Age and Gender Effects on Cardiomyocyte Apoptosis in the Normal Human Heart

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Background. Animal studies have suggested that apoptosis could play a significant role in the myocardial aging process. Although no information is available in humans, the paradigm that cardiomyocyte apoptosis is increased in the aged human heart has been widely propagated. Moreover, it is unknown whether gender differences may influence cardiomyocyte apoptosis.

Methods. Cardiomyocyte apoptosis was compared between subjects ranging in age from 21 to 93 years (22 men and 19 women), free of any cardiovascular disease, who died of either violent or natural causes. Strict inclusion and exclusion criteria were used to ensure that the selected hearts accurately represented normal aging.

Results. Apoptosis was detected using the TdT-mediated dUTP digoxigenin nick end labeling (TUNEL) technique (controls for TUNEL included negative staining for splicing factor SC-35 and for Ki-67 antigen). The percentage of cardiomyocyte death ranged from 0% to 0.0437%, with no correlation with the age of the subject (p = .85). However, the percentage of apoptosis was threefold higher in men than in women (0.0133% ± 0.0030% vs 0.0042% ± 0.0008%, respectively; p < .01).

Conclusions. Our results in humans do not support the hypothesis that aging influences the percentage of cardiomyocyte apoptosis. However, gender appears to be an important determinant of the occurrence of apoptosis.

There is increasing evidence that myocyte death by apoptosis occurs in the diseased human heart (1–7). Recent animal studies have shown that cardiac myocyte apoptosis occurs soon after birth (8) and may be involved in the age-related loss of cardiac myocytes (9). Although no information is available in humans, the paradigm that cardiac myocyte apoptosis is increased in the aged heart has been proposed in several review articles (10,11). Moreover, it is unknown whether gender differences may influence cardiomyocyte apoptosis in the normal human heart. This study was therefore designed to investigate the occurrence of cardiac myocyte apoptosis in subjects ranging from 21 to 93 years, free of any cardiovascular disease, who died of either violent or natural causes.

Methods

In Paris, all suspicious deaths are investigated at the Institut Médico-Légal de Paris (Institute of Forensic Medicine of Paris). The bodies are stored at 0°C before autopsy while medicolegal investigations are conducted. Only those hearts that were in an excellent state of preservation at autopsy were included in the present study.

Pre-autopsy Inclusion Criteria

We included subjects with no known cardiovascular disease who died outside the hospital of either violent or natural causes. No subject was taking chronic medications.

Autopsy Inclusion Criteria

The hearts were considered normal and were included in the present study if they met the following conditions: (i) the weight was normal (≤400 g in men and <350 g in women), (ii) the valves were normal (no significant calcifications or leaflet thickening), (iii) there was no morphologic evidence of significant coronary artery disease (no major epicardial coronary artery narrowed ≥75% in a cross-sectional area by an atherosclerotic plaque), and (iv) the four cavities had normal wall thickness and normal volume. Histology was performed to detect (and exclude the cases with) significant fibrotic scars or myocarditis.

A mean of 2480 autopsies for suspicious death are performed per year at the Institute, among which 80 autopsies are performed in subjects ≥75 years old. The vast majority of old subjects could not be included because they suffered cardiovascular disease or they were found days after the suspected date of death. Therefore, according to the strict inclusion criteria cited above, we identified 41 normal hearts (i.e., representing the normal aging process). There were 22 men (52.3 ± 4.1 years old) and 19 women (66.0 ± 6.2 years old) ranging from 21 to 93 years of age.

The hearts were fixed in 10% buffered formalin. Several defined myocardial samples (~15 mm × 25 mm) comprising the whole thickness of the myocardium were obtained from the anterior, lateral, and posterior regions of the left ventricle; the septum; and the right ventricle of each heart and were embedded in paraffin. The sampling procedure
was performed rigorously by an expert pathologist (P.F.) according to a pre-defined protocol. This ensured a very good reproducibility of the sampling procedure.

In Situ Detection of Cell Death

In situ detection of cell death was done using the sensitive TdT-mediated dUTP digoxigenin nick end labeling (TUNEL) technique with particular caution in the use of proteinase K and the enzyme terminal deoxynucleotidyl transferase (TdT) to avoid nonspecific staining. We have previously shown that TUNEL positivity is associated with structural modifications that are characteristic of apoptosis (1). Optimal staining was obtained with 20 μg/ml of proteinase K for 5 minutes. The stained nuclei were negative for the proliferation marker Ki-67 and for the splicing factor SC-35 (12), indicating that no DNA or RNA synthesis took place in the nuclei stained by TUNEL. Nuclear staining for TUNEL was abolished after the omission of TdT. Five sections from both ventricles were analyzed. Cardiac myocytes were easily identified as striated cells at high magnification (×400 or more) and by staining with an anti-α- sarcomeric actin antibody. An apoptotic index was calculated as the following ratio: 100 × (the total number of apoptotic myocyte nuclei)/(the total number of myocyte nuclei).

DNA Extraction and Electrophoresis

Myocardial samples from the left ventricle were fixed in 70% ethanol, and DNA extraction was performed according to an established method for the detection of apoptotic death (13). Equal quantities of DNA were loaded into 1.5% agarose gels containing 0.5 μg/ml ethidium bromide. Electrophoresis was then conducted at 80 volts for 2 hours.

Statistical Analysis

Values are expressed as mean ± SEM. Data were compared using a one-way analysis of variance. Simple regression analysis was performed when indicated. A p value of <.05 was considered statistically significant.

RESULTS

The mean ratio of heart weight to body weight correlated significantly with the age of the subject (r = 0.34, p = .05). The mean ratio was 0.57% ± 0.02% in men and 0.52% ± 0.03% in women (p = .26). The mean interval between death and autopsy was 2.37 ± 0.23 days. Time interval between death and autopsy did not correlate with the age of the subject (p = .83) and did not differ between men and women (p = .84). Table 1 details the causes of death.

Detection of Apoptosis

Several myocardial regions were examined, including the anterior, lateral, and posterior regions of the left ventricle, the septum, and the right ventricle. A mean of 81,003 ± 3824 myocyte nuclei were analyzed in each heart. An example of an apoptotic cardiomyocyte nucleus is shown in Figure 1, and the percentage of apoptotic cardiomyocytes is reported for each subject in Table 2. Only 2 subjects (2 men, 53 years old) had markedly elevated apoptotic indexes of about 0.2% and 2%. Interestingly, these 2 subjects experienced severe prolonged hypoxia (severe acute asthma in one and mechanical failure of an oxygen apparatus in the other) and died despite several hours of treatment and attempted resuscitation. In the remaining subjects, the percentage of cardiomyocyte death ranged from 0% to 0.0437% (mean 0.0133 ± 0.0029%) and was homogenous among the different myocardial regions. We found no significant correlation between age and the apoptotic index (Figure 2; p = .43). This remained true when the analysis was performed separately in men (p = .76) or women (p = .33). However, the percentage of apoptotic cardiomyocytes was found to be threefold higher in men than in women (0.0133% ± 0.0030% vs 0.0042% ± 0.0008%, respectively; p < .01). Analysis of individual values (Figure 3) showed that the difference between men and women was essentially due to a subgroup of seven men that had values four- to eightfold higher than the average value in women. The percentage of apoptosis was not related to the death–autopsy interval (p = .85).

DNA was extracted from the left ventricular myocardium of Subjects 8, 10, 12, 16, 24, and 33 (Table 2) and was sub-

![Figure 1](image1.png)

**Figure 1.** Myocardial sections from the heart of an 82-year-old subject (Subject 30). TUNEL (TdT-mediated dUTP digoxigenin nick end labeling) staining was performed as described in the Methods section. A, a normal cardiomyocyte nucleus that counterstained blue by use of hematoxylin and did not stain for TUNEL. B, an apoptotic cardiomyocyte nucleus that stained brown for TUNEL, indicating the presence of fragmented DNA.
jected to electrophoresis in an agarose gel. DNA laddering
was not detected in these samples (data not shown). This re-
sult was not unexpected because the level of apoptotic death
is low, and it is widely recognized that the method lacks
sensitivity for detecting less than 2% of apoptotic cells.

**DISCUSSION**

Our study shows that an increase in apoptosis is unlikely
to be involved in the cardiac aging process in humans. How-
ever, gender differences may influence the level of car-
diomyocyte apoptosis in the normal human heart.

Aging is associated with a substantial increase in both
morbidity and mortality from cardiovascular (and other) dis-
ases, and this may be explained in part by the increased
prevalence of disease with age (14,15). Whether aging per se
(without associated diseases) contributes to specific organ
dysfunction, particularly cardiac dysfunction, is a matter of
intense debate. Several animal studies have demonstrated
that the aged heart exhibits normal cellular and global physi-
ological responses (16–19), and it has been shown that left
ventricular dimensions and heart function at rest are pre-
served with aging in humans (20,21). However, the aged
heart has been shown to adapt poorly to a superimposed me-
chanical load (22–25). Aging has also been shown to be an
independent risk factor for increased mortality and morbidity
after an acute myocardial infarction in humans (26).

Several investigations have been performed to identify
the pathophysiological mechanisms responsible for such a
reduced adaptive capacity of the aged heart (15,27,28).
However, few studies have examined the potential role of
myocyte loss in these age-related alterations (9,29–31). Myo-
cyte loss has been shown to occur in the aged rat heart and
precede the occurrence of ventricular dysfunction, sug-

<table>
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<th>Subject Number</th>
<th>Age (Gender)</th>
<th>Cause of Death</th>
<th>Percentage of CM Apoptosis</th>
<th>Apoptotic Nuclei/Total Nuclei</th>
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</tr>
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</table>

**Figure 2.** The relation between the cardiac apoptotic index (%) and the age of the subject at the time of death. No correlation was observed between age and the apoptotic index ($p = .85$).

**Figure 3.** Individual values of the cardiac apoptotic index (%) in men and women. The mean apoptotic index was found to be three-fold higher in men than in women ($0.0133 \pm 0.0030\%$ vs $0.0042 \pm 0.0008\%$, respectively; $p < .01$). Analysis of individual values showed that the difference between men and women was essentially due to a subgroup of seven men whose values were four- to eightfold higher than the average value in women.
suggested a potential link between the two phenomena (18). Aging in humans is associated with a significant decrease in the absolute number of cardiomyocytes in the male heart, with increased cell volume (hypertrophy) of the remaining myocytes (32,33). Although no decrease in the number of cardiomyocytes was observed in the aged female heart (33), Anversa and Kajstura suggested that this may result from the increased regenerative capacity of myocytes in women rather than from the absence of cell death (11). Interestingly, however, the etiology and mechanisms of myocyte death in the aging human heart were not determined.

Recent studies have demonstrated the presence of apoptotic cardiomyocyte death under various pathological conditions in humans (1–7). Only one study, performed using Fisher rats, reported increased apoptotic cardiomyocyte death with aging (9). The authors concluded that the increased apoptotic myocyte death may be responsible, at least in part, for the age-associated detrimental alterations in the heart (18). On the basis of animal studies (9) and speculations on a possible relationship between senescence and apoptosis (34), it has been proposed in review articles that cardiomyocyte apoptosis could be increased in the aged human heart (10,11). However, no specific study has addressed this issue. In our study, apoptotic cardiomyocyte death was detected in the hearts of subjects from the different age groups, but we observed no differences in the percentage of apoptotic cell death according to age, even when this analysis was performed separately in men and women. This conclusion was derived from the direct examination of an important number of myocyte nuclei in each group of subjects (Table 2). The possibility that a significant increase in apoptotic cell death actually occurs in the aged human heart but was overseen in this study is unlikely for several reasons. First, regional variations in the rate of apoptotic cell death have been shown to occur in animals, with a minor involvement of the septum in comparison with other regions of the heart (9). However, cell loss that occurs with aging in the human heart is not restricted to a particular region of the myocardium (32). Nevertheless, we analyzed different regions of the myocardium, including the septum, and the results were homogenous throughout the heart. Second, although we did not calculate the absolute number of cardiomyocytes, it is unlikely that cell loss did not occur in any of the aged hearts we studied given the reported rate of cardiomyocyte loss that normally occurs with aging in humans (32). Despite this, we detected no increase in the percentage of apoptosis in the aged hearts. Third, Olivetti and colleagues have shown that myocyte loss is not restricted to a particular period in life but follows a relatively constant and linear rate (32), ruling out the possibility that we missed a particular “apoptotic window.” Finally, in agreement with in vitro studies showing that prolonged anoxia induces cardiomyocyte apoptosis (35,36), we detected high levels of cardiomyocyte apoptosis in 2 subjects who experienced prolonged anoxia before death. This suggests that the technique used to detect apoptosis in our study did not lack sensitivity. On the other hand, it is unlikely that high levels of apoptosis were detected in the younger subjects of this study, artifically masking a difference between young and old subjects. Indeed, we used a stringent TUNEL technique for the detection of apoptotic nuclei (12), and the percentages of cardiomyocyte apoptosis reported in our study are very similar to those obtained by other investigators in normal subjects with comparable ages (37).

Our finding that the rate of cardiomyocyte apoptotic death is not increased with aging does not contradict observations of myocyte loss in the aged heart. Indeed, gradual myocyte loss may be the result of continuous cell death (and insufficient regeneration) occurring at a constant rate throughout the life span. Moreover, other forms of cell death, yet unrecognized, may also be present. Finally, the finding of increased apoptotic myocyte death in 2 subjects who died after prolonged anoxia further underscores the necessity of making a strict and careful selection of subjects before attributing any change in the rate of apoptotic myocyte death to aging per se.

Another important finding in this study is the threefold lower percentage of cardiomyocyte apoptosis in women compared with men. Analysis of the individual distribution of apoptosis between men and women (Figure 3) shows that this is due to an increased apoptosis in 7 out of 20 men (35%), whereas the percentage of apoptosis in the remaining 13 men is similar to that in the women. The 7 men with high apoptotic indexes do not differ from the other subjects in terms of age at death, cause of death, interval between death and autopsy, or autopsy findings. This difference in apoptotic levels between men and women may help to explain, at least in part, the more important final loss of cardiac myocytes that is observed in men compared with women (33). Therefore, we believe that gender differences may influence the susceptibility to apoptosis in the human heart.

It could be argued that the mean time interval of 2.37 ± 0.23 days between deaths and autopsies in the present study was rather long. This time is necessary to ensure that only suspicious (unexplained) deaths are included. Moreover, we found no correlation between the time interval and the apoptotic index. It is also a strength of our study to have included only those hearts that represented normal aging. Indeed, in most other studies, hearts are obtained from in-hospital deaths. Such patients are likely to suffer cardiovascular diseases and to take various drugs that may modify myocyte functions or directly influence myocyte apoptosis.

In conclusion, our results in humans do not support the hypothesis that aging influences the percentage of cardiomyocyte apoptosis. However, our findings do not rule out the possibility of an increased susceptibility to apoptotic stimuli with aging under pathological conditions (e.g., ischemia, inflammation, and oxidant stress) as it has recently been reported in Gso transgenic mice, a model of chronically enhanced β-adrenergic signaling (38). In contrast, gender appears to be an important determinant of the occurrence of apoptosis.

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