrointestinal hemorrhage, two from cerebral hemorrhage, and one from pulmonary thromboembolism.

Ki-67 Labeling and Mitotic Index

Ki-67 is a nuclear antigen expressed in all phases of the cell cycle except G_0 .⁷ Ki-67 is apparent mainly in the late S phase, increases further in G_2 , persists during prophase and metaphase,⁷ and decreases in anaphase and telophase. Ki-67 is preferable to thymidine, bromodeoxyuridine, and proliferating-cell nuclear antigen for labeling, because it is not involved in DNA repair.¹⁶ Expression of Ki-67 is a requirement for cells to traverse the cell cycle and undergo cell division.^{7,8} All types of proliferating human cells express Ki-67.^{16,17} Ki-67 was measured in myocyte nuclei of control and infarcted hearts by confocal microscopy (Fig. 1A, 1B, 1C, and 1D).^{1,3,18,19} In comparison with myocytes from normal hearts, the number of Ki-67-positive nuclei in myocytes from hearts with myocardial infarction was 84 times as high in samples from the border zone and 28 times as high in samples from the distant myocardium ($P<0.001$ for both comparisons) (Fig. 1E).

Although the expression of Ki-67 in myocytes after infarction by itself challenges the assumption that the heart is a postmitotic organ,^{4,6,20} we found further evidence of myocyte division. During mitosis, microtubules form the mitotic spindle, allowing each chromatid to be pulled toward the spindle pole by the kinetochore microtubules. This process occurs in anaphase and lasts only a few minutes.²¹ The arrange-

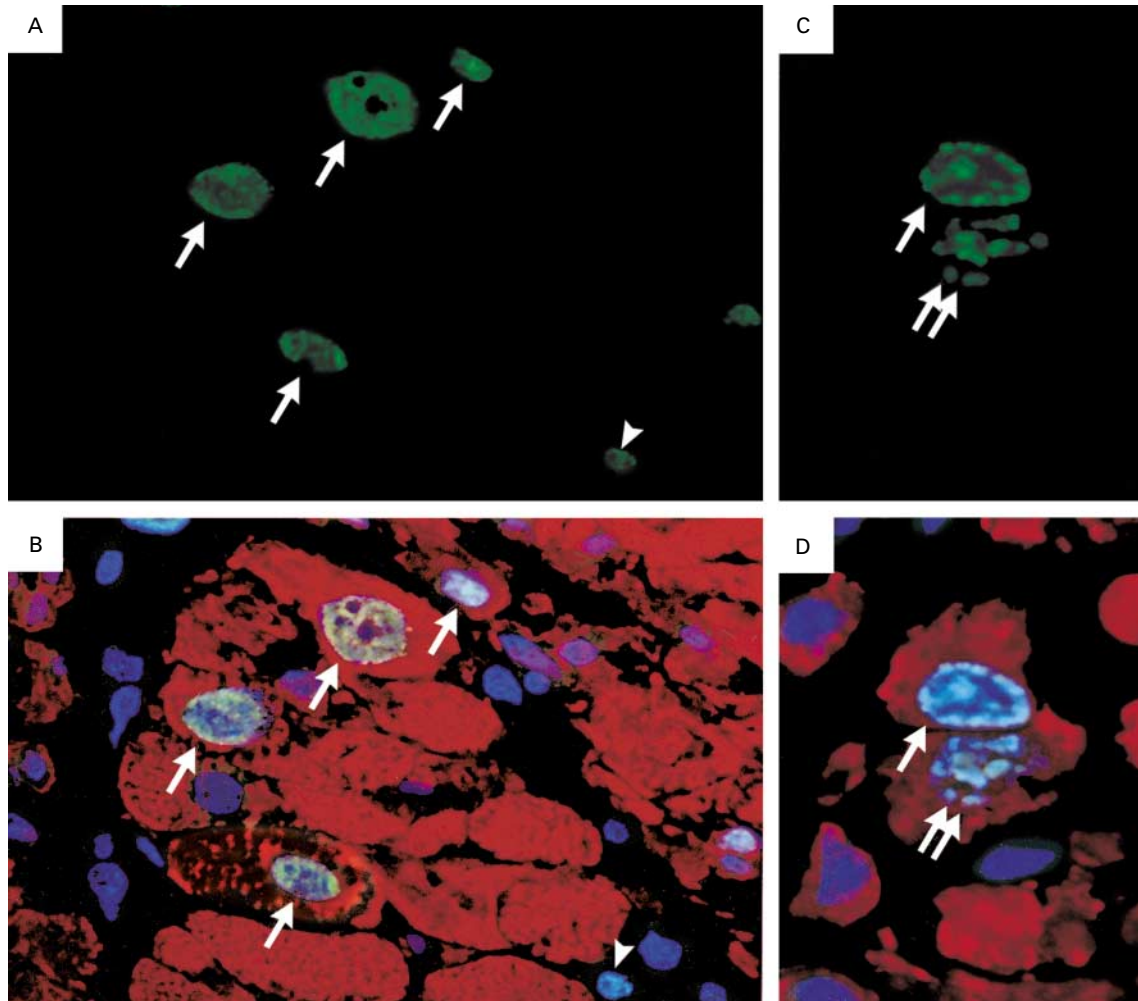
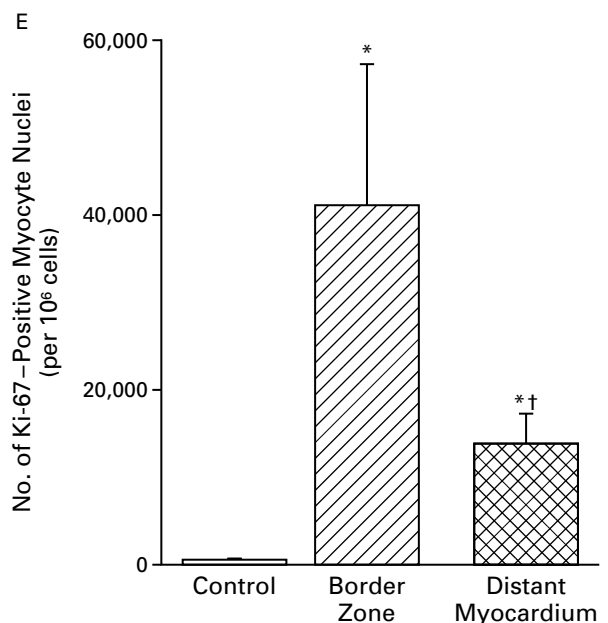


Figure 1. Ki-67 Labeling of Cycling Myocytes in an Infarcted Heart.

In Panels A and C, green fluorescence documents localization of Ki-67 in nuclei (arrows and arrowhead). In Panels B and D, red fluorescence shows staining of myocyte cytoplasm by sarcomeric α -actin antibody, and bright fluorescence shows labeling of myocyte nuclei (arrows) and nonmyocyte nuclei (arrowhead) by a combination of propidium iodide and Ki-67. Ki-67 labeling of a myocyte nucleus in metaphase is evident in Panels C and D (double arrows). Panels A and B show cells from the border zone, and Panels C and D cells from the distant myocardium. (Panels A, B, C, and D, $\times 800$.) Panel E shows the effects of infarction on the mean (\pm SD) number of Ki-67-labeled myocyte nuclei. The asterisks indicate $P < 0.001$ for the comparison between the infarcted hearts and the control hearts; the dagger indicates $P < 0.001$ for the comparison between the distant myocardium and the border zone in the infarcted hearts.



ment of microtubules in the mitotic spindle of dividing myocytes was detected on microscopical examination (Fig. 2). In addition, accumulation of actin and its assembly in the contractile ring were identified with the use of sarcomeric α -actin antibody (Fig. 3). Myocyte division was in the process of completion and actin was condensed in a narrow region, delineating a groove between the two forming daughter cells. Images of nuclear mitotic division (Fig. 4) and cytokinesis (Fig. 5) were also obtained, strengthening the notion that Ki-67 labeling in nuclei represents multiplying myocytes.

As a direct quantitative estimate of the extent of myocardial repair, we calculated a myocyte mitotic index. Staining with antibody to sarcomeric α -actin is specific for I bands of cardiac and skeletal muscle cells and does not affect other actin isoforms.²² Therefore, the distinction between myocyte and nonmyocyte nuclei is extremely simple: interstitial cells are not stained

by α -sarcomeric actin, and only their nuclei can be seen on staining with propidium iodide (Fig. 2D, 4A, 4B, 4C, and 5B). The same approach was used for Ki-67 labeling (Fig. 1B and 1D). Myocyte mitotic indexes are shown in Figure 6. In comparison with normal hearts, hearts with myocardial infarction have 70 times as many myocytes undergoing mitosis in the border zone and 24 times as many in the distant myocardium ($P < 0.001$ for both comparisons). The fact that the value was 2.9 times as high in the border zone as in the distant myocardium ($P < 0.001$) is consistent with the higher level of Ki-67 expression in the border zone.

DISCUSSION

Our results indicate that the adult heart has a subpopulation of myocytes that are not terminally differentiated; these myocytes evidently reentered the cell cycle and underwent nuclear mitotic division early af-

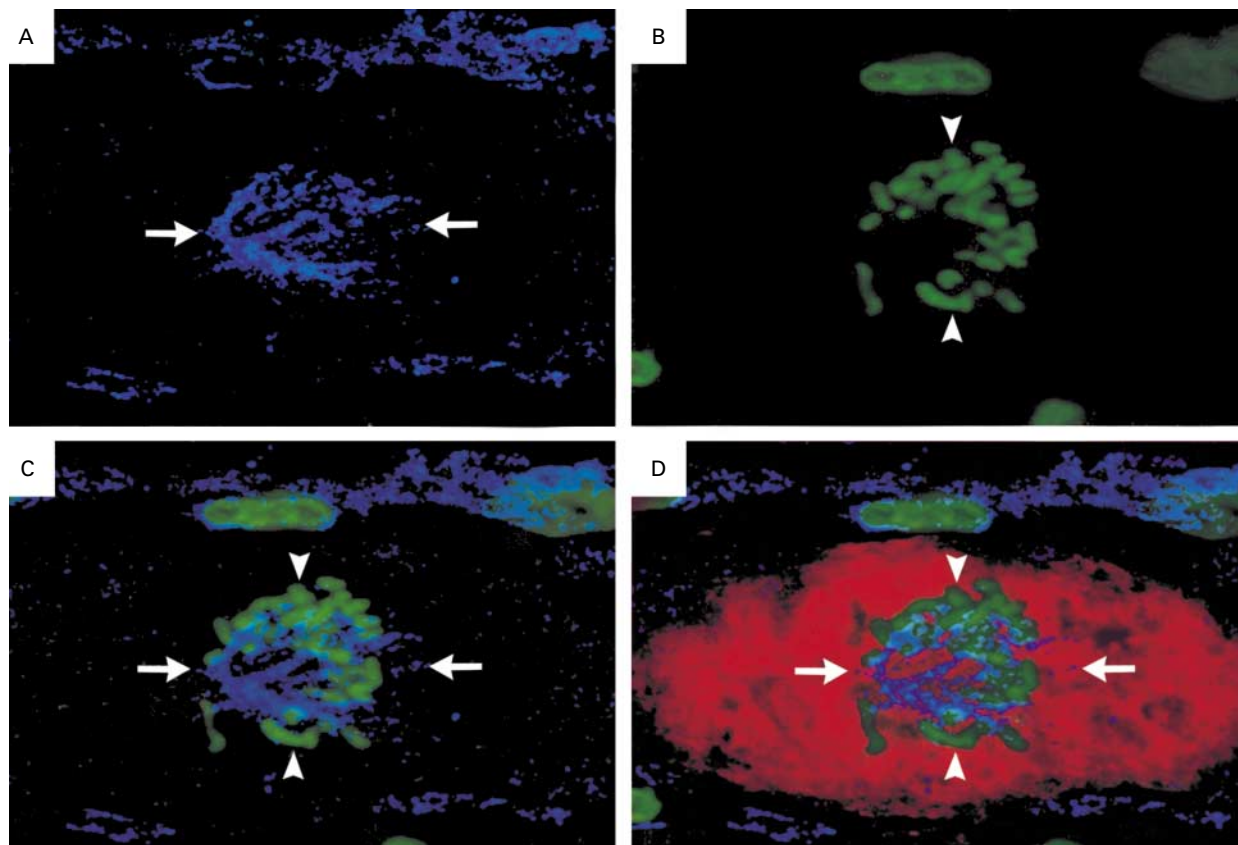


Figure 2. Identification of Mitotic Spindles in Dividing Myocytes from Infarcted Hearts ($\times 2000$).

In Panel A, blue fluorescence indicates the organization of tubulin in the mitotic spindle (arrows). Panel B depicts a nucleus in metaphase, indicated by the green fluorescence of propidium iodide (arrowheads). In Panel C, green and blue fluorescence shows the combination of tubulin and metaphase chromosomes (arrows and arrowheads). Panel D shows staining of myocyte cytoplasm by antibody against sarcomeric α -actin (red fluorescence), tubulin labeling (blue fluorescence), and chromosomes in metaphase (green fluorescence) (arrows and arrowheads).

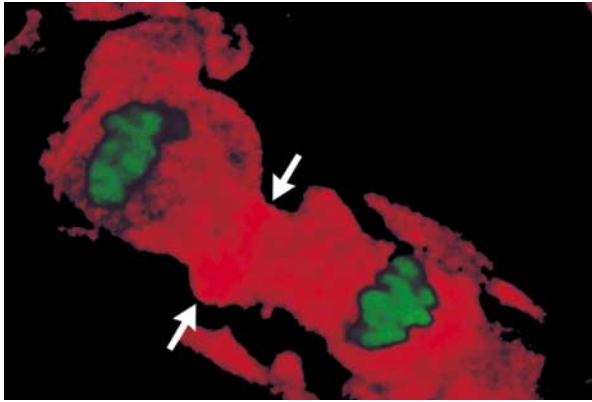


Figure 3. A Myocyte in the Process of Cytokinesis.

Accumulation of actin (arrows) in the region of cytoplasmic division and cell separation is shown; red fluorescence shows staining of myocyte cytoplasm by antibody against sarcomeric α -actin, and green fluorescence shows propidium iodide labeling of chromosomes ($\times 2000$).

ter infarction. The number of cycling myocytes was significantly larger in the zone bordering the infarct than in the distant myocardium. In laboratory animals, conditions that mimic coronary artery disease are characterized by DNA replication and myocyte division.^{3,23} These responses peak 7 to 14 days after coronary-artery restriction and decrease with time.²³ A similar phenomenon may occur in humans, suggesting that prolonged heart failure may progressively affect the mitotic activity of myocytes. Multiplication of myocytes is markedly attenuated as the length of time after myocardial infarction increases.¹

DNA synthesis in myocyte nuclei has been measured experimentally on the basis of incorporation of nucleotides such as [³H]thymidine and bromodeoxyuridine or labeling by proliferating-cell nuclear antigen, which is implicated in the transition from G₁ to S phase.^{3,4,20,24} However, these findings have been questioned as indicators of cell proliferation.^{3,4} The detection of myocyte nuclei that are positive for thymidine and bromodeoxyuridine does not indicate whether DNA synthesis is coupled with nuclear hyperplasia, ploidy formation, or DNA repair. Furthermore, thymidine and bromodeoxyuridine cannot be injected into humans except in unusual circumstances.²⁵ Limitations apply to staining of proliferating-cell nuclear antigen in cell nuclei. Proliferating-cell nuclear antigen is a cofactor of DNA polymerase δ , which is implicated in DNA synthesis, cell-cycle progression, and DNA repair.⁷ The last property may explain only in part the high level of expression of this protein in the nuclei of myocytes in terminally decompensated human hearts.²⁶ In fact, the reported values most likely overestimated the actual number of replicating my-

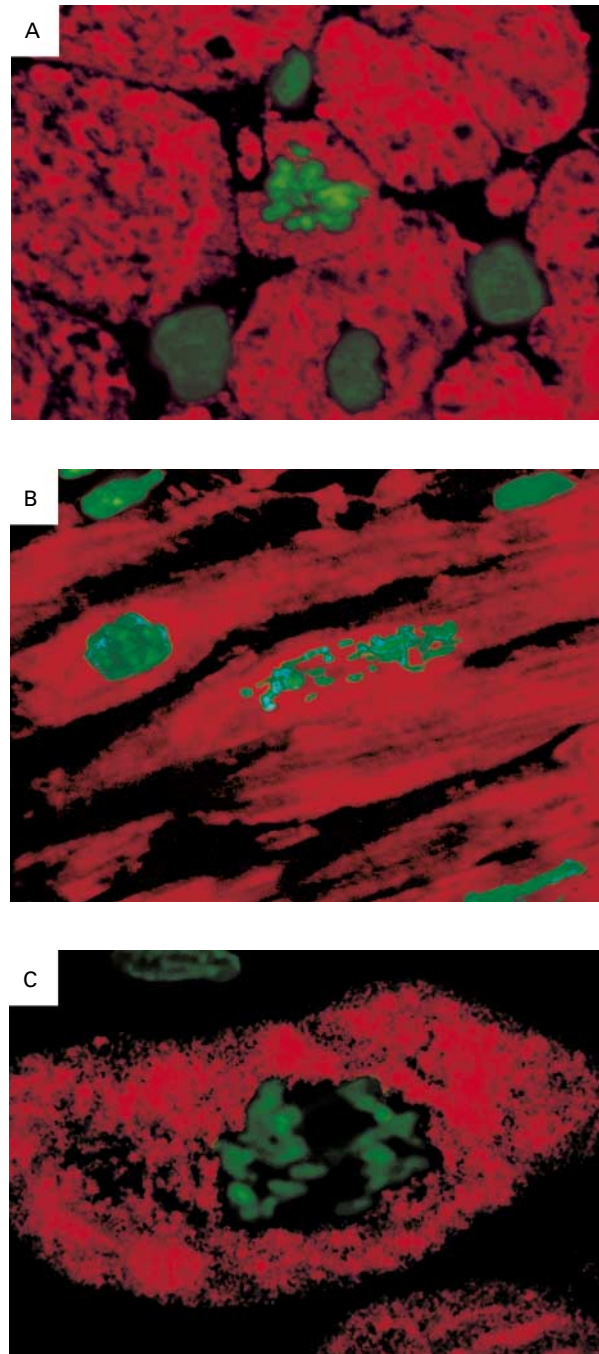


Figure 4. Mitotic Myocyte Nuclei in Infarcted Hearts.

Panels A, B, and C demonstrate the combination of labeling of myocyte cytoplasm by antibody against sarcomeric α -actin (red fluorescence) and staining of metaphase chromosomes by propidium iodide (green fluorescence). (Panels A and B, $\times 1200$; Panel C, $\times 2000$.)

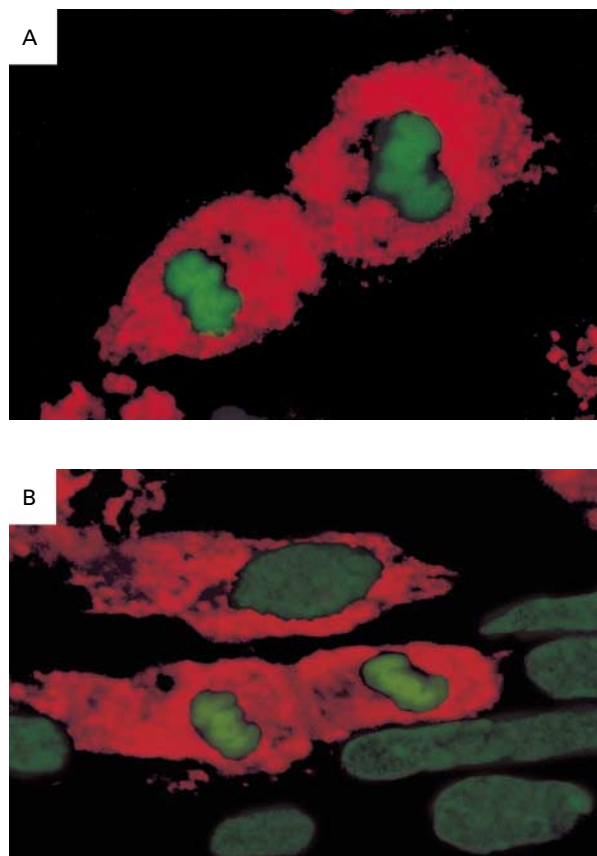


Figure 5. Myocyte Cytokinesis in Infarcted Hearts ($\times 1500$). Cytokinesis is shown in Panels A and B by the combination of labeling of nuclei by propidium iodide (green fluorescence) and staining of myocyte cytoplasm by antibody against sarcomeric α -actin (red fluorescence). The divided nuclei mirror each other in the newly formed myocytes.

ocytes. We overcame these difficulties by using Ki-67 as a marker of cell proliferation. There is not a single example of a Ki-67-positive cell that cannot divide.^{7-9,16,17} Biochemically, Ki-67 is an essential element of the outer dense fibrillar compartment of the nucleolus, where it acts as an efficiency factor in the rapid production of ribosomes for the increased metabolic requirements of dividing cells.⁸ Structurally, Ki-67 is a molecule of 395 kd that contains a motif typical of several transcription factors.²⁷ Ki-67 has a preference for binding to adenine- and thymidine-rich sequences similar to the consensus site of *p53*.⁹ This competition emphasizes the role of Ki-67 in cell replication.

The observation that mitotic indexes of nearly 800 and 300 myocyte nuclei per 10^6 cells characterize the acute myocardial response to infarction raises some crucial questions. The infarcted heart is frequently discussed as proof of the inability of myocytes to reenter the cell cycle and reconstitute muscle mass.^{6,20} How-

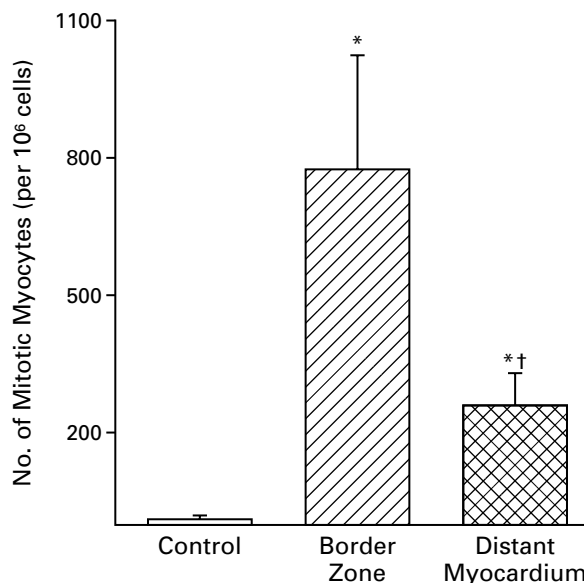


Figure 6. Effects of Infarction on the Mean (\pm SD) Number of Mitotic Myocytes.

The asterisks indicate $P < 0.001$ for the comparison between the infarcted hearts and the control hearts; the dagger indicates $P < 0.001$ for the comparison between the distant myocardium and the border zone in the infarcted hearts.

ever, myocytes in the infarcted area die in a few hours, and ischemic damage occurs in the vascular and non-vascular components of the interstitium.¹³ The formation of new myocardium in the infarcted region by myocyte growth alone is impossible. We found that mitotic activity occurred in myocytes in the border zone and the more distant myocardium, where tissue oxygenation was largely maintained.²⁸ The possibility that karyokinesis was not followed by myocyte cytokinesis is unlikely. Unlike the myocytes of rodents and dogs,^{23,29,30} ventricular myocytes in humans are predominantly mononucleated.³¹ Studies of dissociated myocytes from 72 normal hearts, 81 hearts with hypertrophy, and 95 hearts with ischemic cardiomyopathy, from subjects ranging from 26 to 93 years of age, found that mononucleated myocytes constituted 75 percent and binucleated cells 25 percent of the cell population. This proportion was not affected by disease, age, or sex.³¹ However, this finding does not exclude the possibility that some binucleation of myocytes occurred after infarction.

Measurements of the proportion of myocytes in the cell cycle by labeling of Ki-67 and expression of the proportion of mitotic myocytes by means of the mitotic index indicate a consistent relation between these two markers of cell growth. The number of cycling myocytes is nearly 50 times as high as the number of mitotic myocytes in both normal and infarcted hearts.

Since mitosis is completed in about 30 minutes,³² the duration of the myocyte cell cycle in vivo should be approximately 25 hours. The normal left ventricle contains 5.5×10^9 myocytes, and this value decreases to an average of 3.8×10^9 after myocardial infarction.³³ A mitotic index of 11 myocytes per 10^6 in the intact ventricle and 520 myocytes per 10^6 in the injured ventricle (775 myocytes per 10^6 in the border zone and 264 myocytes per 10^6 in the distant myocardium; mean, 520 myocytes per 10^6) implies that 60,500 myocytes are in mitosis in the normal left ventricle and 1,976,000 in the infarcted left ventricle. If the level of proliferation measured up to 12 days after coronary-artery occlusion persisted, the 1.7×10^9 myocytes lost as a result of infarction would be replaced in 18 days (myocytes per day, $1.98 \times 10^6 \times 48 = 95 \times 10^6$; myocytes per 18 days, $95 \times 10^6 \times 18 = 1.7 \times 10^9$). This calculation assumes that mitosis lasts 30 minutes (24 hours = 48 half-hours) and that replicating myocytes divide only once during this period.

A relevant issue is the origin of cycling myocytes in normal and diseased hearts. These proliferating cells could derive from resident cardiomyocytes or from circulating stem cells that reach the spared myocardium after infarction. However, in the absence of stimulation by several cytokines, the number of circulating stem cells is very low.³⁴⁻³⁶ Moreover, circulating stem cells move to the area of injury³⁶ without infiltrating the viable tissue.³⁷ Recently, we showed that bone marrow-derived stem cells, injected into the border of a myocardial infarct, homed to the infarcted zone and did not move into the remaining nonaffected portion of the ventricular wall.³⁷ Injury and large numbers of stem cells seem to be required for these cells' migration, multiplication, and differentiation into the cell lineages of the damaged heart or other organs.^{37,38}

Although a cardiac stem cell has not yet been identified, such primitive undifferentiated cells may be present, and the dividing myocytes may be their progeny. This phenomenon occurs in the brain.^{5,36} As in the damaged brain,³⁶ repair of the necrotic myocardium may involve interventions that promote the migration of endogenous, exogenous, or both types of stem cells to the infarcted region. Whether this therapeutic approach is superior to transplantation of myoblasts³⁹ or fetal cardiomyocytes⁴⁰ remains an important question. Hypertrophy and proliferation of myocytes do not prevent ventricular remodeling and the onset and evolution of cardiac failure after severe ischemic injury. Restoration of the infarcted myocardium, even in part, might interfere with the progression of the structural and functional alterations of the diseased heart,³⁷ thus delaying irreversible ventricular dysfunction.

In summary, our results challenge the dogma that the heart is a postmitotic organ. Myocyte proliferation may be a component of the growth reserve of the human heart; this mechanism could replace damaged myocardium. The presence of cell division in the non-

diseased part of the heart suggests a continuous turnover of cells during the life span of the organism. The belief that myocardial infarction constitutes the most obvious demonstration of the incapacity of ventricular myocytes to replicate must be reconsidered.

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