Myocyte Cell Loss and Myocyte Cellular Hyperplasia in the Hypertrophied Aging Rat Heart

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To determine the effects of age on the myocardium, the functional and structural characteristics of the heart were studied in rats at 4, 12, 20, and 29 months of age. Mean arterial pressure, left ventricular pressure and its first derivative (dP/dt), and heart rate were comparable in rat groups up to 20 months. During the interval from 20 to 29 months, elevated left ventricular end-diastolic pressure and decreased dP/dt indicated that a significant impairment of ventricular function occurred with senescence. In the period between 4 and 12 months, a reduction of nearly 19% in the total number of myocytes was measured in both ventricles. In the subsequent ages, similar decreases in myocyte cell number were found in the left ventricle, whereas in the right ventricle, the initial loss was fully reversed by 20 months. Moreover, from 20 to 29 months, a 59% increase in the aggregate number of myocytes occurred in the right ventricular myocardium. In the left ventricle, a 3% increment was also seen, but this small change was not statistically significant. These estimations of myocyte cellular hyperplasia, however, were complicated by the fact that cell loss continued to take place with age. The volume fraction of collagen in the tissue, in fact, progressively increased from 8% and 7% at 4 months to 16% and 22% at 29 months in the left and right ventricles, respectively. In conclusion, myocyte cellular hyperplasia tends to regenerate the ventricular mass being lost with age in the adult mammalian rat heart. (Circulation Research 1990;67:871–885)

Physiological and biochemical investigations have shown that the aging myocardium is characterized by changes in contractile behavior paralleled by a decrease in adenosine 3'-phosphatase activity and an isoenzyme shift from V₅ to V₆. The latter has been found to be accompanied by prolongation of contraction duration and a reduced velocity of shortening and relengthening of the myocardium. Whether the combination of these effects represents a successful adaptation of the heart or an indication of myocardial dysfunction has been a matter of controversy. On the one hand, the mechanical and biochemical alterations may be viewed as processes that would tend to reduce the amount of energy required for ventricular pump function, but on the other hand, a prolonged diastolic and/or systolic phase of the cardiac cycle may result in a prolonged systolic and/or diastolic stress on the myocardium evoking a myocyte cellular hypertrophic response. Moreover, the load on the heart may be further enhanced by the mechanism of myocyte cell loss that accompanies the evolution of life in the rat model. However, measurements of global cardiac hemodynamics have failed so far in providing a demonstration that pump function becomes significantly impaired in aging. The absence of ventricular dysfunction and failure in the aging heart may depend on the fact that critical intervals of postnatal life were not examined or that the reserve capacity of the myocardium is not fully exhausted in senescence, providing the basis for a preservation of contractile performance. Therefore, the present investigation was undertaken to determine the effects of aging on the functional and structural properties of the myocardium in Fischer 344 rats. Because of the differences in load sustained by the left and right sides of the heart throughout life, the quantitative morphological analysis of the myocardium was conducted in both the left and right ventricles. Rats were examined at 4, 12, 20, and 29 months after birth, since these animals are considered to be young adults at 4–6

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months, fully mature adults at 10–12 months, aged at 20 months, and senescent at 27–30 months.

Materials and Methods

Animals

Hearts of male Fischer 344 rats (National Institutes of Health colony, Harlan Sprague-Dawley, Indianapolis) were studied at 4, 12, 20, and 29 months of age. Each group consisted of 10 rats.

Functional Measurements

Before cardiac arrest, the rats were anesthetized with chloral hydrate (300 mg/kg body wt i.p.), and physiological measurements of systemic blood pressure, intraventricular pressures, and dP/dt were obtained. After tracheostomy, the femoral artery was cannulated with a polyethylene catheter, and blood pressure was measured with a P23ID transducer (Gould, Cleveland). The right carotid artery was cannulated with a microtip pressure transducer catheter (Millar, Houston) connected to a Gould ES 2000 chart recorder. The transducer was then advanced into the left ventricle for the evaluation of ventricular pressures and dP/dt. Standard limb lead II of the electrocardiogram was recorded with subcutaneous needle electrodes to determine heart rate.

Fixation Procedure

At the completion of the hemodynamic measurements, the abdomen was opened, and the abdominal aorta was cannulated below the renal arteries with a polyethylene catheter (PE-200) filled with phosphate buffer (pH 7.4) and heparin (100 IU/ml). The catheter was first sealed in place with a ligature and then connected to a perfusion apparatus. In rapid succession, the heart was arrested in diastole by an intravenous injection of 1 ml cadmium chloride (100 mM), the chest was opened, the right atrial appendage was cut, and the coronary vasculature was perfused at a pressure equal to the in vivo measured mean arterial pressure. After perfusion with buffer for 3 minutes, the coronary bed was perfused for 15 minutes with a solution containing 2% paraformaldehyde and 2.5% glutaraldehyde.

Tissue Sampling

After the fixation procedure, the heart and great vessels were excised, and the weights of the left ventricle including that of the septum and the right ventricle were recorded. The corresponding volumes of myocardium of each ventricle were determined by dividing their respective weights by the specific gravity of muscle tissue (1.06 g/ml). Twenty-five specimens extending from the epicardial to the endocardial surface of each ventricle were obtained from each heart. These tissue pieces were postfixed in osmium tetroxide, dehydrated in acetone, and embedded in araldite.

Morphometric Analysis

Ten randomly chosen tissue blocks from each ventricle were sectioned at a thickness of 0.75 µm and stained with toluidine blue. Morphometric sampling at a magnification of ×1,000 consisted of counting the number of myocyte nuclear profiles [N(n)] in a measured area [A] of tissue sections in which cardiac muscle fibers were cut transversely. A square tissue area of 10,036 µm² was delineated in the microscopic field by an ocular reticule containing 42 sampling points (model 105844, Wild Heerbrugg Instruments, Inc., Farmingdale, N.Y.). A total of 25 such fields was evaluated in the endocardial and epicardial regions of each ventricle of each animal to determine the number of nuclear profiles per unit area of myocardium [N(n)A] and the volume fraction of myocytes [V(m)A], collagen, and other interstitium (i.e., capillaries, veins, arteries, fibroblasts, and endothelial cells) in the myocardium of the inner and outer layers of the wall.

Nuclear length [D(n)] was determined in the endocardial and epicardial regions of each ventricle from 50 measurements each made at a magnification of ×1,250 in longitudinally oriented myocytes viewed with a microscope having an ocular micrometer accurate to 0.5 µm. Ten blocks with myofibers sectioned parallel to their length were cut, sections at approximately 2 µm in thickness were collected and stained, and 10 measurements of nuclear length were recorded from each tissue section for each region of the wall.

From the estimation of N(n)A and D(n), the number of myocyte nuclei per unit volume of myocardium [N(n)V] was computed using the following equation:

\[ N(n)\text{V} = N(n)A / D(n) \]

Myocyte cell volume per nucleus in the inner and outer layers of the wall of each ventricle [V(m)A] was obtained from V(m)V divided by N(n)V:

\[ V(m)\text{A} = V(m)\text{V} / N(n)\text{V} \]

The total number of myocyte nuclei in each ventricle [N(n)T] was then derived from the product of N(n)V and the total ventricular volume [V(T)]:

\[ N(n)\text{T} = N(n)\text{V} \times V(T) \]

In this case, N(n)V was evaluated by averaging the values obtained in the inner and outer layers of the wall in each ventricle. A similar approach was used when regional morphometric values of volume fraction of tissue components, numerical density of nuclei, nuclear length, and cell volume per nucleus were expressed as indicators of the entire wall. Finally, initial estimates of nuclear numerical densities per unit area of myocardium and per unit volume of tissue were subsequently recalculated in terms of area and volume of myocytes to minimize differences in the volume composition of the myocardium. With
such an analysis, the myocyte compartment was used as a uniform reference point.

Absolute volumes of tissue components in each ventricle were evaluated from the products of total ventricular myocardial volume and their respective values per unit volume. The theoretical aspects and practical applications of the morphometric procedure briefly summarized above have recently been described in detail.12

Preparation of Isolated Ventricular Myocytes

Myocytes were isolated according to the procedure originally described by Wittenberg and Robinson,13 which was recently modified by Wittenberg et al.14 Fischer 334 rats were heparinized and killed by cervical dislocation. Hearts were excised and placed on a stainless steel cannula for retrograde perfusion through the aorta.

The solutions were supplements of modified commercial MEM Eagle Joklik (DMC317, K.C. Biological, Vesinet, France). HEPES-MEM contained (mM) NaCl 117, KCl 5.7, NaHCO3 4.4, KH2CO3 1.5, MgCl2 1.7, HEPES 21.1, glucose 11.7, and L-glutamine 2, together with amino acids, vitamins, and 21 milliunits/ml (9 × 10−8) insulin. The pH was adjusted to pH 7.2 with NaOH. This solution was 292 mosm, which is isomolar with rat serum, and in the standard procedure contained no added calcium; measured calcium activity was 5 μM. Resuspension medium was HEPES-MEM supplemented with 0.5% bovine serum albumin, 0.3–1.0 mM calcium chloride, and 10 mM taurine adjusted to 292 mosm.

The cell isolation procedure consisted of three main steps:

1) Low calcium perfusion. Blood washout and collagenase (selected type II, Worthington Biochemical Corp., Freehold, N.J.) perfusion of the heart were carried out at 32°C with HEPES-MEM gassed with 85% O2-15% N2. Calcium chloride (0–30 μM) was added to HEPES-MEM.

2) Mechanical tissue dissociation. After removing the heart from the cannula, the left ventricle inclusive of the interventricular septum and the right ventricular free wall were separated and minced. Collagenase-perfused tissue was subsequently shaken in resuspension medium containing creatine, collagenase, and 0.3 mM calcium chloride. Supernatant cell suspensions were washed and resuspended in resuspension medium.

3) Separation of intact cells. Intact cardiac cells were enriched by centrifugation through Percoll (Pharmacia Fine Chemicals, Upsala, Sweden). Approximately 106 cells were suspended in 10 ml isotonic Percoll (final concentrations, 41% in resuspension medium) and centrifuged for 10 minutes at 34g. Intact cells were recovered from the pellet, washed, and then fixed in glutaraldehyde-paraformaldehyde mixture diluted 1:2 in phosphate buffer. Smears were made, and the cells were stained with hematoxylin. The distinction of nuclei in the cells was then counted. The numbers of hearts prepared in this manner at each age interval for the left ventricle were as follows: five at 4 months, six at 12 months, four at 20 months, and eight at 29 months. Corresponding numbers of hearts for the right ventricle were four, four, five, and eight, respectively.

Sampling Size

The magnitude of sampling in this investigation was selected on the basis of previous work performed in our laboratory and the principle of Poisson statistics. The latter can be used as a reasonable guideline for morphometric data collection since it provides a somewhat more conservative estimate of necessary counts than more specific formulations derived for point counts and profile counts. By assuming that biological variability among animals in a given experimental group is approximately 10%, counting errors in each animal should also be limited by the same order of magnitude for the least frequent structure. In the present study, the area of myocardium sampled yielded an average of 355, 208, 220, and 208 nuclear profile counts for each ventricle of rats at 4, 12, 20, and 29 months of age. Corresponding sampling errors for these values are 5.3%, 6.9%, 6.7%, and 6.9%, respectively. In the right ventricle, the numbers of nuclear profiles counted were 408, 270, 271, and 258; these imply sampling errors of 5.0%, 6.1%, 6.1%, and 6.2%. Significantly smaller sampling errors were obtained for volume fraction determinations. Sampling errors for nuclear counts in isolated myocytes were 5.1%, 4.5%, 4.4%, and 4.8% for the left ventricle at 4, 12, 20, and 29 months. Sampling errors for the right ventricle were 4.6%, 3.8%, 5.2% and 4.9%. The nested analysis of variance performed after the code was broken demonstrated that the number of blocks sampled, the number of nuclei measured, and the number of sampling points and profile counts were in excess of what would have been the minimum required for optimum efficiency.

Data Collection and Analysis

All tissue samples were coded, and the code was broken at the end of the experiment. Results are presented as mean±SD computed from the average measurements obtained from each rat. The data regarding the number of cells in the ventricles were computed from the product of the number of nuclei in the ventricles and the distribution of nuclei in the cell populations. Statistical significance for comparisons between two measurements within the wall of each ventricle was determined using the paired Student’s t test. Statistical significance in multiple comparisons among independent groups of data, in which analysis of variance and the F test indicated the presence of significant differences, was determined by the Bonferroni method.20 Values of p<0.05 were considered to be significant.
Results

Physiological Measurements

The changes in heart rate, mean arterial pressure, left ventricular systolic and end-diastolic pressures, and dP/dt in rats at 4, 12, 20, and 29 months after birth are illustrated in Table 1. These physiological parameters were found to remain substantially constant up to 20 months. However, at 29 months, heart rate was decreased with respect to the other age intervals, and the differences were found to be statistically significant (p<0.0001). On the other hand, left ventricular end-diastolic pressure was increased by 3.57, 3.75, and 2.74-fold in comparison with values obtained in rats at 4, 12, and 20 months. These augmentations were all statistically significant (p<0.0001). During the period from 4 to 29 months, left ventricular end-diastolic pressure increased from 6.3±3.0 to 22.5±9.5 mm Hg. Moreover, at the later age, left ventricular dP/dt was substantially depressed. When comparisons with the other rat groups were made, an average decrease in dP/dt of 31% was measured at 29 months (from p<0.01 to p<0.0001). Thus, the marked elevation in left ventricular end-diastolic pressure, in combination with the depression in dP/dt, indicated that severe ventricular dysfunction had developed in rats at 29 months of age.

Cardiac Weights

Table 2 shows the changes in weight of the heart, left ventricle including the interventricular septum, and right ventricle as a function of age. Left ventricular weight increased by 31% (p<0.0001) from 4 to 12 months, remained unchanged from 12 to 20 months, but expanded again by 18% (p<0.0001) from 20 to 29 months. A comparable behavior was noted for the right side of the heart, although the increases in right ventricular mass from 4 to 12 months and from 20 to 29 months were of greater magnitude; the right ventricle was augmented in weight by 46% (p<0.001) and 47% (p<0.0005), respectively. No weight change in the right ventricle occurred from 12 to 20 months. These effects on the left and right ventricles provoked first a 33% (p<0.0001) enlargement of the heart from 4 to 12 months, followed by a second increase of 24% (p<0.0001) from 20 to 29 months. Since body weights in these rats were 305±22, 459±25, 463±27, and 312±35 g at 4, 12, 20, and 29 months, the increases in the ratios of heart weight, left ventricular weight, and right ventricular weight to body weight at 29 months were greater than those measured in terms of absolute heart and ventricular weight gains (data not shown).

Qualitative Observations

Light microscopic analysis of tissue sections revealed the presence of focal areas of interstitial and replacement fibrosis across the ventricular wall. Although scattered lesions of these types were noted at 4 months, they increased markedly with age, mostly at 20 and 29 months (Figure 1). Both ventricles were affected, and the impact of these changes resulted in a substantial accumulation of collagen within the myocardium, which was quantitatively analyzed (see below). Moreover, these foci of replacement and interstitial fibrosis were more numerous in the subendocardial than in the subepicardial region of the ventricular wall at each age interval studied.

Table 1. Changes in the Hemodynamic Characteristics of Fischer 344 Rats as a Function of Age

<table>
<thead>
<tr>
<th>Age (mo)</th>
<th>4</th>
<th>12</th>
<th>20</th>
<th>29</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (beats/min)</td>
<td>410±26</td>
<td>400±23</td>
<td>391±20</td>
<td>340±19*</td>
</tr>
<tr>
<td>MAP (mm Hg)</td>
<td>97±11</td>
<td>98±10</td>
<td>96±10</td>
<td>94±9.5</td>
</tr>
<tr>
<td>LVEDP (mm Hg)</td>
<td>6.3±3.0</td>
<td>6.0±3.1</td>
<td>8.2±3.1</td>
<td>22.5±9.5*</td>
</tr>
<tr>
<td>LVPSP (mm Hg)</td>
<td>135±15</td>
<td>136±12</td>
<td>133±14</td>
<td>126±15</td>
</tr>
<tr>
<td>+dP/dt (mm Hg/sec)</td>
<td>12.1±1.70</td>
<td>12.9±1.90</td>
<td>13.4±2.10</td>
<td>9.2±1.7†</td>
</tr>
</tbody>
</table>

Values are mean±SD. MAP, mean arterial pressure; LVEDP, left ventricular end-diastolic pressure; LVPSP, left ventricular peak systolic pressure; +dP/dt, peak rate of left ventricular pressure development (x10⁶).

*Significantly different (p<0.0001) from those measured at 4, 12, and 20 months of age.
†Significantly different from those measured at 4 (p<0.01), 12 (p<0.001), and 20 (p<0.0001) months of age.

Table 2. Changes in the Gross Cardiac Characteristics of Fischer 344 Rats as a Function of Age

<table>
<thead>
<tr>
<th>Age (mo)</th>
<th>4</th>
<th>12</th>
<th>20</th>
<th>29</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart weight (mg)</td>
<td>749±43</td>
<td>999±50*</td>
<td>957±99*</td>
<td>1,189±53†</td>
</tr>
<tr>
<td>LVW (mg)</td>
<td>613±41</td>
<td>801±60*</td>
<td>761±74*</td>
<td>901±33‡</td>
</tr>
<tr>
<td>RVW (mg)</td>
<td>136±10</td>
<td>198±15‡</td>
<td>196±41‡</td>
<td>288±50§†</td>
</tr>
</tbody>
</table>

Values are mean±SD. LVW, left ventricular weight; RVW, right ventricular weight.

*Significantly different (p<0.0001) from that measured at 4 months of age.
‡Significantly different from those measured at 12 (p<0.001) and 20 (p<0.0001) months of age.
§Significantly different (p<0.005) from that measured at 4 months of age.
FIGURE 1. Semithin sections of araldite-embedded tissue blocks of the left (panels A, B, and C) and right (panels D, E, and F) ventricular myocardium of rats at 4 (panels A and D), 20 (panels B and E), and 29 (panels C and F) months of age. The extent of myocardial injury in the form of interstitial and replacement fibrosis increased qualitatively with age in the subendocardial region of both ventricles. Toluidine blue staining; magnification ×225 (panels A, B, and C) and ×265 (panels D, E, and F).
Volume Composition of the Myocardium

The changes in the volume composition of the left and right ventricular myocardium with age are shown in Figures 2 and 3. The fractional volumes of myocytes and collagen in the epicardium and endocardium of the left ventricle of rats at 4, 12, 20, and 29 months of age are depicted in Figure 2. These separate determinations were subsequently combined to yield average values across the ventricular wall. It was apparent that the myocyte compartment occupied a larger fraction of the tissue in the epicardium than in the endocardium (Figure 2A). Moreover, this difference became significantly greater with age. The volume fraction of myocytes in the endocardium was 3\% \ (p<0.007), 5\% \ (p<0.0005), 6\% \ (p<0.05), and 8\% \ (p<0.009) lower than that measured in the epicardium at 4, 12, 20, and 29 months. It should also be noted that a progressive decrease in the volume percent of myocytes in the tissue occurred with age in the inner and outer regions of the left ventricle. From 4 to 29 months, this tissue component became reduced by 12\% \ (p<0.0001) and 7\% \ (p<0.005) in the endocardium and epicardium, respectively (Figure 2A). The average value in the wall diminished by 9\% \ (p<0.0001), being a combination of the regional changes.

As illustrated in Figure 2B, alterations in the opposite direction of those seen for the myocyte compartment of the myocardium occurred in the collagen volume fraction of the tissue. This quantitative tissue constituent was consistently higher in the endocardium than in the epicardium and markedly increased with age (Figure 2B). At 4 months, collagen comprised 9.1\% and 7.0\% of the inner and outer layers of the wall, whereas corresponding regional values at 29 months were 18.8\% and 13.3\%. These changes reflected a 2.1-fold \ (p<0.0001) and a 1.9-fold \ (p<0.0001) increase throughout the entire period of investigation. The average value across the wall expanded by 2.0-fold \ (p<0.0001) during the same age interval.

An identical analysis of the changes in the volume composition of the myocardium of the right ventricle as a function of age is shown in Figure 3. In a manner similar to that observed in the left ventricle (Figure 2), the volume fraction of myocytes in the right myocardium was consistently higher in the epicardium than in the endocardium (Figure 3A). In contrast to the left ventricle, however, this regional difference did not increase with age, since the reductions in the volume percent of myocytes in the inner and outer layers of the wall were of comparable magnitudes. From 4 to 29 months, this tissue component decreased by 16\% \ (p<0.0001) and 17\% \ (p<0.0001) in the endocardium and epicardium, respectively. These reductions of the myocyte compartment were accompanied by a 2.82-fold \ (p<0.0001) and a 3.58-fold \ (p<0.0001) increase in the collagen concentration within the tissue (Figure 3B). When average values across the wall were
considered, it was found that the volume percent of myocytes decreased by 17% ($p<0.0001$), whereas the relative amount of collagen in the myocardium expanded by 220% ($p<0.0001$).

When the data obtained in the left and right ventricles are compared, additional observations can be made. At 4 months, the right ventricular myocardium was found to contain an average 15% ($p=NS$) less collagen than left myocardium. In contrast, at 29 months, the right myocardium showed a 36% ($p<0.002$) greater concentration of collagen than the left myocardium.

**Number of Myocyte Nuclei in the Ventricles**

The primary measurements that were used for the determination of the number of myocyte nuclei in the myocardium of the left and right ventricles are illustrated in Figures 4 and 5. The numerical densities of myocyte nuclei are expressed per unit area or unit volume of myocytes instead of per unit area or volume of myocardium. As indicated in "Materials and Methods," the myocyte compartment of the tissue was used as a reference point for these estimations to minimize the effects produced by the changes in the volume composition of the myocardium in the layers of the ventricular wall as a function of age (Figures 2 and 3).

The number of myocyte nuclear profiles per square millimeter of myocytes in the left ventricle was similar in the endocardial and epicardial regions of the wall throughout (Figure 4A). During the interval from 4 to 29 months, however, this parameter decreased by 32% ($p<0.0001$) in the endocardium and by 33% ($p<0.0001$) in the epicardium, resulting in an average reduction across the wall of 32.5% ($p<0.001$). Nuclear length values did not show consistent regional differences across the wall (Figure 4B). With aging, nuclear length became longer, and this alteration was apparent at the two later age periods. From 4 to 29 months, the longitudinal diameter of myocyte nuclei increased by 17% ($p<0.0001$), 11% ($p<0.0001$), and 14% ($p<0.0001$) in endocardial myocytes, epicardial myocytes, and across the wall, respectively.

The changes in the number of myocyte nuclei per unit area of myocytes and in nuclear length with age...
provoked a decline in the numerical density of myocyte nuclei per unit volume of myocytes (Figure 4C). During the entire period of investigation, this quantity decreased by 42% \((p<0.0001)\) and 40% \((p<0.0001)\) in the inner and outer layers of the ventricle and by 41% \((p<0.0001)\) across the wall. It should be pointed out, however, that these reductions occurred mostly from 4 to 12 months, since at this time a 38% \((p<0.0001)\), 41% \((p<0.0001)\), and 40% \((p<0.0001)\) decrease in the numerical density of myocyte nuclei per cubic millimeter of myocytes was measured in the endocardium, epicardium, and the entire wall, respectively.

Figure 5 illustrates the changes in the numerical density (Figure 5A) and length (Figure 5B) of myocyte nuclei in the right ventricle. In contrast to the observations made in the left ventricle, regional differences were found at all age intervals. The number of myocyte nuclei per square millimeter of myocytes was 20% \((p<0.003)\), 28% \((p<0.0001)\), 32% \((p<0.005)\), and 22% \((p<0.007)\) greater in the endocardium than in the epicardium at 4, 12, 20, and 29 months, respectively. Moreover, from 4 to 12 months, this parameter decreased by 30% \((p<0.0001)\) in the endocardium, 35% \((p<0.0001)\) in the epicardium, and 32% \((p<0.0001)\) in the entire wall. However, from 12 to 29 months, these reductions were followed by corresponding increases of 26% \((p<0.001)\), 33% \((p<0.02)\), and 29% \((p<0.0001)\). Because of these two opposite effects, values at 29 months were not statistically significantly different from those at 4 months. Average nuclear length was found to be increased consistently throughout the wall from 4 to 12 months, but this nuclear property did not change thereafter.

Figure 5 illustrates last the results obtained with respect to the number of myocyte nuclei per cubic millimeter of aggregate myocyte volume (Figure 5C). As seen on a per unit area basis, consistent differences were present between the inner and outer layers of the wall. Values in the endocardium were greater than those in the epicardium by 18% \((p<0.01)\) at 4 months, 28% \((p<0.0001)\) at 12 months, 32% \((p<0.007)\) at 20 months, and 21% \((p<0.008)\) at 29 months.

Two relevant changes were seen with aging. The numerical density of myocyte nuclei per unit volume of myocytes decreased from 4 to 12 months but subsequently increased from 12 to 29 months. The decreases consisted of 37% \((p<0.0001)\) in the endocardium, 42% \((p<0.0001)\) in the epicardium, and 39% \((p<0.0001)\) in the whole wall. Magnitudes of corresponding increases were 29% \((p<0.001)\), 35% \((p<0.05)\), and 32% \((p<0.0005)\).

Figure 6 illustrates the effects of age on the aggregate number of myocyte nuclei in the ventricles. In comparison with rats at 4 months, the total number of myocyte nuclei in the left ventricle (Figure 6A) decreased by 20% \((p<0.001)\) at 12 months, 26% \((p<0.0005)\) at 20 months, and 20% \((p<0.001)\) at 29 months. An 8% increase was noted from 20 to 29 months, but this change was not statistically significant.

In the right ventricle (Figure 6B), a 21% \((p<0.05)\) loss of myocyte nuclei was measured during the interval from 4 to 12 months. However, from 12 to 29 months, an 80% \((p<0.0001)\) increase in the total number of nuclei was detected. In comparison with
the values at 4 and 20 months, the 29-month-old right ventricle contained 42% (p<0.0001) and 62% (p<0.0001) more myocyte nuclei.

**Myocyte Cell Volume**

Figures 7 and 8 show the measurements of myocyte cell volume per nucleus obtained in the left and right ventricles and their changes as a function of age. In the left ventricle, this cellular parameter was not different in the endocardial and epicardial regions of the wall at any of the age periods examined (Figure 7). However, myocyte cell volume per nucleus increased by 57% (p<0.0001) and 64% (p<0.0001) in the endocardium and epicardium during the interval from 4 to 12 months. The subsequent changes in myocyte cell volume per nucleus as a function of age did not produce any further increase in cell size, although values at 20 and 29 months were significantly greater than those seen at 4 months.

In the right ventricle (Figure 8), myocyte cell volume per nucleus was consistently greater in the epicardium than in the endocardium throughout. From 4 to 12 months, myocyte cell volume per nucleus was augmented by 58% (p<0.0001) and 72% (p<0.0001) in the endocardium and epicardium, respectively. However, a progressive decrease in this cellular parameter was observed with age. From 12 to 20 months, myocyte cell volume per nucleus was reduced by 15% (p<0.01) and 7% (p=NS) in the inner and outer layers of the ventricle, resulting in an overall reduction of 13% (p<0.02) across the wall. When the data at 12 months were compared with those at 29 months, reductions of 22% (p<0.0001), 25% (p<0.02), and 25% (p<0.0001) were obtained in endocardial myocytes, epicardial myocytes, and the cells assumed to be representative of the entire wall, respectively.

Figure 9 shows the changes in the absolute amounts of myocytes, collagen, and other interstitium in the entire left (Figure 9A) and right (Figure 9B) ventricles with age. It can be seen that in the left ventricle the aggregate myocyte volume expanded by 31% (p<0.0001) from 4 to 12 months but that it decreased by 13% (p<0.005) from 12 to 20 months. Myocyte volume increased again by 17% (p<0.0005) during the last age interval from 20 to 29 months. Throughout the entire period of study, this component was augmented by 34% (p<0.0001). In contrast, the volume of collagen increased consistently with age resulting in an increment of 2.98-fold (p<0.0001) from 4 to 29 months. The remaining portion of the interstitium also increased steadily with age. In the right ventricle, from 4 to 29 months, the expansion of the myocyte compartment (1.75-fold; p<0.0001) was markedly less than that of collagen (6.82-fold; p<0.0001).

**Myocyte Cell Number**

Table 3 shows first the number of myocyte nuclei counted in cells isolated from the left ventricular myocardium of rats at 4, 12, 20, and 29 months of age. The analysis of the distribution of nuclei in the different populations of myocytes indicated that the
fraction of nuclei associated with mononucleated cells increased by almost fourfold \((p<0.0001)\) from 4 to 20 months but that it decreased by 43% \((p<0.01)\) from 20 to 29 months. In contrast, the percentage of nuclei associated with binucleated myocytes decreased by 8% \((p<0.0001)\) from 4 to 20 months, and it increased by 3% \((p<0.05)\) from 20 to 29 months. In a similar fashion, the percentage of mononucleated myocytes was augmented by nearly 3.7-fold \((p<0.0001)\) from 4 to 20 months, subsequently diminishing by 41% \((p<0.001)\) in the 20–29 month interval. Binucleated cells, however, first decreased by 14% \((p<0.0001)\) and the increased by 8% \((p<0.005)\) during the same age periods. At 20 months, a small percentage of nuclei \((0.33\pm0.65\%)\) was found to be associated with trinucleated cells, accounting for 0.2±0.4% of the myocyte population. At 29 months, the percentage of nuclei associated with trinucleated cells \((1.59\pm0.44\%)\) and tetrinucleated cells \((0.42\pm0.28\%)\) accounted for 1.0±0.27% and 0.21±0.14% of myocytes, respectively.

The knowledge of the distribution of nuclei in myocytes in combination with the morphometric estimation of the total number of myocyte nuclei in the left ventricular myocardiun allows the computation of the aggregate number of mononucleated and binucleated cells in the ventricle as a function of age (Figure 10). Mononucleated cells increased by 184% \((p<0.0001)\) from 4 to 20 months. However, a 38% \((p<0.001)\) reduction in this quantity occurred from 20 to 29 months. The changes in the binucleated cell population followed a pattern opposite that observed in terms of mononucleated myocytes. Binucleated cells decreased by 21% \((p<0.001)\) from 4 to 12 months and 32% \((p<0.0001)\) from 4 to 20 months but increased by 11% from 20 to 29 months. This latter change, however, was not statistically significant. In addition, at 20 and 29 months of age, small quantities of trinucleated and tetrinucleated cells were present. When the cell populations were added to yield the absolute number of myocytes in the left ventricle, it could be seen that myocyte loss occurred from 4 to 12 months, whereas in the subsequent age intervals, the increases in mononucleated cells first and binucleated cells later were capable of maintaining the total number of cells in the ventricle nearly constant. The aggregate numbers of trinucleated cells at 20 and 29 months were \(0.11\pm0.71\times10^6\) and \(0.59\pm0.051\times10^6\). Tetrinucleated cells at 29 months were \(0.16\pm0.024\times10^6\) cells.

Table 4 illustrates that aging of the right ventricle was associated with a 2.13-fold \((p<0.05)\) increase in the fraction of mononucleated cells from 4 to 20 months, which was followed by a 12% decrease from 20 to 29 months. However, this diminution was not statistically significant. The percentage of binucleated myocytes was augmented by nearly fourfold \((p<0.0001)\) from 4 to 20 months, subsequently decreasing by 41% \((p<0.001)\) in the 20–29 month interval. In the 29-30 month interval, the percentage of nuclei associated with trinucleated cells \((1.59\pm0.44\%)\) and tetrinucleated cells \((0.42\pm0.28\%)\) accounted for 1.0±0.27% and 0.21±0.14% of myocytes, respectively.
Nuclei associated with trinucleated cells was seen to decrease by 7% (p<0.05) from 4 to 20 months, remaining constant from 20 to 29 months. At 20 months, the percentage of nuclei associated with trinucleated cells was 0.2±0.45%, which accounted for 0.12±0.27% of myocytes. At 29 months, the percentage of nuclei associated with trinucleated and tetranucleated cells was 1.38±0.71% and 0.73±0.50%, which accounted for 0.87±0.45% and 0.34±0.23% of myocytes.

The computation of the aggregate number of mononucleated cells in the ventricle revealed that this cell population consistently increased with age, leading to an overall 2.65-fold (p<0.0001) increase from 4 to 29 months (Figure 11). Binucleated cells first decreased by 24% (p<0.01) from 4 to 12 months but subsequently increased during the other two time periods. From 12 to 29 months, binucleated cells were augmented by 77% (p<0.0001), so that the right ventricular myocardium at this age possessed 35% (p<0.0001) and 60% (p<0.0001) more binucleated myocytes than that at 4 and 20 months, respectively. Hyperplasia of mononucleated and binucleated cells as a function of age resulted in an overall 45% (p<0.0001) increase in the total number of cells in the right ventricle from 4 to 29 months of age (Figure 11). The number of trinucleated cells at 20 and 29 months was 0.020±0.014×10⁶ and 0.228±0.0369×10⁶. Tetranucleated cells at 29 months were 0.121±0.0191×10⁶.

**Discussion**

**Ventricular Function and Aging**

The results of the present study demonstrate that the aging process induces a significant impairment of ventricular hemodynamics that becomes apparent only during the late stages of life in the rat model. On the basis of the current observations, no changes were seen up to 20 months after birth in terms of heart rate, mean arterial and ventricular pressures, and dP/dt. On the other hand, in senescent rats at 29 months, alterations occurred in left ventricular end-diastolic pressure and dP/dt, indicating that significant deterioration of left ventricular pump function had developed during the interval from 20 to 29 months of age. This is in agreement with recent observations.

Although the findings obtained in our study provide experimental evidence of severe global myocardial dysfunction and failure with age in rats, a decrease in the intrinsic mechanical adaptive capacity of the aging heart has previously been shown in this animal model. Furthermore, alterations in the biochemical and electrophysiological properties of the myocardium combined with a reduced ability to respond to a prolonged systolic and diastolic...
overload have been documented. Similarly, coronary blood flow hemodynamics and coronary vascular resistance become impaired as a function of age,\textsuperscript{24} but differences have been found between strains of rats.\textsuperscript{25–27} It should be pointed out, however, that these modifications in muscle contractile behavior, biochemistry, and coronary flow have been found to develop much earlier in life than those detected here with respect to left ventricular performance. Thus, it may be suggested that these tissue properties continue to deteriorate with the progression of life, leading first to a depressed cardiac functional reserve and subsequently to overt failure and death. It should be emphasized, however, that collagen accumulation as a function of age may result in changes in the compliance characteristics of the myocardium which, in turn, may lead to diastolic and ultimately systolic dysfunction and failure.\textsuperscript{28}

### Myocyte Loss and Aging

The data in this investigation demonstrate that a significant loss of myocytes occurred with age in the left and right ventricular myocardium. Such a phenomenon was of almost identical magnitude in the two ventricles, but it persisted longer in the left than in the right side of the heart. In both ventricles, it became apparent during the period from 4 to 12 months, when a 19% reduction in the total number of myocytes was measured. In the subsequent age intervals, similar decreases in myocytes were found in the left ventricle, whereas in the right ventricle, the initial loss was almost fully reversed by 20 months. Although the proportion between mononucleated and binucleated myocytes has been reported to remain constant in the adult rat heart,\textsuperscript{29–31} the present findings in Fischer 344 rats indicate that the phenomenon of cell loss affects exclusively binucleated cells so that a change in the relative percentage of these two myocyte populations occurs with aging. Thus, muscle cell loss participates in the aging process of the heart, and this detrimental effect precedes the occurrence of ventricular dysfunction. Similar results, restricted to the left ventricle, have previously been shown in Sprague-Dawley rats,\textsuperscript{9} and these observations provide supportive evidence for the possibility that a comparable phenomenon may occur in the human heart as well.\textsuperscript{32}

The mechanisms responsible for the loss of myocytes with areas of interstitial and replacement fibrosis in the myocardium are at present unknown. However, local ischemia is a likely possibility since coronary vascular reserve and coronary vascular resistance are both compromised in aging Fischer 344 rats.\textsuperscript{24} Moreover, these alterations have been observed to occur at the identical age interval during which myocyte loss begins to take place in this animal model.\textsuperscript{24} On a structural basis, capillary density is reduced in the aging myocardium.\textsuperscript{33–35} The decreased concentration of capillaries produces a reduction in the endothelial surface accessible for oxygen exchange in the tissue and a greater average diffusion distance for oxygen transport to the myocytes. These capillary characteristics in association with the impairment in coronary blood flow hemodynamics may constitute the principal factors implicated in cell loss and collagen accumulation in the ventricles with aging. It should be recognized, however, that this is a complex problem and that there is no proof for ischemia as the causative event in the occurrence of myocyte loss.

### Myocyte Cellular Hyperplasia and Aging

The current results demonstrate that, after an initial 19% loss of muscle cells in the left and right ventricles from 4 to 12 months, no further reduction in cell number occurred from 12 to 20 months. The overall number of myocytes in the ventricles was preserved at this time through an increase in the aggregate number of mononucleated cells, which was capable of compensating for the loss of binucleated cells with aging. During the period from 4 to 20 months, the 32% decrease in binucleated cells in the left ventricle was accompanied by a 2.84-fold increase in mononucleated myocytes. In the right ventricle, the 24% loss of binucleated cells from 4 to 12 months was associated with a 68% increase in mononucleated cells. In the subsequent interval, a modest increase in both populations of cells was also measured in the right myocardium. However, the increases in mononucleated cells in both ventricles do not necessarily imply myocyte cellular hyperplasia via nuclear mitotic division. Mononucleated cells may rise from ordinary mitosis or the division of binucleated cells. The later possibility has to be considered since the total number of myocyte nuclei in the ventricles at 20 months was not significantly increased with respect to the preceding age period. A similar limitation exists in the interpretation of the data collected in the left ventricle at 29 months, in which the increase in binucleated cells was paralleled by a decrease in mononucleated cells, suggesting that fusion of mononucleated cells may have occurred during senescence.

Although the results in the left ventricular myocardium up to 29 months and in the right myocardium up to 20 months do not demonstrate with certainty that myocyte cellular hyperplasia, mediated by nuclear mitotic division, may occur with aging, the data in the right ventricle at 29 months strongly support the concept that such a phenomenon may take place late in life in the aged, senescent myocardium. The total number of myocytes in the 29-month-old right ventricle was 45%, 77%, and 58% greater than that at 4, 12, and 20 months, respectively. The number of mononucleated cells was 16%, 59%, and 42% higher than that of the preceding time intervals. Binucleated cells were also 35%, 77%, and 60% more numerous than at 4, 12, and 20 months. Finally, the aggregate number of myocyte nuclei in the ventricle was increased by 42%, 80%, and 62% with respect to the values at 4, 12, and 20 months.
Although these data appear to indicate that myocyte cellular hyperplasia was restricted to the right myocardium, the magnitudes of cellular proliferation stated above can be considered to be only minimal indexes of the actual extent of this cellular growth mechanism in the ventricles. The simultaneous presence of myocyte loss complicates the estimation of the real amount of newly formed cells in the myocardium by any methodological procedure currently available. The process may initiate much earlier in life and affect both ventricles, but these events may be obscured by the concurrence of cell death.\textsuperscript{6,36–38} The observation that the volume fraction of collagen in the myocardium continues to increase with age in the left and right ventricles and the fact that these changes are paralleled by an augmentation in the number of foci of replacement fibrosis across the wall strongly support the concept that myocyte cell death accompanies the progression of the aging process of the heart. Thus, myocyte loss results in an underestimation of myocyte cellular hyperplasia in the tissue, whereas myocyte hyperplasia leads to an underestimation of the magnitude of myocyte death in the myocardium.

It is well established that, once myocyte proliferation ceases by the age of weaning in the rat, both physiological and induced postnatal myocardial growth occur principally through hypertrophy of myocytes.\textsuperscript{39} DNA synthesis in cardiac muscle cells has been considered to come to an end between 2 and 6 weeks after birth in the mammalian heart.\textsuperscript{39,40} In contrast, a hyperplastic component has been observed in cardiac enlargement produced in neonatal animals by nutritional anemia\textsuperscript{41} or after aortic banding in rats early after birth.\textsuperscript{40} More recently, evidence has been accumulated that adult atrial\textsuperscript{42} and ventricular\textsuperscript{43,44} myocytes can be stimulated and that nuclear hyperplasia can be induced in cardiac muscle cells. Moreover, in vitro studies clearly demonstrate that DNA synthesis can be evoked in adult atrial and ventricular myocytes,\textsuperscript{45,46} pointing to the possibility that cardiomyocytes may retain their capacity to proliferate throughout life. Such a potential reserve mechanism has been suggested to be operative in the human heart.\textsuperscript{47} Moreover, growing information supports the view that nuclear hyperplasia reflects a comparable magnitude of cellular hyperplasia.\textsuperscript{43,48} In addition, myocyte cellular hyperplasia can be elicited in vitro by different growth factors and tissue-type plasminogen activator,\textsuperscript{46} strengthening the concept that cellular proliferation may be induced in the adult heart in vivo. This cellular process may be present in the senescent myocardium in the attempt to maintain pump function in a failing heart, and it may constitute the ultimate response of the myocardium before intractable ventricular dysfunction and death supervene.

The methodological approach employed here for the analysis of the changes in cell size and number as a function of age has been able to document that myocyte hypertrophy and hyperplasia occur in the aging myocardium. Although light microscopic autoradiography of tritiated thymidine–labeled tissue would have been helpful in confirming DNA synthesis in myocyte nuclei, limitations of this methodology include the inability to demonstrate nuclear division and/or cytokinesis. Moreover, it is impossible by this technique to distinguish whether DNA synthesis is due to nuclear hyperplasia or ploidy formation.

**Cardiac Hypertrophy and Aging**

It is a general belief that, as the heart reaches senescence, it undergoes a modest degree of hypertrophy.\textsuperscript{1} However, based on the current results, heart weight, as well as the weights of its major subdivisions, did not consistently increase with age, a pattern previously observed in Fischer 344 rats.\textsuperscript{2,6} Moreover, the changes in the volume composition of the myocardium with the progression of life clearly indicate that measurements of organ hypertrophy by weight changes are a crude, often misleading, oversimplification of the growth mechanisms at the tissue level of organization. In this regard, during the interval from 12 to 20 months, no change occurred in the weight of the left ventricle, whereas the aggregate volume of the myocyte mass was found to be reduced by 13%, suggesting atrophy of this compartment of the myocardium more than hypertrophy. The same applies to the ventricular hypertrophic response observed from 20 to 29 months in which the accumulation of myocyte volume (17%) was much less than that of collagen (69%). A similar disproportionate growth adaptation was also seen in the right ventricle, since myocyte volume expanded by 39% and collagen expanded by 107%.

Changes in myocyte cross-sectional area,\textsuperscript{35} mean myocyte cell volume per nucleus,\textsuperscript{8} and the average volume of collagenase dissociated myocytes\textsuperscript{29} have previously been used to characterize aging-induced cardiac hypertrophy at the cellular level of organization. The findings in the present study, however, demonstrate that myocyte cellular hyperplasia participates in the hypertrophic response of the myocyte compartment of the myocardium during senescence. Thus, measurements of myocyte cell volume alone may result in a significant underestimation of the extent of myocyte growth occurring with age. A similar limitation has been observed experimentally in long-term pressure-overload hypertrophy\textsuperscript{3,44,46} and in humans with markedly enlarged hearts.\textsuperscript{47} The mechanisms responsible for the processes of myocyte hypertrophy and hyperplasia as a function of age are difficult to identify. However, the continued loss of myocytes in the ventricles can be expected to generate a greater workload on the remaining myocytes, which may serve as a chronic mechanical stimulus for cellular growth.\textsuperscript{8,36,37} Such a condition of cellular overloading has been claimed to be operative in experimental cardiomyopathies\textsuperscript{49,50} or after myocardial infarction.\textsuperscript{37} Evidence in the current study that the greater expansion of the myocyte compartment of...
the myocardium occurred in the presence of left ventricular failure further supports this possibility.

**Left and Right Ventricles and Aging**

Results here demonstrate that aging effects are quantitatively different in the two ventricles. Although the extent of myocyte loss was comparable in the left and right ventricles, the magnitude of myocyte cellular hyperplastic response was greater in the right than in the left side of the heart. Moreover, in spite of the fact that collagen accumulated to a larger extent in the right myocardium, a deficit in myocyte mass was never observed, contrasting the finding in the left ventricle at 20 months. During the 25-month interval examined, the myocyte compartment increased by 1.75-fold in the right ventricle, and this increment was 2.2-fold greater than that of the myocyte component of the left ventricle. Thus, the right myocardium possesses a growth reserve that markedly exceeds that of the left myocardium. Whether these differences are dependent upon the greater pressure load sustained by the left ventricle throughout life, which may exhaust the compensatory capacity of the tissue at an earlier age, cannot be established at present. However, the current observations, in agreement with previous reports,8 tend to support the concept that the detrimental effects of aging appear first on the left side of the heart, leading to the occurrence of left side dysfunction and failure.

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