Myocyte Death in the Failing Human Heart Is Gender Dependent

Sabrina Guerra, Annarosa Leri, Xiaowei Wang, Nicoletta Finato, Carla Di Loreto, Carlo Alberto Beltrami, Jan Kajstura, Piero Anversa

Abstract—Cardiovascular disease is delayed and less common in women than in men. Myocyte death occurs in heart failure, but only apoptosis has been documented; the role of myocyte necrosis is unknown. Therefore, we tested whether necrosis is as important as apoptosis and whether myocyte death is lower in women than in men with heart failure. Molecular probes were used to measure the magnitude of myocyte necrosis and apoptosis in 7 women and 12 men undergoing transplantation for cardiac failure. Myocyte necrosis was evaluated by detection of DNA damage with blunt end fragments, whereas apoptosis was assessed by the identification of double-strand DNA cleavage with single base or longer 3’ overhangs. An identical analysis of these forms of cell death was performed in control myocardium. Heart failure showed levels of myocyte necrosis 7-fold greater than apoptosis in patients of both sexes. However, cell death was 2-fold higher in men than in women. Heart failure resulted in a 13-fold and 27-fold increase in necrosis in women and men, respectively. Apoptosis increased 35-fold in women and 85-fold in men. The differences in cell death between women and men were confirmed by the electrophoretic pattern of DNA diffusion and laddering of isolated myocytes. The lower degree of cell death in women was associated with a longer duration of the myopathy, a later onset of cardiac decompensation, and a longer interval between heart failure and transplantation. In conclusion, myocyte necrosis and apoptosis affect the decompensated human heart; each contributes to the evolution of cardiac failure. However, the female heart is protected, at least in part, from necrotic and apoptotic death signals. ( Circ Res. 1999;85:856-866.)

Key Words: apoptosis ■ necrosis ■ heart failure ■ sex ■ cardiomyopathy

Cardiovascular diseases are delayed and less frequent in women than in men.1,2 The hormonal profile differs between women and men, and estrogens may exert their protective effects on the heart at multiple levels. Estrogen replacement in postmenopausal women reduces the risk of cardiovascular events,3 and hypertension affects a more limited number of premenopausal women than men of a comparable age.4 Similarly, heart failure affects a more limited number of premenopausal women than men of a comparable age.4,5 The female heart is less susceptible to death signals and possesses an inherent ability to counteract the activation of the endogenous cell death pathway. Cell death occurs by apoptosis, necrosis, and the combination of both.11,12 Although the pathogenesis of heart failure remains unclear, myocyte apoptosis may be 1 of the critical factors involved.5,6 Experimentally, myocyte apoptosis has been implicated in the transition from compensated to decompensated hypertensive hypertrophy7 and in the acute restructuring of the wall and chamber dilation of the postinfarcted heart.8 Prevention of cell death attenuates the impact of ischemic damage on ventricular anatomy and performance.9,10 A relevant question is whether the myocardium in women is less susceptible to death signals and possesses an inherent ability to counteract the activation of the endogenous cell death pathway. Cell death occurs by apoptosis, necrosis, and the combination of both.11,12 The possibility that myocyte death is reduced in women is consistent with observations in the aging heart. The male heart loses 64×10⁶ myocytes per year during adulthood and senescence; the female heart does not.13 However, the cause of myocyte loss with aging remains to be identified because methods for the detection of apoptosis and necrosis in humans have not previously been available. The current study tested the hypothesis that apoptotic, necrotic, and apoptotic-necrotic myocyte death differ in the failing hearts of women and men.

Hearts from female and male patients who were undergoing cardiac transplantation were examined, and each form of cell death was measured. In this population, impairment in ventricular function is essentially identical. This allowed us to document whether cell death is reduced in women, despite the similarity of the overload. Probes capable of identifying DNA damage,14,15 as reflected by myocyte apoptosis and/or necrosis, were used for the first time in the human heart. These evaluations were complemented with the electrophoretic analysis of low-molecular-weight DNA obtained from isolated myocytes. In a subset of patients, the fraction of myocytes showing morphological changes consistent with apoptosis or necrosis was assessed by electron microscopy.

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Materials and Methods

Patients
Anatomical and functional properties of the heart were measured by 2-dimensional echocardiography in 26 patients, 9 women and 17 men, before transplantation. Pressure measurements were determined by cardiac catheterization. Three women had ischemic cardiomyopathy, and 6 had dilated cardiomyopathy. Eight men had ischemic cardiomyopathy, and 9 had dilated cardiomyopathy. Samples of the left ventricle were fixed in formalin and embedded in paraffin. Control myocardium was obtained from 5 women and 5 men at autopsy13 and surgically from the papillary muscles of 4 women and 4 men who had mitral stenosis.

Myocyte Apoptosis and Necrosis

A Taq probe was prepared as previously described14,16. Digoxigenin-labeled probes were ligated to DNA using the T4 ligase. Sections were incubated with anti-digoxigenin and exposed to FITC-labeled goat anti-mouse IgG. For confocal microscopy, sections were stained with anti-digoxigenin and exposed to FITC-labeled antibodies. For double-labeling, sections were processed first with anti-Pfu and then with TdT. To distinguish TdT from Pfu staining of nuclei, TdT was performed with rhodamine-labeled extravidin.

Single-base 3’ overhangs are identified by a polymerase chain reaction–generated Taq polymerase probe that possesses complementary structures (Figure 1A). Using the T4 ligase, this probe interacts only with damaged DNA exhibiting single-base 3’ overhangs. The TdT assay recognizes staggered ends in the DNA with ≥1 and ≤4 bases of 3’ overhangs. TdT links several molecules of biotinylated deoxyuridine 5-triphosphate to 3’ overhangs, and this tail is visualized by extravidin labeled with FITC (Figure 1B). Thus, apoptosis mediated by DNase I (Taq)14,18 or by both DNase I and DNase II (TdT)14,18 can be identified. During necrosis, the release of lysosomal proteases degrades histones, resulting in the loss of DNA protection and exposure to endonucleases and exonucleases.19 Enucleases produce double-strand DNA cleavage with recessed 3’ overhangs, and these are recognized by the Pfu probe, which contains blunt ends (Figure 1C). To confirm that Pfu-positive nuclei reflect myocyte necrosis with disruption of the sarcolemma, sections were stained by Pfu and vinculin. Vinculin is more prominent in costameres, but it clearly defines the continuity of the sarcolemmal surface.20

Myocyte DNA Gel Electrophoresis
Left ventricular myocytes were isolated from 3 control and 7 failing hearts using the methodology for enzymatic dissociation of myocytes in the dog heart.17 The presence of low-molecular-weight DNA fragments was then determined.6,16,17

Electron Microscopy of Myocyte Apoptosis and Necrosis
Samples from 6 failing hearts were fixed, processed for electron microscopy, and analyzed.5,16

Statistical Analysis
Results are presented as mean±SD. Significance at P<0.05, between 2 or multiple groups, was determined by Student’s t test and the Bonferroni method.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results

Heart Samples
Hearts from 26 patients, 9 women and 17 men, who underwent cardiac transplantation, were studied (Table). All patients were treated with inotropic drugs, diuretics, and angiotensin-converting-enzyme inhibitors. The age of the 9 women varied from 41 to 61 years. Ten autopsy hearts were used as controls. Samples were collected 1 to 7 hours after death in 5 women (aged 54±11 years) and 5 men (aged 46±16 years). These specimens were used for the detection of apoptosis by TdT and Taq probes. Only 1 female and 1 male heart could be used for baseline measurements of necrosis by Pfu because they were available within 2 hours after death. Additional sampling consisted of 8 papillary muscles obtained from patients of comparable age (4 women aged 58±2 years and 4 men aged 57±6 years), who had valve replacement for mitral stenosis. These were not healthy individuals. Although we recognize this limitation, a more appropriate control tissue was not found.

Myocyte Apoptosis
Internucleosomal DNA cleavage was measured separately in female and male hearts by TdT and Taq polymerase assays. Taq polymerase yields products with single-base 3’ over-
Clinical, Functional, and Anatomical Characteristics of Patients

<table>
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<tr>
<th>Variable</th>
<th>Women</th>
<th>Men</th>
<th>Normal Value</th>
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<tr>
<td>No. of patients with</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Idiopathic dilated cardiomyopathy</td>
<td>6</td>
<td>9</td>
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<td>Ischemic cardiomyopathy</td>
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<td>Age, y</td>
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<td>379±115</td>
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<td>275–325</td>
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<td>Left ventricular diameter, mm</td>
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<td>Systolic</td>
<td>58±6 (n=8)</td>
<td>61±6 (n=16)</td>
<td>20–35</td>
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<td>Diastolic</td>
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<td>72±7 (n=16)</td>
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<td>Wall thickness/chamber diameter</td>
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<td>0.24±0.05 (n=16)</td>
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<td>Ejection fraction, %</td>
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<td>23±7 (n=16)</td>
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<td>3835±1191</td>
<td>4246±1088 (n=16)</td>
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<td>2292±544 (n=16)</td>
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<td>Mean pulmonary artery wedge pressure, mm Hg</td>
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<td>Right ventricular end-diastolic pressure, mm Hg</td>
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<td>0–8</td>
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<td>Duration of disease, mo</td>
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<td>Onset of disease to heart failure, mo</td>
<td>77±83</td>
<td>54±56</td>
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<tr>
<td>Time from heart failure to transplantation, mo</td>
<td>24±24</td>
<td>14±11</td>
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Results are presented as mean±SD.

By guest on December 14, 2017

hangs. The TdT reaction with a fluorescent probe identifies staggered ends of the cleaved DNA, but it does not distinguish between single-base or longer ends, which are recognized by enzymes from lysosomes to produce DNA fragments with blunt ends. During necrosis, the release of endonucleases and exonucleases from lyso- some produces DNA fragments with blunt ends. 14,16 TdT reaction with a fluorescent probe identifies necrotic myocytes in vivo and in vitro. 23 Myocardial infarction is characterized by both necrotic and apoptotic myocyte death, as seen in Figures 5A through 5C, which illustrate Pfu-positive nuclei in the infarcted region of a male heart 4 days after the acute event. Cells were also TdT positive. A Pfu-labeled necrotic myocyte is shown in Figures 5D through 5F in a woman affected by dilated cardiomyopathy. Double-labeling with Pfu and TdT was done to detect necrotic-apoptotic myocytes in pathological hearts. Two cells were positive for both stainings: 1 in a man (Figure 6) and 1 in a woman with ischemic cardiomyopathy.

Quantitatively, myocyte necrosis involved counting 439 881 and 465 844 myocyte nuclei in female and male pathological hearts, respectively. In controls, 231 015 nuclei were examined (Figure 7). Results from hearts with ischemic and dilated cardiomyopathy were comparable, and they were included in a single group in each sex. Values in female and male control myocardium were also similar and combined. With heart failure, myocyte necrosis was nearly 2-fold (P<0.001) higher in men than in women. Moreover, with respect to baseline, this form of cell death increased 13-fold (P<0.002) and 27-fold (P<0.0001) in female and male diseased hearts, respectively. Examination of a large number of myocyte profiles by confocal microscopy showed that the majority of cells had continuity of the plasma membrane (Figure 8A). When sarcolemmal disruption was detected by vinculin staining, nuclei were Pfu-positive (Figure 8, B through D). In 200 myocytes, 100 each from the male and female failing hearts, Pfu labeling of nuclei was associated with membrane damage in all cases. This indicated that double-strand cleavage of DNA with blunt ends occurred exclusively in combination with membrane injury.

Myocyte DNA Agarose Gel Electrophoresis

Figure 9 illustrates, by confocal microscopy, enzymatically dissociated myocytes that were used to detect DNA diffusion...
(ie, necrosis), DNA laddering (ie, apoptosis), and the simultaneous presence of both. In myocytes from a female heart with ischemic cardiomyopathy, laddering was barely detectable, and it was restricted at 200 bp (Figure 10A, lane 2). In a similar male heart, DNA diffusion was apparent, and laddering was visible at 200, 400, and 600 bp (Figure 10A, lane 3). Moderate DNA diffusion was seen in a sample from a female heart with dilated cardiomyopathy (Figure 10A, lane 4); DNA laddering was not noted. Additional cases of DNA damage in myocytes from failing hearts are shown in Figure 10B. DNA diffusion and a laddering pattern with different intensities at 200, 400, and 600 bp were observed in male hearts with ischemic (Figure 10B, lanes 3 and 5) and dilated (Figure 10B, lanes 6 and 7) myopathies. The preparation of female myocytes shown previously (Figure 10A, lane 2) was included for comparison with male myocytes. In all cases of heart failure, male myocytes showed greater levels of DNA diffusion and laddering than female myocytes.

Electron Microscopy of Myocyte Apoptosis and Necrosis

Electron microscopy of 5 tissue blocks in each of 4 male hearts with cardiac failure included the analysis of 1752, 1478, 1533, and 1697 myocyte profiles. In the 2 female failing hearts, 1882 and 1684 myocyte profiles were examined. The fraction of cells with morphological changes characteristic of apoptosis (ie, chromatin margination, condensation, clumping and discontinuity of nuclear...
membranes; Figure 11, A and B) was determined; 3, 6, 2, and 5 myocytes with these properties were found in the 4 male hearts, and 2 and 1 in the 2 female hearts. Percentages of apoptosis in men were 0.17%, 0.41%, 0.13%, and 0.29%, and percentages of apoptosis in women were 0.11% and 0.06%. The same sampling was used to compute necrotic cells. Myocytes with membrane discontinuity and diffuse swelling (Figure 11C), severe disruption of mitochondrial cristae and swelling, and almost complete disorganization of cell structure were observed. These morphological alterations with unspecific nuclear damage reflected cell necrosis. In men, 18, 20, 9, and 16 myocytes had some of these aspects (Figure 11C). In the 4 cases, myocyte necrosis was 1.03%, 1.35%, 0.59%, and 0.94%.

Figure 3. Detection of myocyte apoptosis in female heart with dilated cardiomyopathy (A through C) and in male heart with ischemic cardiomyopathy (D through F). A and D illustrate nuclei stained by PI (red); B and E depict, respectively, Taq and TdT labeling of apoptotic nuclei (green); C and F show α-sarcomeric actin staining of myocyte cytoplasm (red) and combination of PI with Taq (C) or TdT (F) labeling of nuclei (yellow). Arrowheads indicate apoptotic nuclei. A through F, ×800.
when cell death was inhibited in hypertensive rats and in myocardial injury in rats and dogs. Interference with cell death in hemodynamics with age, systemic hypertension, and ischemia plays a critical role in the initiation of ventricular dysfunction. These observations suggest that the chronic loss of myocytes in the failing heart, but its magnitude differed significantly in women and men. Myocyte necrosis and apoptosis were markedly lower in women than in men. The reduced incidence of cell death in women was apparent, although the disease and cardiac failure were present for a longer time. Higher myocyte death in men was associated with a shorter duration of the myopathy and an earlier onset of heart failure. In women, the average times from the beginning of the morbid state to impairment in function and from diagnosis to transplantation were 77 and 24 months, respectively. In men, these intervals were 54 and 14 months, respectively. The entire length of the disease at transplantation was 101 and 68 months in women and men, respectively. These observations suggest that the chronic loss of myocytes plays a critical role in the initiation of ventricular dysfunction and its progression to severe cardiac decompensation. Recent clinical results are consistent with this possibility. The extent of cell death correlates with alterations in ventricular hemodynamics with age, systemic hypertension, and ischemic injury in rats and dogs. Interference with cell death in the surviving myocardium after infarction or coronary constriction decreases ventricular loading, chamber dilation, and hypertrophy in mice. Similar findings have been obtained when cell death was inhibited in hypertensive rats and in failing dogs.

The mechanism responsible for reduction in myocyte death in women with cardiac failure is unknown. The hormonal profile differs in women and men, and estrogens phosphorylate insulin-like growth factor-1 (IGF-1) receptors, improving cell survival. Although it was not possible to determine whether women in this study were on estrogen-supplemented therapy, changes in circulating levels of IGF-1 and IGF binding proteins (IGFBP) exerted a less significant role than their corresponding tissue concentrations. In this regard, a complete dissociation has been found between the adaptation of the systemic and local IGF-1 and IGFBP in pathological states. IGF-1 was not reduced in the myocardium with uremia, despite extreme levels of IGFBPs.

Estrogen may enhance the phosphorylation of IGF-1R in myocytes, because these cells can produce the sex hormone throughout life. Aging is characterized by a reduction in the circulating precursor pool of estrogen, androstenedione, and testosterone in women more than men. However, the ability to generate estrogen is higher in female myocytes, even in the presence of lower circulating amounts of C19 precursors. As a consequence, estrogen receptors increase. Additionally, the stimulation of the IGF-1/IGF-1 receptor system enhances the expression of antiapoptotic gene products, such as Bcl-2 and Bcl-X, and decreases the induction of proapoptotic proteins, such as Bax. Changes in these regulators of cell death may dictate the cell reaction to a death stimulus. IGF-1 activates the phosphatidylinositide-3’-OH kinase/Akt pathway, which suppresses cell death. Estrogen and IGF-1 stimulate NO, which promotes vasodilation and anti-thrombotic and antiinflammatory responses. These factors increase tissue oxygenation and may counteract necrotic death signals in women.

### Discussion

**Sex and Myocyte Death**

Results in the current study indicate that myocyte death occurred in the failing heart, but its magnitude differed significantly in women and men. Myocyte necrosis and apoptosis were markedly lower in women than in men. The reduced incidence of cell death in women was apparent, although the disease and cardiac failure were present for a longer time. Higher myocyte death in men was associated with a shorter duration of the myopathy and an earlier onset of heart failure. In women, the average times from the beginning of the morbid state to impairment in function and from diagnosis to transplantation were 77 and 24 months, respectively. In men, these intervals were 54 and 14 months, respectively. The entire length of the disease at transplantation was 101 and 68 months in women and men, respectively. These observations suggest that the chronic loss of myocytes plays a critical role in the initiation of ventricular dysfunction and its progression to severe cardiac decompensation. Recent clinical results are consistent with this possibility. The extent of cell death correlates with alterations in ventricular hemodynamics with age, systemic hypertension, and ischemic injury in rats and dogs. Interference with cell death in the surviving myocardium after infarction or coronary constriction decreases ventricular loading, chamber dilation, and hypertrophy in mice. Similar findings have been obtained when cell death was inhibited in hypertensive rats and in failing dogs.

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**Necrosis, Apoptosis, and Heart Failure**

Myocyte apoptosis occurs in end-stage cardiac failure. However, apoptosis involves, at most, 1% of myocytes; this value may challenge the impact of this phenomenon on the final outcome of the pathological state. Low levels of apoptosis were confirmed in the present study in the decompenated heart; levels were 0.18% in men and 0.08% in women. Importantly, myocyte necrosis comprised 1.2% of myocytes in men and 0.5% in women, which exceeded apoptosis in both sexes. Although the number of necrotic myocytes was several-fold greater than apoptotic myocytes in the male and female myocardium, the time required for the completion of each form of cell death is unknown. Labeling of DNA strand-breaks in myocyte nuclei by TdT or Taq corresponds to the early phases of apoptosis and does not provide information on the sequence of events taking place in the cell after nuclear fragmentation. Similarly, the recognition of myocyte necrosis by Pfu leaves unanswered the question concerning the duration of the necrotic process. In vitro studies in various model systems have shown that apoptosis may be completed in a period ranging from 30 minutes to 2 hours. However, no indication exists of the time required for myocyte apoptosis. An identical limitation applies to myocyte necrosis; this form of cell death has been claimed to reach its final stage in 1 to 2 days in infarcted rats. This period is necessary for the cell to be engulfed by surrounding macrophages. Apoptosis may be much faster than necrosis, suggesting that the higher value of myocyte necrosis in the failing heart may not reflect a significant difference in the number of cells dying by these 2 distinct mechanisms.

If the assumption is made that at any time, nearly 1.5% of myocytes are experiencing cell death, the heart should rapidly disappear. This contention does not consider 2
critical points: (1) myocyte proliferation does occur in the failing heart, and (2) these hearts are in the terminal phase of decompensation. The current findings do not characterize the mechanisms by which ventricular dysfunction deteriorates chronically; they show the end-stage, premortal condition of the disease. Ongoing cell death was documented by 6 different techniques: apoptosis by Taq and TdT labeling; necrosis by Pfu labeling and vinculin distribution; apoptosis and necrosis by electron microscopy; and apoptosis and necrosis by DNA laddering and diffusion of low-molecular-weight DNA in isolated myocyte preparations.

The cause of myocyte death with cardiac failure remains to be identified. Additionally, it is not apparent why apoptosis and necrosis occur simultaneously in ischemic and dilated cardiomyopathy. Alterations in coronary blood

Figure 5. Male infarcted heart at 4 days (A through C) and female heart with dilated cardiomyopathy (D through F). A and D, Nuclei stained by PI (red). B and E, Pfu-labeling of necrotic nuclei (green). C and F, α-sarcomeric actin staining of myocyte cytoplasm (red) and combination of PI and Pfu-labeling of nuclei (yellow). A through C, ×200; D through F, ×800.
flow are severe in the decompensated heart, and these defects in coronary perfusion and tissue oxygenation may trigger necrotic and apoptotic myocyte death. Transient ischemia activates myocyte apoptosis, but sustained reductions in coronary blood flow result in myocyte necrosis, which exceeds apoptosis. Although the primary event differs in ischemic and dilated cardiomyopathy, foci of replacement fibrosis and collagen accumulation are present in the noninfarcted myocardium and throughout the ventricular wall of the dilated myopathy. These pathological processes parallel the abnormalities in blood supply to the myocardium. In the current study, myocyte apoptosis was the result of double-strand cleavage of the DNA with single base overhangs, which occur only through the activation of DNase I. Systemic and local factors may increase cytosolic Ca and trigger apoptosis. Importantly, end-diastolic pressure is elevated in heart failure, and sarcomere stretching upregulates the myocyte renin-angiotensin system, leading to the formation and release of angiotensin II. Activation of the angiotensin II AT receptor subtype effector pathway may increase intracellular Ca, stimulate DNase I, and ultimately induce myocyte apoptosis. This is consistent with the beneficial effects of inhibition of the systemic and local renin-angiotensin system on heart failure.

Detection of Apoptotic and Necrotic Cell Death
Recently developed molecular probes have allowed the identification of double-strand cleavage of nuclear DNA with staggered or blunt ends. These forms of DNA damage correspond to apoptosis and necrosis, respectively. Additionally, vinculin localization in the plasma membrane permits the unequivocal recognition of membrane rupture, a feature of cell necrosis. The combination of these stainings with confocal microscopy provides the simultaneous visualization of discontinuity of the plasma membrane, morphological changes in chromatin and nuclear structure, and the detection of typical forms of DNA injury. The electrophoretic pattern of DNA complements, on a biochemical level, these histochemical methods. Although these approaches for the measurement of cell death have been emphasized, electron microscopy has been proposed as an alternative method of investigation. The major problem is the difference in sam-

Figure 6. Myocyte apoptosis (TdT) and necrosis (Pfu) in male heart with ischemic cardiomyopathy. A through C indicate, numerically, 3 nuclei. A, only nuclei 1 and 3 are labeled by TdT (red). Note fragmentation in nucleus 1. Nucleus 2 is not stained in A. B, nuclei 2 and 3 are labeled by Pfu (green), whereas nucleus 1 is not stained. Thus, nucleus 1 is affected by apoptosis only and nucleus 2 by necrosis only. Nucleus 3 is undergoing apoptosis (red) and necrosis (green). C represents combination of A and B and shows α-sarcomeric actin staining of myocyte cytoplasm (red). A through C, ×1200.

Figure 7. Effects of heart failure (HF) on myocyte necrosis as measured by Pfu in female (F) and male (M) hearts. Results are mean±SD. C indicates control hearts; *, difference from control value; and **, difference between men and women with heart failure.
planning between electron microscopy and confocal microscopy. In electron microscopy, an average section is 0.2 mm² in area and 70 nm in thickness, whereas in confocal microscopy, an average section is 150 mm² in area and 5 μm in thickness. The latter can be examined by optical sectioning. This is particularly relevant when the magnitude of cell death is, at most, 1%. On this basis, electron microscopy requires an extravagant number of sections and pictures to maintain a sampling error within 10%. Because of this limitation, the values obtained by electron microscopy in this study were not shown statistically (mean±SD).

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