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Myocyte proliferation in end-stage cardiac failure in humans

(mitotic index/cytokinesis)

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ABSTRACT **Introduced several decades ago, the dogma persists that cardiac myocytes are terminally differentiated cells and that division of muscle cells is impossible in the adult heart. More recently, nuclear mitotic divisions in myocytes occasionally were seen, but those observations were challenged on the assumption that the rate of cell proliferation was inconsequential for actual tissue regeneration. Moreover, mitoses were never detected in normal myocardium. However, the analysis of routine histologic preparations constituted the basis for the belief that myocytes were unable to reenter the cell cycle and divide, ignoring the limitations of these techniques. We report here by confocal microscopy that 14 myocytes per million were in mitosis in control human hearts. A nearly 10-fold increase in this parameter was measured in end-stage ischemic heart disease (152 myocytes per million) and in idiopathic dilated cardiomyopathy (131 myocytes per million). Because the left ventricle contains 5.8×10^9 myocytes, these mitotic indices imply that 81.2×10^3, 882×10^3, and 760×10^3 myocytes were in mitosis in the entire ventricular myocardium of control hearts and hearts affected by ischemic and idiopathic dilated cardiomyopathy, respectively. Additionally, mitosis lasts less than 1 hr, suggesting that large numbers of myocytes can be formed in the nonpathologic and pathologic heart with time. Evidence of cytokinesis in myocytes was obtained, providing unequivocal proof of myocyte proliferation.**

It is a general contention that cardiac myocytes are unable to divide in the adult heart (1, 2). However, quantitative results suggest that an increase in myocyte number occurs with severe myocardial hypertrophy (3, 4), but because mitoses in myocytes were not identified, this deficiency led to disbelief of these morphometric results. The occasional detection of nuclear mitotic divisions in the pathologic heart (5, 6), was considered of no value in terms of actual regeneration of myocardial mass. Additionally, mitoses were never observed in control myocardium. Similarly, documentation of cytokinesis in myocytes was lacking. Ischemic and idiopathic dilated cardiomyopathies in humans are characterized structurally by severe myocardial scarring consisting of multiple sites of replacement fibrosis and diffuse interstitial fibrosis (7–10). Moreover, areas of segmental fibrosis are present in all cases of ischemic myopathies (7, 9). Segmental, replacement, and interstitial fibrosis are the consequence of myocyte necrosis. However, a discrepancy exists between the extensive collagen accumulation and the modest reduction in the number of ventricular myocytes in the postinfarcted human heart (9). The deposition of 1 mm^3 of collagen reflects the loss of 50×10^3 muscle cells (11), and the magnitude of fibrosis in end-stage ischemic cardiomyopathy would imply a nearly 90% decrease in the total number of left ventricular myocytes (9). Conversely, decreases of less than 30% have been reported (9). This discrepancy is even more apparent in idiopathic dilated cardiomyopathy in which myocardial fibrosis is associated with preservation of the number of myocytes in the ventricles (10).

Understanding of the cellular basis of wall restructuring in the diseased heart is complicated further by the documentation that programmed myocyte cell death occurs with ventricular decompensation (12, 13). Apoptosis does not result in tissue fibrosis; dying myocytes are removed from neighboring cells in the absence of an inflammatory reaction (14). These phenomena, indicating severe ongoing necrotic and apoptotic myocyte death, point to the possibility that myocytes are not terminally differentiated and cell proliferation may be stimulated in the pathologic heart. Immunocytochemistry and confocal microscopy were used here to measure a mitotic index in myocytes of hearts obtained from patients undergoing cardiac transplantation as a result of chronic ischemic heart disease and dilated cardiomyopathy. Hearts collected at autopsy were used as controls.

MATERIALS AND METHODS

Cardiac Characteristics. Twenty-seven patients undergoing cardiac transplantation, 12 for ischemic and 15 for idiopathic dilated cardiomyopathy, were studied. The first group included 11 males and one female, with an average age of 52 ± 9 years, and the second 11 males and four females, with an average age of 55 ± 11 years. Nine control hearts, seven males and two females, with an average age of 48 ± 15 years, were collected at autopsy within 15 hr after death; death occurred from causes other than cardiovascular disease.

Mitotic Index. In the 27 explanted and nine control hearts, specimens comprising the entire thickness of the anterior and posterior aspects of the left ventricular wall were obtained halfway between the apex and the base of the heart. Samples were fixed in formalin and embedded in paraffin. Sections were stained with propidium iodide (20 μg/ml) and α-sarcomeric actin antibody (clone 5C5, Sigma) to visualize DNA and myofibrillar structures. These sections were examined by confocal microscopy (MRC-1000, Bio-Rad) with an optical section thickness of 0.57 μm. The percentage of myocyte nuclei undergoing mitosis was obtained by sampling a number of myocyte nuclei, varying from 12,000 to 67,000. Values in control hearts were 75,000 and 230,000. The evaluation of a mitotic index in interstitial cells included seven cases with ischemic cardiomyopathy, five with dilated cardiomyopathy, and four control hearts. In each pathologic and normal heart 30,000 and 100,000 nuclei were sampled, respectively.

Data Collection and Analysis. Results are presented as mean ± SD. Significance between two measurements was determined by the Student's *t* test, and in multiple comparisons was evaluated by the Bonferroni method (15). $P < 0.05$ was considered significant.

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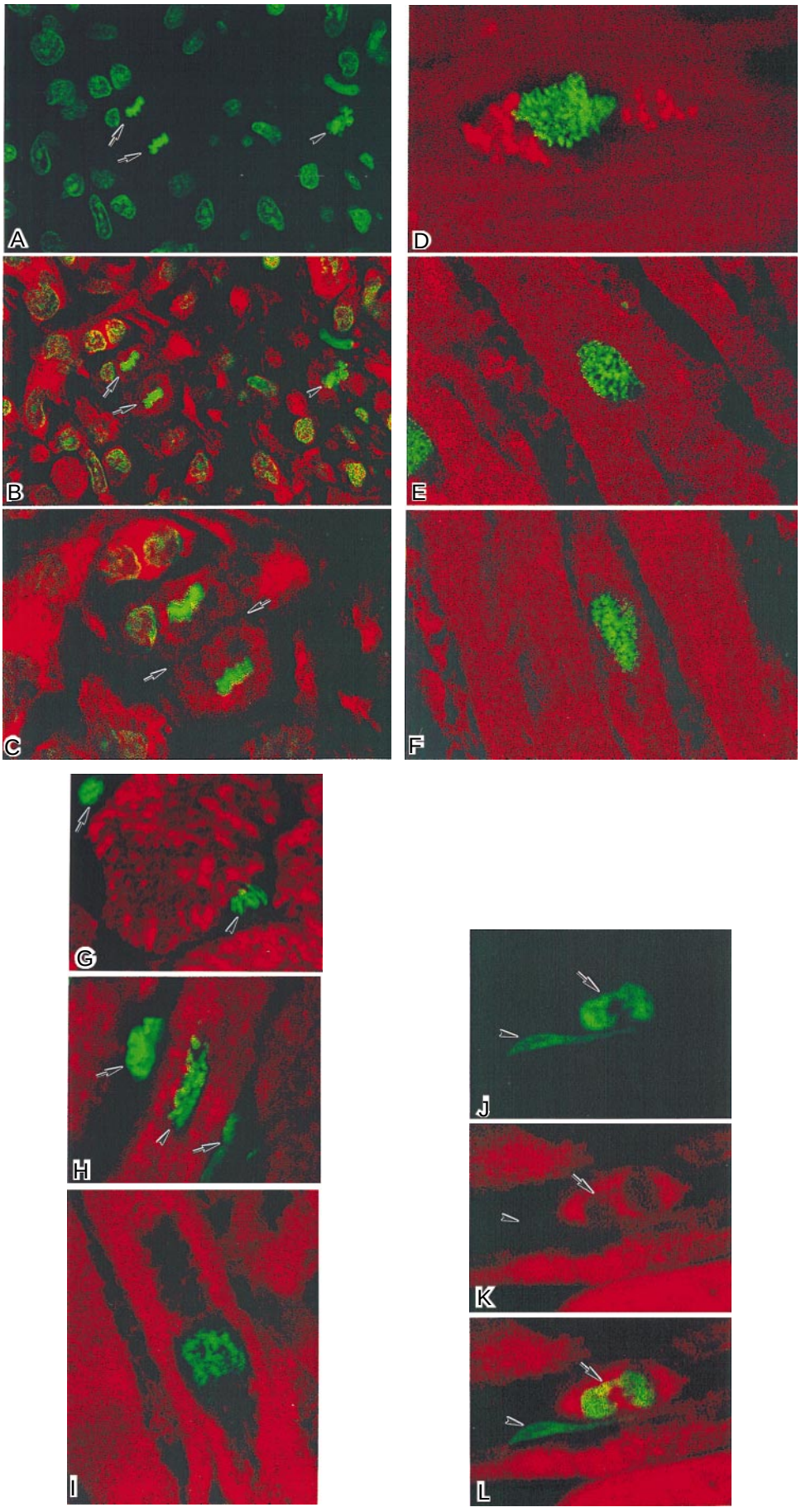

FIG. 1. (*Legend appears at the bottom of the opposite page.*)

RESULTS

Patients. All patients had New York Heart Association functional class III or IV. Left and right ventricular weights were 189 ± 26 and 62 ± 18 g, respectively, in controls, 281 ± 51 and 110 ± 33 g, respectively, in ischemic cardiomyopathy, and 362 ± 129 and 96 ± 36 g, respectively, in dilated cardiomyopathy. The 49% ($P < 0.05$) and 92% ($P < 0.001$) increase in left ventricular weight, and 77% ($P < 0.01$) and 55% ($P < 0.05$) increase in right ventricular weight with ischemic and dilated cardiomyopathy, respectively, were significant. Tissue sampling of the left ventricle was restricted to regions in which areas of scarring were not macroscopically visible. However, small foci of replacement fibrosis and diffuse interstitial fibrosis were present in the tissue sections from pathologic hearts. Areas of reparative and interstitial fibrosis occasionally were seen in the left ventricle of control hearts.

Confocal Microscopy. Sections of myocardium were labeled with propidium iodide and α-sarcomeric actin antibody; this antibody is specific for I bands of cardiac and skeletal muscle cells and does not react with other actin isoforms (16). Confocal microscopy allowed an accurate identification of mitosis in myocytes. Chromosomes were depicted by the green color assigned to propidium iodide fluorescence, and myofibrillar structures were recognized by the red color assigned to the fluorescence of α-sarcomeric actin antibody labeling. Fig. 1 *A–C* illustrates a nucleus in mitosis and two daughter cells at the completion of cytokinesis in a patient affected by dilated cardiomyopathy. In this latter example, the aggregates of chromosomes mirror each other in the two newly generated myocytes. Fig. 1*E* shows a late prophase that is characterized by the preservation of nuclear shape in the absence of nuclear membrane. Two more myocyte nuclei exhibiting metaphase chromosomes are depicted in Fig. 1 *D* and *F*. The initial separation of chromosomes in Fig. 1*D* may correspond to late metaphase or onset of anaphase. These three mitotic figures were found in a case of ischemic cardiomyopathy, dilated cardiomyopathy, and a control heart, respectively. A mitotic image in an interstitial cell and three additional mitoses in myocytes are depicted in Fig. 1 *G–L*. Undifferentiated cytoplasm surrounding the nucleus undergoing division (Fig. 1*I*) was observed in 52% of the cases (74 of 142). Organelles break up into small fragments to allow more uniform distribution of these components in the two daughter cells. The distinction between myocyte and nonmyocyte nuclei was extremely simple, because interstitial cells were not stained by α-sarcomeric actin antibody and only the nucleus could be identified by propidium iodide staining (Fig. 1 *G*, *H*, and *J–L*). This was apparent in nondividing and dividing interstitial cells (Fig. 1*G*). There was no apparent difference in the localization of mitoses in the anterior and posterior aspects of the left ventricle in control and pathologic hearts.

The average area of myocardium examined by confocal microscopy in each patient was 609 ± 240 mm^2 in controls, 327 ± 193 mm^2 in ischemic cardiomyopathy, and 341 ± 194 mm^2 in dilated cardiomyopathy. Corresponding numbers of myocyte nuclei counted were 141,136 ± 56,699, 38,854 ± 16,766, and 36,013 ± 17,134. Values for myocyte mitotic figures were 1.8 ± 0.6, 5.1 ± 3.5, and 4.3 ± 2.0, respectively. These data allowed the computation of a myocyte mitotic index in each group (Fig. 2). In normal left ventricles, an average of 14 myocytes per million cells were undergoing mitosis, but a much higher mitotic index was measured in pathologic hearts. In ischemic cardiomyopathy, 152 myocytes per million were dividing, and in dilated cardiomyopathy, 131 myocytes per million were in mitosis. The small difference between the two groups of patients with cardiac failure was not significant, yielding an average value of 140 proliferating myocytes per million cells. In comparison with healthy myocardium, cardiac failure was characterized by a 10-fold increase in the number of dividing myocytes ($P < 0.0001$). No gender difference in this parameter could be detected. The mitotic index in interstitial cells was 18 ± 13 per million cells in controls ($n = 4$) and 106 ± 42 per million cells in failing hearts ($n = 12$; seven ischemic and five idiopathic myopathies). With respect to myocytes (140 ± 50; $n = 27$), the 24% lower value in interstitial cells was not significant.

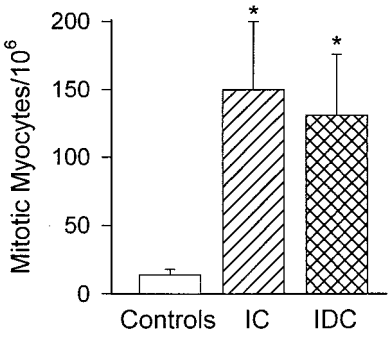

FIG. 2. Mitotic index in myocytes from control hearts (Controls), and hearts affected by ischemic cardiomyopathy (IC) and idiopathic dilated cardiomyopathy (IDC). Results are presented as means ± SD. *, $P < 0.00l$. Controls: $n = 9$; IC, $n = 12$; IDC, $n = l5$.

DISCUSSION

Cardiac Hypertrophy. In the early 1920s, anatomical studies emphasized the difficulties of detecting mitotic figures in myocytes and, on this basis, introduced the concept that muscle cell proliferation is absent in the adult, fully differentiated, mammalian myocardium (1). Moreover, experimental results of acute cardiac hypertrophy in rodents demonstrated the inability of myocytes to reenter the cell cycle, synthesize DNA, and undergo mitotic division (17–19). These observations were responsible for the creation of the dogma that, shortly after birth, ventricular myocytes withdraw permanently from the cell cycle and are destined to die without further replication. Such a contention was challenged by the morphometric findings of Linzbach in the mid-1950s, suggesting that myocyte

FIG. 1. (*On the opposite page*) Left ventricular myocardial section of a patient affected by end-stage dilated cardiomyopathy. Large field area illustrated by propidium iodide labeling only (green; *A*) and by a combination of propidium iodide and α-sarcomeric actin staining of the myocyte cytoplasm (red; *B*). Arrowhead indicates a myocyte nucleus in metaphase, and arrows indicate a myocyte at completion of cytokinesis. Myocyte cytokinesis is shown at higher magnification in *C* by a combination of propidium iodide and α-sarcomeric actin antibody staining (green and red, respectively). (*D–F*) Three mitotic figures in the center of myocytes corresponding to a patient with ischemic cardiomyopathy (*D*), dilated cardiomyopathy (*E*), and control left ventricle (*F*). Staining in *D–F* consists of a combination of propidium iodide and α-sarcomeric actin antibody. The punctate red staining in *D* corresponds to lipofuscin. (*G*) Two interstitial cell nuclei (arrow and arrowhead) (green), one of which is in mitosis (arrowhead). The cytoplasm of these nonmyocytes is not visible because it is not stained by α-sarcomeric actin antibody. (*H*) A mitotic figure in a myocyte from a patient with dilated myopathy is apparent (arrowhead); two interstitial cell nuclei (green) also are noted (arrows). These interstitial cells are not α-sarcomeric actin positive. (*I*) A mitotic figure in a center of a myocyte in which a large area of undifferentiated cytoplasm (negative to α-sarcomeric actin staining) surrounds the dividing nucleus is illustrated (patient with ischemic cardiomyopathy). (*J–L*) Patient with dilated cardiomyopathy. Two nuclei by propidium iodide (*J*, green), myocyte cytoplasm labeled by α-sarcomeric actin (*K*, red), and a combination of these two images (*L*). Note the myocyte nucleus in late telophase (arrows) and the nondividing interstitial cell nucleus (arrowheads). Magnifications: (*A* and *B*) ×700; (*C*) ×1,300; (*D*) ×2,700; (*E*, *F*, *H*, and *I*) ×1,500; (*G*) ×1,200; (*J–L*) ×1,600.

cellular hyperplasia occurs with cardiac failure in humans (3). Recent data have supported Linzbach's hypothesis and confirmed that the number of ventricular myocytes nearly doubles in the decompensated human heart (4, 20, 21).

Quantitative investigations failed to document mitosis in myocytes, favoring criticisms on the application of stereological principles to the analysis of changes in cell number of the heart. The lack of mitosis prompted complex explanations for the mechanism of cell proliferation, involving the longitudinal splitting of myocytes in the proximity of nondividing nuclei (3, 4). This phenomenon would result in a decrease in the number of nuclei per cell. However, the proportion of mononucleated and binucleated myocytes does not change in the human heart (22). If cells are not terminally differentiated, they are in a G_0 state and, at stimulation, reenter the cell cycle and undergo karyokinesis and cytokinesis (23). It is only through this growth process that an increase in the number of myocytes and myocardial regeneration can occur. Earlier observations (5) and the current data are consistent with this possibility because karyokinesis and cytokinesis have been demonstrated in myocytes of failing hearts.

Myocyte Proliferation. According to the dogma, ventricular myocytes are terminally differentiated cells and their life span corresponds to that of an individual or animal. In the case of human beings, the number of myocytes attains an adult value a few months after birth (24), and the same myocytes are believed to contract 70 times per min throughout life. Because a certain fraction of the population reaches 100 years of age or more, an inevitable consequence of the dogma is that cardiac myocytes may be immortal, functionally and structurally. Such an assumption contradicts the concept of cellular aging and programmed cell death, and the logic of a slow turnover of cells with the progression of life in the mammalian heart. The latter may be a very likely possibility because the male heart loses 64×10^6 myocytes per year from 17 to 89 years (25), indicating that cell death occurs with age, in the absence of cardiac pathology. Moreover, human ventricular myocytes can reenter the cell cycle and synthesize DNA in spite of the lack of any physiologic load (26). These observations, together with the documentation that 14 myocytes per million are in mitosis under normal conditions, point to myocyte aging and a continuous renewal of cells in the heart.

The ability of myocytes to proliferate and replace dying cells is markedly enhanced in the failing myocardium in which 140 myocyte nuclei per million were found in mitosis. The mitotic indices measured in the normal and pathologic heart require some comments to appreciate the magnitude of cell regeneration that can be achieved with these levels of mitosis. In a 45-year-old man with 5.8×10^9 myocyte nuclei in the left ventricle, a mitotic index of 14 nuclei/10^6 implies that 81,200 myocyte nuclei are in mitosis in the entire ventricle. In most cell systems, mitosis is completed in less than 1 hr (23), indicating that nearly 0.71×10^9 myocyte nuclei are produced in 1 year in the unaffected left ventricle. Because in the left ventricular myocardium 74% of myocytes are mononucleated and 26% are binucleated (22), 0.61×10^9 new myocytes are formed. Conversely, in the same ventricle, in the presence of failure, 812×10^3 myocyte nuclei are in mitosis, resulting in an accumulation of 7.12×10^9 myocyte nuclei in 1 year. The proportion of mononucleated and binucleated myocytes is not influenced by aging, cardiac hypertrophy, and ischemic cardiomyopathy (22), implying that this increase in nuclei may reflect the generation of 6.19×10^9 new myocytes in the decompensated ventricle. Moreover, mitotic figures, similar to those presented here, have never been found positive for apoptosis, excluding myocyte death at this stage of the cell cycle (unpublished results). It should be acknowledged that myocyte apoptosis (13) appears to exceed the level of myocyte proliferation detected here. However, a comparison between these two events currently is impossible because the time required for the completion of the apoptotic process in myocytes is unknown. The same problem exists for the duration of the cell cycle. At present, no information is available concerning the length of any of the phases of the cell cycle in neonatal and adult myocytes *in vitro* and *in vivo*.

On the basis of the current results, myocyte proliferation compensates, at least in part, for the massive myocyte cell death that occurs acutely after myocardial infarction and during the evolution of the ischemic myopathy (7, 9, 12, 13). Similarly, cell death characterizes idiopathic dilated cardiomyopathy (8, 12, 13), but cell regeneration maintains the number of myocytes relatively constant in the diseased heart (10). Myocyte hypertrophy constitutes an additional growth reserve mechanism and cells can nearly double in size in end-stage cardiac failure (3, 4, 9, 10). However, both cellular growth processes are unable to normalize the elevated diastolic load on the myocardium and/or decrease ventricular dilation. Myocytes elongate more than they expand in diameter (10, 11) and new myocytes may be added in series, providing the structural template for the increase in cavitary volume. If this pattern of cell proliferation is operative in the decompensated heart and the lateral insertion of cells with mural thickening is minimal, myocyte hyperplasia has to be regarded as a contributory factor to ventricular deadaptation and terminal failure. At present, this is an unresolved issue.

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