Senescence and Death of Primitive Cells and Myocytes Lead to Premature Cardiac Aging and Heart Failure

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Abstract—Chronological myocardial aging is viewed as the inevitable effect of time on the functional reserve of the heart. Cardiac failure in elderly patients is commonly interpreted as an idiopathic or secondary myopathy superimposed on the old heart independently from the aging process. Thus, aged diseased hearts were studied to determine whether cell regeneration was disproportionate to the accumulation of old dying cells, leading to cardiac decompensation. Endomyocardial biopsies from 19 old patients with a dilated myopathy were compared with specimens from 7 individuals of similar age and normal ventricular function. Ten patients with idiopathic dilated cardiomyopathy were also analyzed to detect differences with aged diseased hearts. Senescent cells were identified by the expression of the cell cycle inhibitor p16INK4a and cell death by hairpin 1 and 2. Replication of primitive cells and myocytes was assessed by MCM5 labeling, myocyte mitotic index, and telomerase function. Aged diseased hearts had moderate hypertrophy and dilation, accumulation of p16INK4a positive primitive cells and myocytes, and no structural damage. Cell death markedly increased and occurred only in cells expressing p16INK4a that had significant telomeric shortening. Cell multiplication, mitotic index and telomerase increased but did not compensate for cell death or prevented telomeric shortening. Idiopathic dilated cardiomyopathy had severe hypertrophy and dilation, tissue injury, and minimal level of p16INK4a labeling. In conclusion, telomere erosion, cellular senescence, and death characterize aged diseased hearts and the development of cardiac failure in humans. (Circ Res. 2003;93:604-613.)

Key Words: aging ■ telomeric shortening and telomerase activity ■ p16INK4a marker of cellular senescence ■ cardiac primitive cells ■ heart failure

Myocardial aging in humans has been studied extensively and two major conclusions have been reached. Aging effects decrease the functional reserve of the heart and loss of myocytes contributes to the attenuation of the response of the old heart to sudden changes in ventricular loading. However, the selection of patients or hearts to be analyzed has always been based on the chronological age of otherwise healthy individuals. This understandable approach has neglected several variables of the aging process that cannot be easily quantified but may have a significant impact on organ and/or organism aging. It is a well-established fact that chronological age and physical age do not necessarily coincide. An 80-year-old man or woman may appear as young as a 60-year-old person. Similarly, organism and organ age do not proceed at the same pace. In general, there is little appreciation of these unpredictable factors and aged patients with cardiac decompensation are diagnosed according to classified diseases, excluding that aging per se can be the etiology of the pathological condition.

To establish whether aging alone results in a dilated myopathy with characteristics different from idiopathic dilated cardiomyopathy (IDC), myocardial biopsies were obtained from old patients with heart failure that appeared to be age-related. The presence of an established marker of cellular senescence, p16INK4a, together with cell death or telomeric shortening, was measured in myocytes. Senescence and death of progenitor cells expressing the stem cell surface antigen c-kit was also evaluated. Identical information was collected from subjects of similar age with no signs of ventricular dysfunction. Moreover, the colocalization of p16INK4a and myocyte death was determined in a group of old patients affected by IDC. These two forms of controls were studied to identify typical aspects of the aging myopathy. To characterize the growth reserve of the age-dependent myopathy, replication of c-kit–positive (c-kitPOS) cells and myocytes was assessed by the expression of MCM5, a nuclear protein implicated in the cell cycle. The degree of cell multiplication was complemented with the measurement of

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telomerase activity and the detection of telomerase in cycling cells.\textsuperscript{12,13,18} This was done because small, telomerase-competent myocytes, possibly originated from progenitor cells, actively divide in the failing heart.\textsuperscript{12,19–21}

Materials and Methods
An expanded version of Materials and Methods can be found in an online data supplement (available at http://www.circresaha.org).

Results
Aging Myopathy
In this and subsequent sections, several criteria were utilized to distinguish the dilated aging myopathy from IDC. The aged diseased heart had a moderate increase in ventricular mass, and LVPT and IVST were within normal range. Conversely, severe hypertrophy was detected with IDC (Table). Additionally, aged patients were 73 years old, an age unusual for IDC. Ventricular dilation and overt left and right ventricular failure were present in aged diseased hearts and in IDC. The duration of the aging myopathy from the onset of symptoms to biopsy was 20 months. In IDC, the only interval available comprised the period from diagnosis to transplantation, 90 months. In aged diseased hearts, coronary angiography was normal and valvular abnormalities were not present. Hypertension (systolic >140 mm Hg; diastolic >90 mm Hg), ischemic heart disease, diabetes mellitus, systemic disorders, or a history of drug and alcohol abuse were absent. Fidelity for IDC was negative, and we excluded viral infection.\textsuperscript{22} Treatment consisted of angiotensin-converting enzyme inhibitors, diuretics, digitalis, and \(\beta\)-blockers. Thus, the aging myopathy differs from IDC; it develops at an older age and shows modest cardiac hypertrophy.

Volume Composition of the Myocardium and Myocyte Size
The relative proportion of myocytes, replacement and interstitial fibrosis, and other interstitium were comparable in myocardial samples from aged hearts and aged diseased hearts. However, both aspects of cardiac fibrosis were higher in patients with IDC (www.circresaha.org). This increase in collagen accumulation with IDC was associated with a corresponding decrease in the percentage of myocytes in the ventricle. The average myocyte cross sectional area was similar in aged (243±69 \(\mu\)m\(^2\)) and aged diseased (217±75 \(\mu\)m\(^2\)) hearts but was significantly larger (422±72 \(\mu\)m\(^2\)) in IDC. The distribution of myocyte cross sectional areas showed a unique feature in aged diseased hearts; a large fraction of cells was less than 100 \(\mu\)m\(^2\) in size. This subpopulation of myocytes was minimal in aged control hearts and was absent in IDC (see online data supplement). The histological analysis excluded that aged diseased hearts had myocarditis, hemochromatosis, and amyloidosis. Thus, at variance with IDC, scarring and reduction in the myocyte compartment do not occur in the aging myopathy that typically shows a population of small developing myocytes.

Cell Senescence and Death
\(c\text{-kit}\textsuperscript{POS}\) cells were identified in all samples; they were 4 to 6 \(\mu\)m in diameter and were mostly dispersed throughout the myocardium (Figure 1A). Their bone marrow origin was excluded by the absence of CD45 labeling, which is the common leukocyte antigen. These cells were also CD34 negative. Primitive cells are considered to be immortal and to be protected by the effects of age.\textsuperscript{23} However, \(p16\textsuperscript{INK4a}\) was detected in \(c\text{-kit}\textsuperscript{POS}\) cells of control and diseased aged hearts (Figures 1B through 1D), and in hearts from patients with IDC. \(p16\textsuperscript{INK4a}\) is a cyclin-dependent kinase inhibitor;\textsuperscript{7–9} it blocks Cdk4 and Cdk6 maintaining the retinoblastoma protein (RB) in its active hypophosphorylated state.\textsuperscript{7–9} \(p16\textsuperscript{INK4a}\)-positive cells cannot reenter the cell cycle and are kept at the G0-G1 transition. Quantitatively, the numerical density of \(c\text{-kit}\textsuperscript{POS}\) cells per 100 mm\(^2\) of myocardium was comparable in aged diseased hearts (21±6) and in hearts affected by IDC (19±7). These values were significantly higher (\(P<0.001\)) than in aged control hearts (9±2). However, 59% of \(c\text{-kit}\textsuperscript{POS}\) cells were labeled by \(p16\textsuperscript{INK4a}\) in aged diseased hearts, whereas only 14% express the kinase inhibitor in aged control hearts. In the presence of IDC, 17% of \(c\text{-kit}\textsuperscript{POS}\) cells expressed \(p16\textsuperscript{INK4a}\) (Figure 1E). Thus, activation and growth of \(c\text{-kit}\textsuperscript{POS}\) cells occurs in aged diseased hearts and hearts with IDC, but in the...
aging myopathy, c-kit^{POS} cells reach senescence and growth arrest. In the aging myopathy, senescence involved also the myocyte compartment; 48% of these cells were labeled by p16^{INK4a}. In aged control hearts and in IDC, only 16% and 15% of myocytes were p16^{INK4a} positive, respectively (Figure 1F).

Apoptosis and necrosis of c-kit^{POS} cells was detected in 10 of 19 cases of aged diseased hearts and cell death was restricted to senescent cells expressing p16^{INK4a} (Figures 2A through 2D). None of the c-kit^{POS} cells in the 7 aged control hearts was undergoing cell death. This difference was statistically significant by chi-square analysis (P<0.02).

Myocyte apoptosis and necrosis in both groups of aged hearts was detected only in p16^{INK4a} positive cells. The level of myocyte death, however, was significantly higher in aged diseased hearts than in controls (see online data supplement). Aged diseased hearts and IDC had similar levels of myocyte apoptosis, but myocyte necrosis was more extensive with IDC. Importantly, the presence of p16^{INK4a} in myocytes was not a determinant factor for the occurrence of cell death in IDC. Only 16±7% of apoptotic and 13±4% of necrotic myocytes were labeled by p16^{INK4a}, indicating that a larger number of dying myocytes with IDC was not linked to p16^{INK4a} (see online data supplement).

In summary, the aging myopathy is characterized by senescence of a large number of primitive cells and mature myocytes and these old cells are more susceptible to death signals linked to apoptosis. This is not the case in IDC in which myocyte necrosis predominates and myocyte death is mostly independent from the expression of the kinase inhibitor p16^{INK4a}.

**Length of Telomeres in Senescent Myocytes**

Telomeric length was measured by confocal microscopy in aged control and aged diseased hearts (Figures 3A through 3D). Normal aging was associated with a widely spread distribution of telomeric length in myocyte nuclei with an average value of 5.6±1.3 kbp; the longest telomeres were 12.5 kbp. Premature aging was characterized by a shift to the

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**Figure 1.** Senescence of undifferentiated c-kit^{POS} cells and mature myocytes. A, Small primitive c-kit^{POS} cell is shown by the localization of green fluorescence on the cell surface (arrow). Nuclei are stained by the blue fluorescence of PI. Myocytes are identified by the red fluorescence of α-sarcomeric actin antibody staining. c-kit^{POS} cell is negative for α-sarcomeric actin. B through D, c-kit^{POS} cells (green fluorescence; arrows) and myocytes (red fluorescence) show by green fluorescent dots p16^{INK4a} labeling of their nuclei (arrowheads). These sections correspond to aged diseased hearts. Percentage of p16^{INK4a}-labeled c-kit^{POS} cells (E) and myocytes (F). Results are mean±SD. *†Significant difference vs aged control hearts and aged diseased hearts, respectively.
left in the length distribution of telomeres in myocyte nuclei with an average value of 3.4 ± 1.1; the longest telomeres were also 12.5 kbp (Figure 3E). The 39% reduction in average telomere length in aged diseased hearts was statistically significant (P < 0.001). Myocyte nuclei with telomeres equal or shorter than 2.5 kbp were p16INK4a positive in both groups of patients (Figures 3F through 3K). However, a larger fraction of myocytes with shorter telomeres was found in failing aged diseased hearts. These findings and the results of cell death and p16INK4a labeling are consistent with the notion that reduction in telomere length, cellular senescence, and cell death are mutually linked and potentiated by pathological aging.

Replicating Lineage Committed c-kitPOS Cells and Amplifying Myocytes
A marker of cell replication, MCM5, was used to identify cycling c-kitPOS cells committed to the myocyte lineage and amplifying myocytes. The expression of MEF2 alone or in combination with α-sarcomeric actin was utilized to recognize c-kitPOS cells differentiating into myocytes. Amplifying myocytes are young maturing cells that have lost stem cell surface antigens, such as c-kit, but rapidly proliferate and contain in their cytoplasm specific contractile proteins.20,21 Cycling amplifying myocytes were identified by the presence of telomerase, MCM5, and α-sarcomeric actin (Figures 4A through 4I); c-kit was absent in these cells. In aged control hearts, MCM5 labeling of c-kitPOS cells with markers of the myocyte lineage was found in 12 of 28 cells. In aged diseased hearts, 62 of 91 myocyte-committed c-kitPOS cells expressed MCM5. Chi-square test demonstrated that this difference was statistically significant (P < 0.02), indicating that a larger fraction of myocyte-committed c-kitPOS cells was activated in the aging myocardium. The recognition that the stem cell surface antigen c-kit was present in cells expressing MEF2 and α-sarcomeric actin established an unequivocal pathway of differentiation of resident primitive cells to ventricular myocytes. Small amplifying myocytes that lost c-kit were also recognized (Figures 4J through 4L). Cells with these characteristics were detected in both aged hearts.

A quantitative analysis was performed in terms of cardiomyocytes expressing telomerase and MCM5. Pathological cardiac aging was characterized by an increase in the number of these rapidly dividing cells. With respect to aged control hearts, the percentage of replicating telomerase competent myocytes increased 14-fold in aged diseased hearts and this difference was statistically significant (Figure 4M).

The colocalization of telomerase and MCM5 in progenitor cells and amplifying myocytes constituted the morphological counterpart of telomerase activity, which was measured biochemically by the TRAP assay (Figure 5). Aged failing hearts had an enzymatic activity 2-fold higher (OD = 12.6 ± 3.6, n = 7) than age-matched controls (OD = 6.4 ± 2.1, n = 3; P < 0.02). Thus, telomerase function was enhanced in telomerase-competent cells of old diseased hearts, and this change occurred in the presence of an increased number of permanently quiescent p16INK4a labeled c-kitPOS cells and myocytes. Thus, pathological aging led to an increase in cellular heterogeneity of the myocardium.

Mitotic myocytes were frequently encountered in aged diseased hearts (Figures 6A through 6D), providing a further link between primitive c-kitPOS cells and amplifying myocytes. Quantitatively, in comparison with aged control hearts, a 22-fold increase in myocyte mitotic index was found in aged diseased hearts (Figure 6E). Thus, replication and differentiation of myocyte-committed c-kitPOS cells and amplifying myocytes, coupled with telomerase activity, constitute the hallmark of self-renewing organs. However, this highly proliferative cellular compartment does not appear to compensate for the much higher degree of myocyte death and the development of an aging myopathy and cardiac failure.

Discussion
Myocardial Aging
Aging is a stochastic process that involves random variables and manifestations; genes, environment, and proba-
Bilistic changes all contribute to define the lifespan of the organism. In spite of the presence of a systemic clock that regulates the aging of the organism as a whole, organs age at a different pace. The independent lifespan of each cell suggests that a major component of aging may occur at the cellular level. Therefore, the question whether an aging myopathy exists has been addressed at this level. Myocardial aging could depend on attenuation of cell growth with accumulation of old cells. The recognition that the human heart possesses replicative potential suggests that an

![Figure 3](image-url)
imbalance between myocyte growth and death can result in a premature increase in the number of senescent myocytes with depressed contractile performance. These structural and mechanical defects may lead ultimately to the onset of ventricular dysfunction and failure. A similar theory of aging has been applied to other organs.

Consistent with this paradigm, the forced entry of primitive cells into an irreversible quiescent state, coupled with an increased number of senescent myocytes, was identified in the aged diseased heart by the expression of p16\(^{\text{INK4a}}\) and very short telomeres. This led to a deficiency in cell regeneration with respect to cell death. Additionally, apoptosis and necrosis of progenitor cells and myocytes were associated with the expression of p16\(^{\text{INK4a}}\), which was found in cells with a 55% telomeric shortening with respect to the average length of telomeres in aged control hearts. By inference, the activation of the endogenous cell death pathway could have been triggered by telomeric shortening. In old patients with ventricular dysfunction, cell death was found exclusively in cells expressing the kinase inhibitor p16\(^{\text{INK4a}}\). Conversely, patients affected by IDC did not show comparable levels of senescent myocytes and cell death was not strictly connected with the expression of p16\(^{\text{INK4a}}\).

**Aging Myopathy**

The phenotype of the aged diseased heart appears to consist of premature senescence of primitive stem-like cells and differentiated myocytes. These cells die mostly by apoptosis and this provides a plausible explanation for the preservation of the volume composition of the myocardium and the absence of tissue damage in the decompensated old heart. Cell necrosis predominates in IDC and ischemic heart disease, in which profound alterations in the structure of the myocardium have been found in combination with diffuse interstitial fibrosis and multiple foci of replacement fibrosis.

The increase in mass of the aged diseased heart is consistent with activation of myocyte formation mediated by the lineage commitment of primitive cells that resulted in a 22-fold increase in myocyte mitotic index. This process reduces the stem cell pool size and attenuates the regenerative reserve of the aged failing heart. Moreover, the percentage of hypertrophied myocytes, 400 \(\mu\)m\(^2\) in cross-sectional area and larger, is similar in healthy and pathological old hearts. As a consequence, the number of larger cells is necessarily higher in the heavier heart. Together, these factors contributed to expand the muscle compartment of aged diseased hearts.

Nearly 50% of ventricular myocytes in the aged diseased heart are p16\(^{\text{INK4a}}\) positive. This marker of cellular senescence
is typically found in myocytes with extreme degree of hypertrophy, which cannot increase further in size or replicate and large old myocytes contract poorly contributing to ventricular dysfunction. Conversely, newly formed small myocytes have been shown experimentally to possess higher peak shortening, and velocity of shortening and relaxating, positively affecting cardiac performance (authors’ unpublished data, 2003).

**Cell Death, Cell Growth, and the Aging Myopathy**

In human cells, severe telomeric shortening stimulates the endogenous cell death pathway by two mechanisms. Erosion of chromosome ends upregulates p16INK4a and p53 alone or in combination. Loss of telomeric DNA is sensed by the cells as a 3’ overhang DNA damage signal, which in turn, activates the DNA damage recognition pathway, resulting in the recruitment of p16INK4a and p53. The cyclin-dependent kinase inhibitor p16INK4a maintains RB in its hypophosphorylated active state inducing growth arrest. Conversely, growth stimuli phosphorylate and inactivate RB so that the cells can traverse the cell cycle and divide. Death signals also inactivate RB by caspase-mediated cleavage of the protein increasing the proficiency of cells to undergo apoptosis. It has recently been shown that p16INK4a can trigger apoptosis through a mechanism that does not require degradation of RB; p16INK4a decreases the mitochondrial membrane potential favoring the release of cytochrome-c and procaspase-9 in the cytoplasmic compartment. This phenomenon is modulated by upregulation of the proapoptotic proteins Bax and Bim independently from p53. However, the simultaneous downregulation of Bcl-2 seems to be controlled by p16INK4a-induced p53 activation.

Although not investigated in this study, the interaction between p53 and critical telomeric shortening promotes apoptotic myocyte death, inadequate myocyte growth and premature cardiac aging in an animal model with ablation of telomerase. The precociously senescent heart typically shows cavitary dilation, wall thinning, ventricular failure, and decreased lifespan, mimicking the phenotype of the aged diseased human heart. These experimental observations strengthen the notion that aging is a determining factor of heart failure in old individuals with ventricular decompensation of unknown origin.

The growth activation of primitive and early committed cells and maturing myocytes detected in the current and previous studies provides further evidence of the regenerating potential of the heart in humans. The recognition that these cells frequently express the stem cell surface antigen c-kit is consistent with the formation of new myocytes by lineage commitment of undifferentiated cells. Cycling primitive cells and rapidly dividing amplifying myocytes are telomerase competent and express proteins present during the cell cycle. This reverse transcriptase postpones growth arrest, delays aging, interferes with cell death and extends cell lifespan. However, detectable levels of telomerase are not sufficient to block telomeric shortening and aging in the hematopoietic system and, as shown here, in the heart as well. A similar pattern of cell growth and differentiation is operative in other self-renewing organs including the brain, the skin, and the intestine.

Therefore, we propose the existence of an aging myopathy in humans based on the identification of early cellular senescence, growth limitation, and enhanced death of primitive and committed cells (Figure 6F). Life leads to the development of a time-dependent disease state of the heart, which varies among individuals of the same age. Chronological age, physical age, and organ age do not necessarily coincide. However, they remain critical interrelated variables of maximum lifespan. Whether age-induced heart failure is the crucial determinant of the lack of increase of lifespan in the Western world in the last 70 years is an important unanswered question.

**Limitations of the Study and Alternative Interpretation**

The recognition of an aging myopathy is challenging. The controversy lies on whether an aging dilated myopathy can be distinguished from an IDC. The distinction has been made in this study on the basis of severe telomeric shortening and the expression of p16INK4a in myocytes and cardiac stem-like cells. Myocytes with telomeres of 2.5 kbp or shorter are p16INK4apositive and highly susceptible to apoptotic death signals. Critically shortened telomeres, p16INK4a inhibition of cell growth, and proficiency to apoptosis have been interpreted as important variables of the aging myopathy and ventricular dysfunction. This notion does not exclude that other factors remain to be identified for a more precise characterization of cardiac aging.

The parameters indicated above are not commonly present in IDC. Additionally, aged diseased hearts show a preservation of the volume composition of the myocardium that is dictated by apoptotic cell death, lack of an inflammatory reaction and absence of collagen accumulation. Conversely, myocyte necrosis, macrophage, and inflammatory cell infiltration and tissue fibrosis are hallmarks of IDC. If patients with aged diseased hearts would represent late stages of IDC, replacement and interstitial fibrosis would predominate, and cell necrosis and inflammation would be evident and diffuse throughout the myocardium. It is more reasonable to assume that over the years an aging myopathy may acquire aspects of IDC. The possibility that an IDC changes with time its phenotype mimicking an aging myopathy seems rather unlikely. For this to occur the fibrotic myocardium should be dissolved and cell death mechanisms should switch from predominantly necrotic to predominantly apoptotic. This shift in phenotype would not involve, however, telomeric shortening and p16INK4a expression. Of relevance, patients with IDC were younger than patients with aging dilated myopathy. This may appear as a problem but it represents the inevitable consequence of the difference in the time of onset and evolution of the two pathological cardiac conditions. Premature aging may mask an underlying IDC. Although this cannot be excluded, it is difficult to envision how p16INK4apositive myocytes with short telomeres would die by necrosis instead of apoptosis, reversing the process.

A few additional comments concerning our study have to be made because they have implications in the interpretation of the
collected results. These are, in fact, limitations that include the modest magnitude of sampling of the myocardium, the variability in the medical management of the patient population, the duration of the disease with its unclear starting point, and the complexity of obtaining an extensive morphological and immunohistochemical analysis in all hearts. Because of these confounding factors, it is important to suggest that these age-associated changes of the myocardium may interact with pathological states developed as a function of age and together participate in the definition of the clinical phenotype. This safer and more conservative interpretation is consistent with the concept of cardiac aging recently emphasized in three review articles.41–43

Figure 4. Aging and cardiac cell replication. Same field is illustrated in A through C, D through F, and G through I. c-kit\textsuperscript{POS} cells (green fluorescence, arrows; A, D, and G) show in their nuclei, stained by the blue fluorescence of PI, MEF2 (yellow fluorescence; A and D) and MCM5 (white fluorescence; B, E, and G). Telomerase protein in the nucleus of two c-kit\textsuperscript{POS} (arrows) and one c-kit\textsuperscript{NEG} (arrowhead) cells, all three labeled by MCM5, is illustrated by magenta fluorescence dots in H. Expression of α-sarcomeric actin in a c-kit–negative, small, amplifying myocyte (asterisk), and matured cells is depicted by red fluorescence in panels C, F, and I. J and K. Two examples of small c-kit\textsuperscript{POS} cells (green, arrows) expressing MEF2 alone (yellow; J) or MEF2 in combination with a thin layer of cytoplasm (α-sarcomeric actin, red; K). L. Two small developing myocytes negative for c-kit and positive for MEF2 (yellow) and α-sarcomeric actin (red; asterisks). These sections correspond to aged diseased hearts. Values of MCM5 and telomerase labeling in myocytes of aged hearts are documented in M. Results are mean±SD. *Indicates a significant difference vs aged control hearts.
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Legends to Figures

Figure 1: Premature Aging, IDC and Composition of the Myocardium. (A) Volume percent of myocytes (M), replacement fibrosis (RF), interstitial fibrosis (IF) and other interstitial structures (O) in the myocardium. Results are mean±SD. *‡Indicate a significant difference vs aged control hearts and aged diseased hearts, respectively. (B) Distribution of myocyte-cross sectional areas in the myocardium. Buckets are 50 μm². Hatched bars correspond to myocytes less than 100 μm². Aged control hearts, n=308; Aged diseased hearts, n=794; IDC, n=322.

Figure 2: Cellular Senescence and Apoptotic and Necrotic Cell Death. Panel A: Apoptosis and necrosis in myocytes. Panel B: Expression of p16INK4a in myocytes dying by apoptosis and necrosis. Results are mean±SD. *‡Indicate a significant difference vs aged control hearts and aged diseased hearts, respectively.

Materials and Methods

Patients

Seventeen men and 2 women, 73±2 years of age, with dilated myopathy of unknown etiology and heart failure New York Heart Association (NYHA) class III and IV were studied. These 19 patients correspond to the group of aged diseased hearts. Echocardiography, cardiac catheterization, coronary angiography and right ventricular endo-myocardial biopsies were obtained in all patients. The institutional review committee approved the study and patients gave informed consent. Echocardiographic measurements included left ventricular end-systolic (LVESD) and end-diastolic (LVEDD) diameters, interventricular septal (IVST) and LV posterior wall (LVPT) thickness, ejection fraction (EF) and fractional shortening (FS). Hemodynamic determinations consisted of cardiac index (CI), LV end-diastolic pressure (LVEDP) and pulmonary artery systolic pressure (PASP). Seven biopsies from the right ventricular free
wall from age-matched patients, 5 men and 2 women, 76±4 years old, were collected during by-pass surgery for chronic stable angina and isolated left anterior descending coronary artery disease. These 7 samples correspond to the group of normal aged hearts. None of the control patients had myocardial infarction. Left and right ventricular function was normal. An additional group of 10 hearts with IDC, obtained from 8 men and 2 women, 61±4 years old, was studied. These 10 hearts, stored in our archive, correspond to the IDC group.

**Myocardial Sampling**

Biopsies, each nearly 3 mm³ in size, were performed in the septal region of the right ventricle of old patients with heart failure. Three-four samples were obtained in each case for histochemistry and routine histology. The extent of myocardial fibrosis and the volume fraction of myocytes in the tissue were evaluated morphometrically. Myocyte diameter was measured in the region of the nucleus and the cross sectional area was computed¹. Two-three additional samples from each patient were frozen and employed for detection of cardiotropic viruses and telomerase activity assay. Viral genomic RNA or DNA was searched for by RTPCR and PCR. Control biopsies were used for comparison.

**Death of Senescent Cardiac Primitive Cells and Myocytes**

Morphologic studies were all done by confocal microscopy²-⁶. Small, primitive c-kitPOS cells were identified utilizing a c-kit antibody⁵ (Dako, Carpinteria, CA). CD45 and CD34 were tested to identify bone marrow cells (Dako). Myocytes were labeled by α-sarcomeric actin antibody (clone 5C5, Sigma, St Louis, MO), and senescent c-kitPOS cells and myocytes² were detected by p16INK4a antibody (F-12, Santa Cruz, Santa Cruz, CA).

Death of c-kitPOS cells and myocytes was measured by hairpin 1 (apoptosis) and hairpin 2 (necrosis) probes⁶. The concomitant presence of p16INK4a and hairpin probes in c-kitPOS cells and myocytes was evaluated in aged control and aged diseased hearts. For these analyses in c-kitPOS cells, 250 and 585 mm² of tissue were examined in aged control and aged diseased hearts, respectively. Corresponding values for myocytes were 288 and
105 mm$^2$. The colocalization of p16$^{\text{INK4a}}$ and hairpin probes in myocytes of IDC hearts was also assessed; 100 mm$^2$ of myocardium were sampled.

**Length of Telomeres in Myocytes**

Telomere length in myocyte nuclei was evaluated in tissue sections by fluorescence in situ hybridization (Q-FISH) and confocal microscopy. A fluorescein isothiocyanate-peptide nucleic acid probe was used. The fluorescent signals measured in lymphoma cells (L5178Y) with short (7 kbp) and long (48 kbp) telomeres (kindly provided to us by Dr. M.A. Blasco, Department of Oncology and Immunology, Universidad Autonoma, Madrid) were utilized to compute absolute length of telomeres. Nuclei were stained for p16$^{\text{INK4a}}$ to correlate p16$^{\text{INK4a}}$ and telomere length. Individual telomere signals in each nucleus were added and divided by the propidium iodide (PI) signal to correct for differences in the nuclear fraction included in the section. Sampling included 50-100 nuclei in each heart of 9 patients and 6 controls.

**Proliferation of Early Committed c-kit$^\text{POS}$ Cells and Amplifying Myocytes**

The number of cycling c-kit$^\text{POS}$ cells differentiating in myocytes and amplifying myocytes was evaluated by MCM5 (Accurate Chemicals, Westbury, NY) antibody. Differentiation of c-kit$^\text{POS}$ cells in myocytes was evaluated by the expression of the myocyte specific transcription factor MEF2 (Santa Cruz) and α-sarcomeric actin. The colocalization of MCM5 and telomerase protein (H-231, Santa Cruz) in nuclei was also determined. MCM5 and telomerase labeling involved sampling of 234 and 398 mm$^2$ of myocardium in aged control and aged diseased hearts, respectively. The mitotic index was evaluated by analyzing the fraction of mitotic figures in 83,108 myocyte nuclei in control hearts and 114,261 myocyte nuclei in diseased aged hearts.

**Telomeric Repeat Amplification Protocol (TRAP) Assay**

Telomerase activity was measured in myocardial biopsies of 3 aged control and 6 aged diseased hearts. Tissue was homogenized in 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate buffer and centrifuged at 4°C. Samples of 1 and 0.5 μg of untreated
and RNase-treated tissue extracts were incubated with \( \gamma^{32}\text{P}\text{ATP}\text{-end-labeled telomerase substrate oligonucleotide (5'-AATCCGTCGAGCAGTT-3')}, \) Taq polymerase and anchored reverse primer (5'-GCGCGC[CTTACC]CTAACC-5') for 30 minutes. HeLa cells were used as control for the specificity of the bands. The optical density of the 6-bp ladder was measured and normalized for PCR efficiency.

**Statistical Analysis**

Results are mean±SD. Significance, \( P<0.05 \), between two measurements was determined by Student's \( t \) test and among multiple measurements by the Bonferroni's method. In all cases, \( n \) corresponds to the number of hearts included in each comparison. The variability among cells and telomere lengths are illustrated in the distribution curves. Categorical data were analyzed by chi-square test.

**REFERENCES**


Volume Composition of the Myocardium

Control    Diseased    IDC

Aged Hearts

Supplementary
Fig. 1A
Chimenti et al.
### Supplementary

**Fig. 2A, B**

Chimenti et al.