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Increased Apoptosis and Myocyte Enlargement with Decreased Cardiac Mass; Distinctive Features of the Aging Male, but not Female, Monkey Heart

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Abstract

We studied gender-specific changes in aging cardiomyopathy in a primate model, *Macaca fascicularis*, free of the major human diseases, complicating the interpretation of data specific to aging in humans. Left ventricular (LV) weight/body weight decreased, $p < 0.05$, in old males, but did not change in old females. However, despite the decrease in LV weight, mean myocyte cross-sectional area in the old males increased by 51%. This increase in myocyte size was not uniform in old males, i.e., it was manifest in only 20–30% of all the myocytes from old males. In old males there was a 4-fold increase in frequency of myocyte apoptosis without any increase in proliferation-capable myocytes assessed by Ki-67 expression. Apoptosis was unchanged in old female monkey hearts, whereas the frequency of myocytes expressing Ki-67 declined 90%. These results, opposite to findings from rodent studies, indicate distinct differences in which male and female monkeys maintain functional heart mass during aging. The old male hearts demonstrated increased apoptosis, which more than offset the myocyte hypertrophy, which interestingly was not uniform, without a significant increase in myocyte proliferation.

Keywords

Aging; Hypertrophy; Apoptosis; Remodeling; Gender

INTRODUCTION

Diminished cardiac function with aging has been demonstrated in rodent models [1–4], but is less clear in humans. A major confounding issue is that diseases in humans that are known to damage the cardiovascular system, e.g., atherosclerosis, hypertension and diabetes, are generally factors that may underlie intrinsic aging cardiomyopathy that contribute to heart

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failure. Animal models do not have this limitation, however, the majority of concepts on aging cardiomyopathy have been derived from studies in rodents [1–4], which have different limitations. In addition to species differences, a major concern is the relatively short life span of rodents. Less is known about gender differences in the aging heart.

In order to investigate gender differences in the intrinsic cardiomyopathy of aging in an animal phylogenetically close to humans, and with a considerably longer life span than rodents, we have undertaken a series of studies in a monkey, the crab eating or long tailed macaque (*Macaca fascicularis*), assessing changes in the myocardium associated with aging [5,6]. Our choice of monkeys for the study of the aging heart was based on three considerations: 1) animals proven free of atherosclerosis, hypertension and diabetes permit the study of any intrinsic changes in hearts free of the consequences of these diseases, 2) the events in monkeys are likely to more closely resemble those in humans given their greater than 90% genetic homology with humans [7] than do the events in rats or other non-primates, and 3) these monkeys live to 25–30 years, roughly 10-fold more than rodents [8].

MATERIALS AND METHODS

The young monkeys were born in captivity at the Simian Conservation Breeding and Research Center in Manila, Philippines. The old monkeys were feral animals captured between the ages of 5 and 7 years and kept at the Center thereafter. The ages of the feral monkeys were estimated at the time of capture based on the eruption of dentition, general appearance, assessment of sexual development, and body weight. Monkeys were fed on a standard primate diet containing 5% to 6% fat, 18% to 25% protein, and 0.2% to 0.3% sodium chloride as previously described [5] and otherwise maintained in accordance with the Guide for the Care and Use of Laboratory Animals [Department of Health and Human Services publication (National Institutes of Health) No. 83–23, revised 1996]. All procedures were carried out according to protocols approved by the Animal Care Committees in Manila and New Jersey Medical School. All monkeys had normal laboratory values for fasting plasma glucose, cholesterol and triglycerides [5]. The old females were either pre-menopausal (still cycling) or peri-menopausal (irregular cycles), which is consistent with prior reports in this species [9–11]. Two young female monkeys and one old male were excluded from the study based on histologic evidence of marked myocardial fibrosis. Myocardial histology in the remainder of the animals used for morphologic studies was normal.

Monkeys were weighed immediately prior to sacrifice, sedated with ketamine (5–10 mg/kg, IM), and euthanized with an overdose of sodium pentobarbital (25–40 mg/kg, IV). The hearts were removed immediately, and heart weights and left ventricle weights (free wall and septum) were obtained. Cross-sections from the mid left ventricle free wall were obtained for histology to include the entire thickness of the myocardium.

Histology

Samples of the left ventricle were fixed either in 4% (W/V) solution of paraformaldehyde (PFA) freshly diluted from 16% PFA (Fisher Scientific, Pittsburgh, PA) in PBS (pH 7.0), or in 10% formalin in PBS. Fixed samples were routinely processed and embedded in paraffin within 3 weeks of the date of sacrifice. The myocardial samples were sectioned at 6 μ m and stained with hematoxylin and eosin. Sections of thoracic aorta from a representative sample of animals were examined histologically and found free of evidence of atherosclerosis, inflammation or cystic medial necrosis as were coronary arteries.

Myocyte Size

Sections were stained with tomato lectin (*Lycopersicon esculentum*) labeled with tetramethyl rhodamine (Invitrogen, Carlsbad, CA), mounted in Vectashield with DAPI (Dako, Carpinteria,

CA), and examined by fluorescence microscopy. Hearts required treatment with proteinase K (Sigma-Aldrich, St. Louis, MO), 20 µg/ml for 30 min at room temperature, before staining. Outlines of myocyte cross-sectionals were traced in the subendocardial and subepicardial myocardium in those regions in which round capillary cross-sectionals were present, indicating an orientation of the long axis of the myocytes close to 90 degrees to the plane of section. Areas of cross-sections of 100–200 myocytes in each animal were obtained from the traces using ImageProPlus software (Media Cybernetics, Bethesda, MD).

TUNEL Staining

The TUNEL procedure (Roche Laboratories, Nutley, NJ) was used to assess myocytes undergoing apoptosis. Prior to carrying out the TUNEL procedure, deparaffinized sections were treated with proteinase K (20 µg/ml) (Sigma-Aldrich) for 5 minutes at room temperature and then washed in PBS. Biotinylated dUTP was detected with 1:200 ExtrAvidin labeled with FITC (Sigma-Aldrich) applied at room temperature for 30 minutes. Sections were mounted in VectaShield with DAPI (Vector Laboratories, Burlingame, CA).

TUNEL positive myocyte nuclei in sections from each animal were counted at a magnification of 400×. Nuclei of myocytes were distinguished from vascular and other interstitial nuclei by staining myocytes with anti- α -sarcomeric actin antibody (Dako). DAPI stained myocyte nuclei were counted in ten fields of measured area, and the total number of myocytes in the sections calculated from the average number of myocytes per mm² and the total area of all sections evaluated for TUNEL-positive cells. Areas were measured using the trace function in ImageProPlus software (Media Cybernetics). The frequency of apoptotic nuclei is reported as the percent of positively stained myocyte nuclei. TUNEL counts were performed blinded to the age and sex of the animals.

Ki-67 Staining

The Ki-67 antigen [12] was stained immunohistochemically on sections from the same samples as those used for TUNEL staining. Antigen retrieval was performed by microwave irradiation at 100 W for 10 minutes in pH 6, 0.01 mM citrate buffer. Rabbit polyclonal anti-human Ki-67 antibody (Dako) at a dilution of 1:300 was used as the primary antibody with 1 hour incubation at 37°C. FITC-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), diluted 1:60, was applied at 37°C for 30 minutes. The primary antibody was omitted in negative controls. Slides were mounted in VectaShield with DAPI. The procedure for determining the percent of Ki-67 positive myocytes was the same as that used for TUNEL-positive cells.

Statistics

Histograms for the myocyte cross-sectional areas were constructed using NCSS software (NCSS). Statistical significance was determined at a level of $p < 0.05$ using ANOVA for multiple group comparisons and Students t-test for comparisons between two groups.

RESULTS

Macaca fascicularis monkeys, young males (YM; 3–9 y.o.), old males (OM; 17–26 y.o.), young females (YF; 2–12 y.o.) and old females (OF; 18–24 y.o.) were studied (Table 1). Body weights in immature (2–4 y.o.) males were less than in young males and females, but left ventricular (LV)/body weight (BW) was almost identical in immature and young males and females; accordingly, the young males and young females included immature and young animals ranging from 2–12 y.o.

Analysis of Body Weight and Heart Weight

No consistent increase occurred in body weights between young adult and old male monkeys (Table 1). In female monkeys there was a small increase of body weight with age. Males generally weighed more than females of comparable age (Table 1). The LV/BW of the old male monkeys (2.04 ± 0.08 g/kg) was less, $p < 0.05$, than the mean LV weight/BW of young males (2.27 ± 0.05 g/kg) (Table 1). There were no significant differences between LV/BW in immature (2.32 ± 0.10 g/kg) and young (2.25 ± 0.05 g/kg) males. LV/BW of female monkeys did not change with age (Table 1).

Myocyte cross-sectional areas

Histograms and their density traces for all myocyte cross-sectional areas in each group of monkeys are shown in Figure 1. There was an average increase of 8% in the mean myocyte cross-sectional with age in the female monkeys (Figure 2). In sharp contrast, the increase in mean myocyte cross-sectional area in old male monkey hearts was 51%. The difference in cross-sectional areas between the old males and the other groups was significant, $p < 0.001$. Analysis of the distribution of myocyte size demonstrated that hypertrophy was not uniform in the old male animals, since it was observed in only 20–30 percent of myocytes in old males.

Cardiomyocyte Apoptosis (TUNEL Technique)

Twenty to eighty thousand myocyte nuclei were analyzed in each heart. The percentages of apoptotic cardiomyocytes present in each of the groups are shown in Figure 2. There was a significantly greater; 4-fold increase of apoptotic cardiomyocytes in old male monkeys when compared with young males, but no significant change occurred in the rate of cardiomyocyte apoptosis in the old female monkeys compared with the young females. Among young monkeys there was no difference when the percent of apoptotic cardiomyocytes in male and female monkeys were compared, whereas among old monkeys, there was a statistically significant ($p < 0.01$) greater incidence of apoptosis in the old male monkeys than in the old females (Figure 2).

Ki-67 Staining

The mean percentage of Ki-67 positive cardiomyocytes in the four groups of monkeys are shown in Figure 2. There was a similar frequency of Ki-67 positive cardiomyocytes among the old male, the young male and the young female monkey groups. In old females the percent of Ki-67 positive myocytes dropped significantly from that found in the other groups ($p < 0.05$).

DISCUSSION

The widely held concept that aging cardiomyopathy involves increased cardiac size is based entirely on rodent studies [1–4]. In contrast, a major finding of the current investigation is that LV/body weight actually declines significantly with age in the male monkey. Further, the effect of age on heart weight differed between male and female monkeys in our sample, i.e., mean female LV/body weight did not change with age. In the presence of decreased cardiac weight, there was marked myocyte hypertrophy in aging males with a 51% increase in mean cross-sectional area, as compared with only an 8% increase in older female monkeys. Although it would be predicted that there would be a uniform shift in the distribution of myocyte size with hypertrophy, interestingly, the distribution was skewed in the aging male, a finding not observed previously, with only 20–30% of myocytes undergoing hypertrophy in the aging male. The considerable differential hypertrophic response we have observed may relate to the increased cell-to-cell variation in gene expression documented in the aging mouse heart [13]. Our conclusions regarding myocyte hypertrophy are based on measurement of cross-sectional area. Myocyte length was not measured, which could also affect myocyte volume calculations.

Given the substantial increase in myocyte cross-sectional areas that occurred in the older male hearts in the face of decreased LV/body weight, it seems likely that the increased rate of apoptosis in the male myocytes accounted for loss of myocytes in aged males, although other mechanisms such as autophagocytosis or necrosis may also play a role. As with myocyte cross-sectional area, there were major gender differences in apoptosis. The 4-fold increase in myocyte apoptosis in old males was not paralleled by a significant increase in apoptosis in old female hearts. These data in aging are consistent with data from patients with heart failure, where apoptosis is greater in males than females [14,15].

While there is considerable evidence from both human and experimental studies that increases in individual myocyte size are responsible for the enlargement in heart mass in cardiac hypertrophy, the presence of adult cardiac stem cells [16,17] and myocyte progenitor cells [18,19] allows for the possibility that such cells together with proliferative myocytes [20] could add to the myocyte cell mass in the presence of work load hypertrophy [21,22], or conditions of significant cell loss as in aging. However, this cannot be supported by the current results in monkeys, where there was no increase in Ki-67 positive myocytes, which are presumed proliferation competent [12], in old males and a decrease in Ki-67 positive myocytes in the females.

A small decrease in heart weight in human males and maintenance with no increase in heart weight in females with aging has been documented by Anversa and colleagues [23] as have differences in the increases in myocyte volume and diameter between males and females parallel to those we have observed in monkeys. The result of that study [23] would predict an increase in apoptosis in aging male hearts, but not females, but this was not measured. In another study in humans, Mallat et al. [24] have reported a greater incidence of TUNEL positive myocytes in young male humans than in females, but unlike our findings in monkeys, no increase in apoptosis with age was found in either sex. Neither of the prior studies in humans measured proliferation of aging myocytes.

In summary, the primate model of aging is a unique model, being phylogenically close to humans, without the complicating influence of other diseases of aging, and having a longer life span than rodents. The decrease in LV mass with age, in aging male primates, is directionally opposite to data obtained from aging rodents, and occurs due to myocyte loss in the absence of significant myocyte proliferation. In contrast, the increase in apoptosis and decrease in LV mass are not features of the aging female heart, which can help explain its relative protection compared with older males.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Lakatta EG. Cardiovascular ageing in health sets the stage for cardiovascular disease. *Heart Lung Circ* 2002;11:76–91. [PubMed: 16352074]
2. Lakatta EG. Arterial and cardiac aging: major shareholders in cardiovascular disease enterprises: Part III: cellular and molecular clues to heart and arterial aging. *Circulation* 2003;107:490–7. [PubMed: 12551876]

3. Lakatta EG, Levy D. Arterial and cardiac aging: major shareholders in cardiovascular disease enterprises: Part II: the aging heart in health: links to heart disease. *Circulation* 2003;107:346–54. [PubMed: 12538439]
4. Lakatta EG, Levy D. Arterial and Cardiac Aging: Major Shareholders in Cardiovascular Disease Enterprises: Part I: Aging Arteries: A “Set Up” for Vascular Disease. *Circulation* 2003;107:139–146. [PubMed: 12515756]
5. Takagi G, Asai K, Vatner SF, Kudej RK, Rossi F, Peppas A, et al. Gender differences on the effects of aging on cardiac and peripheral adrenergic stimulation in old conscious monkeys. *Am J Physiol Heart Circ Physiol* 2003;285:H527–34. [PubMed: 12860563]
6. Yan L, Ge H, Li H, Lieber SC, Natividad F, Resuello RR, et al. Gender-specific proteomic alterations in glycolytic and mitochondrial pathways in aging monkey hearts. *J Mol Cell Cardiol* 2004;37:921–9. [PubMed: 15522269]
7. Roth GS, Mattison JA, Ottinger MA, Chachich ME, Lane MA, Ingram DK. Aging in rhesus monkeys: relevance to human health interventions. *Science* 2004;305:1423–6. [PubMed: 15353793]
8. Bonadio, C. *Macaca Fascicularis*. Web, AD., editor. 2000. (On-line)
9. Appt SE. Usefulness of the monkey model to investigate the role soy in postmenopausal women's health. *Ilar J* 2004;45:200–11. [PubMed: 15111739]
10. Kavanagh K, Koudy Williams J, Wagner JD. Naturally occurring menopause in cynomolgus monkeys: changes in hormone, lipid, and carbohydrate measures with hormonal status. *J Med Primatol* 2005;34:171–7. [PubMed: 16053494]
11. Qiu H, Depre C, Ghosh K, Resuello RR, Natividad F, Rossi F, et al. Mechanism of Gender-Specific Differences in Aortic Stiffness with Aging in Non-Human Primates. *Circulation*. 2007In press
12. Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. *J Cell Physiol* 2000;182:311–22. [PubMed: 10653597]
13. Bahar R, Hartmann CH, Rodriguez KA, Denny AD, Busuttil RA, Dolle ME, et al. Increased cell-to-cell variation in gene expression in ageing mouse heart. *Nature* 2006;441:1011–4. [PubMed: 16791200]
14. Biondi-Zoccai GG, Baldi A, Biasucci LM, Abbate A. Female gender, myocardial remodeling and cardiac failure: are women protected from increased myocardiocyte apoptosis? *Ital Heart J* 2004;5:498–504. [PubMed: 15487266]
15. Guerra S, Leri A, Wang X, Finato N, Di Loreto C, Beltrami CA, et al. Myocyte death in the failing human heart is gender dependent. *Circ Res* 1999;85:856–66. [PubMed: 10532954]
16. Anversa P, Kajstura J, Leri A, Bolli R. Life and death of cardiac stem cells: a paradigm shift in cardiac biology. *Circulation* 2006;113:1451–63. [PubMed: 16549650]
17. Nadal-Ginard B, Kajstura J, Leri A, Anversa P. Myocyte death, growth, and regeneration in cardiac hypertrophy and failure. *Circ Res* 2003;92:139–50. [PubMed: 12574141]
18. Pfister O, Mouquet F, Jain M, Summer R, Helmes M, Fine A, et al. CD31- but Not CD31+ cardiac side population cells exhibit functional cardiomyogenic differentiation. *Circ Res* 2005;97:52–61. [PubMed: 15947249]
19. Oh H, Bradfute SB, Gallardo TD, Nakamura T, Gaussin V, Mishina Y, et al. Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. *Proc Natl Acad Sci U S A* 2003;100:12313–8. [PubMed: 14530411]
20. Anversa P, Leri A, Beltrami CA, Guerra S, Kajstura J. Myocyte death and growth in the failing heart. *Lab Invest* 1998;78:767–86. [PubMed: 9690556]
21. Rakusan K, Hrdina PW, Turek Z, Lakatta EG, Spurgeon HA, Wolford GD. Cell size and capillary supply of the hypertensive rat heart: quantitative study. *Basic Res Cardiol* 1984;79:389–95. [PubMed: 6237640]
22. Anversa P, Palackal T, Sonnenblick EH, Olivetti G, Meggs LG, Capasso JM. Myocyte cell loss and myocyte cellular hyperplasia in the hypertrophied aging rat heart. *Circ Res* 1990;67:871–85. [PubMed: 2145091]
23. Olivetti G, Giordano G, Corradi D, Melissari M, Lagrasta C, Gambert SR, et al. Gender differences and aging: effects on the human heart. *J Am Coll Cardiol* 1995;26:1068–79. [PubMed: 7560601]

24. Mallat Z, Fornes P, Costagliola R, Esposito B, Belmin J, Lecomte D, et al. Age and gender effects on cardiomyocyte apoptosis in the normal human heart. *J Gerontol A Biol Sci Med Sci* 2001;56:M719–23. [PubMed: 11682581]

Figure 1.

Normalized histograms and density traces for cross-sectional areas of myocytes from young (black bars) and old (grey bars) males (top) and females (bottom). At least 750 areas were measured for each histogram. The small fraction of hypertrophied myocytes is shown by the distributions at the right, without corresponding values for young animals.

Figure 2.

(Top panel) Effects of age and gender on mean cardiac myocyte cross-sectional area in monkeys (YM, n=12; OM, n=9; YF, n=7; OF, n= 6). Note the significant increase in cross-sectional area in old male monkeys, *p<0.001 vs. other groups; the frequency of apoptotic myocytes (middle panel) (YM, n=10; OM, n=9; YF, n=9; OF, n= 11) and Ki-67 positive cells (bottom panel) (YM, n=6; OM, n=11; YF, n=7; OF, n= 11) are compared among the 4 groups. *p<0.05 vs. all other groups.

Table 1

LV/Body Weight (BW)

Age Group	n	Age Range (Years)	Body Weight (kg)	LV/BW (g/kg)
Young Males	19	3 – 9	5.41±0.40	2.27±0.05
Old Males	22	17 – 26	6.48±0.27 [*]	2.04±0.08 [*]
Young Females	17	2 – 12	3.60±0.19	2.36±0.13
Old Females	22	18 – 24	4.54±0.15 [*]	2.29±0.13

^{*}
p<0.05 Old vs. Young