Age-related changes in lamin A/C expression in cardiomyocytes

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Submitted 31 October 2006; accepted in final form 16 May 2007

LAMIN A AND C (A/C) are type V intermediate filaments encoded by the LMNA gene that form the nuclear lamina (17). The functions of lamin A/C are to support the inner nuclear envelope and to participate in DNA repair, signal transduction, mesenchymal stem cell differentiation, mitosis, and apoptosis (9, 17, 24). LMNA mutations (“laminopathies”) lead to a reduction in lamin A/C expression and diverse phenotypes such as familial cardiomyopathies and accelerated aging syndromes. Normal aging is associated with reduced expression of lamin A/C in osteoblasts and dermal fibroblasts but has not been assessed in cardiomyocytes. Our objective was to compare the expression of lamin A/C in cardiomyocytes of old (24 mo) versus young (4 mo) C57Bl/6J mice using a well-validated mouse model of aging. Lamin B1 was used as a control. Immunohistochemical and immunofluorescence analyses showed reduced expression of lamin A/C in cardiomyocyte nuclei of old mice (proportion of nuclei expressing lamin A/C, 9% vs. 62%, P < 0.001). Lamin A/C distribution was scattered peripherally and perinuclear in old mice, whereas it was homogeneous throughout the nuclei in young mice. Western blot analyses confirmed reduced expression of lamin A/C in nuclear extracts of old mice (ratio of lamin A/C to B1, 0.6 vs. 1.2, P < 0.01). Echocardiographic studies showed increased left ventricular wall thickness with preserved cavity size (concentric remodeling), increased left ventricular mass, and a slight reduction in fractional shortening in old mice. This is the first study to show that normal aging is associated with reduced expression and altered distribution of lamin A/C in nuclei of cardiomyocytes.

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Quantification of lamin A/C and B1 expression by immunohistochemistry. After dissection and fixation, young and old heart samples were embedded in low-melting point paraffin in a Shandon Citadel 2000 automatic tissue processor (Shandon Scientific, Runcorn, UK). Coronal and transverse sections (4 mm) were mounted on silane-coated glass slides (Fischer Scientific, Springfield, NJ). Paraffin was removed with three washes of xylene and rehydrated with washes of graded ethanol (80%-50%-30%) and PBS. Nonspecific binding was blocked by an addition of goat serum for 1 h. Sections were then incubated with mouse monoclonal IgM lamin A/C antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for either 4 h at room temperature or 8–24 h at 4°C. Sections incubated with mouse monoclonal IgG lamin B1 antibody (Santa Cruz Biotechnology) were used as controls. After sections were washed with PBS, hydrogen peroxide-complexed rabbit anti-mouse IgG was added to the sections at room temperature for 30 min, followed by a 30-min incubation with 0.6% hydrogen peroxide ± chromogen. Immunohistochemical staining was performed using the human avidin-biotin complex staining system (Santa Cruz Biotechnology). Lamin-positive cells showed a brown nucleus with punctate brown staining from the peroxidase-labeled antibody and blue counterstaining from the hematoxylin. Lamin A/C expression was calculated as the number of cardiomyocyte nuclei positively stained for lamin A/C (brown-stained nuclei) divided by the total number of cardiomyocyte nuclei (brown- and blue-stained nuclei) in each of 10 randomly chosen high power fields (HPF).

Quantification of lamin A/C by immunofluorescence. Heart sections were treated as described for immunohistochemistry, omitting the final step involving treatment of cells with hydrogen peroxide. After fixation in 4% paraformaldehyde, sections were washed with PBS and then incubated in PBS with 10% blocking serum for 20 min to suppress nonspecific binding of IgG. Sections were incubated with mouse monoclonal IgM lamin A/C antibody (Santa Cruz Biotechnology) with 1.5% blocking serum overnight at 4°C and then incubated with fluorescein-conjugated secondary antibody (FITC, Santa Cruz) diluted to 2 μg/ml in PBS with 1.5% blocking serum for 45 min. Nuclei were counterstained using propidium iodine (2 μg/ml). Control slides were incubated with rabbit IgG according to the manufacturer’s instructions, and triplicate tests and control slides were included in immunofluorescence. Lamin A/C-positive cells showed intense nuclear green fluorescence, whereas negative controls showed only faint nuclear or cytoplasmic fluorescence. The number of positive cardiomyocyte nuclei divided by the total number of cardiomyocyte nuclei in each field was calculated as described for immunohistochemistry.

Quantification of lamin A/C and B1 expression by Western blot analysis. Nuclear extracts were obtained after suspending the heart pieces in two volumes of buffer containing 10 mM EDTA, 0.5 mM phenylmethylsulfonl fluoride, and protease inhibitor cocktail diluted according to the manufacturer’s instruction (Complete protease inhibitor, Boehringer-Mannheim, Laval, QC, Canada). The homogenate was clarified by centrifugation at 25,000 g for 20 min at 4°C, and the nuclear pellet was resuspended in 20 mM HEPES (pH 7.9), 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonl fluoride, and 0.5 mM dithiothreitol. Following a further 20-min centrifugation at 25,000 g, nuclear extracts (supernatant) were dialyzed for 5 h against 20 mM HEPES (pH 7.9), 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonl fluoride, and 0.5 mM dithiothreitol. Protein content was determined with a protein assay kit (Bio-Rad, Mississauga, ON, Canada), and samples were then aliquoted and stored at –80°C. For Western blot analyses, nuclear extracts were resuspended in SDS electrophoresis buffer (Bio-Rad, Hercules, CA), proteins were separated on SDS-polyacrylamide gels, and the proteins were electrotransferred to Immobilon-P polyvinylidene difluoride membranes. After being blocked with PBS containing 0.1% Tween 20 and 10% nonfat dry milk, membranes were incubated overnight at 4°C using a monoclonal antibody directed against lamin A/C and a second monoclonal antibody against lamin B1 (Santa Cruz). Specific staining was revealed after washing, and incubating with horseradish peroxidase-conjugated IgG rabbit anti-mouse antibodies was followed by enhanced chemiluminescence using Lumi-GLO reagents (Kirkegaard and Perry, Gaithersburg, MA). The lamin A/C signals were quantified by densitometry and normalized according to lamin B1 signals.

Statistical analysis. All results are expressed as means ± SE of three replicate determinations, and statistical comparisons are based on one-way ANOVA or Student’s t-tests. A P value of <0.05 was considered significant and ≤0.05 was considered nonsignificant (NS).

RESULTS

The young (n = 5) and old (n = 5) C57BL/6J mice were representative of the litter and did not have identifiable diseases. Dissection of the murine hearts revealed that the mean mass of the isolated hearts was 189 ± 15 mg in old mice and 167 ± 15 mg in young mice (P < 0.05). Microscopic inspection of the hematoxylin and eosin-stained sections did not reveal any gross pathology in the cardiac tissues (Fig. 1, A and B). The number of cardiomyocytes per HPF was inferior in old mice compared with young mice (40–62 vs. 65–80 cells/HPF, P < 0.05), whereas the cell size was similar (108 vs. 110 μm, P = NS).

Echocardiographic results are shown in Table 1. When compared with young mice, old mice demonstrated increased LV wall thickness with preserved LV cavity size resulting in increased relative wall thickness (0.38 ± 0.05 vs. 0.29 ± 0.06, P < 0.05) suggestive of concentric remodeling. Accordingly, LV mass was increased in old mice (121 ± 19 vs. 93 ± 16 mg, P < 0.05). Fractional shortening was slightly reduced in old mice (46.3 ± 3.9% vs. 48.3 ± 3.8%, P < 0.05).

Immunohistochemical analyses showed that the expression of lamin A/C but not lamin B1 was reduced in cardiomyocyte nuclei of old mice (Fig. 1, C–F). The proportion of nuclei positively stained for lamin A/C was 9 ± 6% in old mice and 62 ± 16% in young mice (P < 0.001). In contrast, the proportion of nuclei positively stained for lamin B1 control remained constant (90 ± 6% in old mice and 96 ± 4% in young mice, P = NS). Immunofluorescence analyses also showed that the expression of lamin A/C was reduced in cardiomyocyte nuclei of old mice (Fig. 2, A–D).

In addition to quantitative changes in lamin A/C expression, qualitative changes in lamin A/C distribution were observed. The distribution of lamin A/C was homogeneous throughout the nuclei in young mice, whereas it was scattered toward the periphery and perinuclear with minimal contact between staining sites in old mice (Fig. 2, E and F).

Western blot analyses confirmed and quantified the reduction in lamin A/C expression in cardiomyocyte nuclei of old mice (Fig. 3). The ratio of lamin A/C to B1 as measured by densitometry was 0.6 in old mice and 1.2 in young mice (P < 0.01).

Finally, changes in lamin A/C expression were observed in other heart cells. Specifically, immunohistochemical analyses suggested that the expression of lamin A/C but not B1 was reduced in vascular endothelial cell nuclei of old mice compared with young mice (Fig. 4).

DISCUSSION

Our study is the first to show that normal aging is associated with a reduced expression of lamin A/C in cardiomyocytes. Moreover, we found that normal aging is associated with a
scattered perinuclear distribution of lamin A/C and may be associated with a reduced expression of lamin A/C in vascular endothelial cells. Thus, age-related changes in lamin A/C expression and distribution denote a novel aging mechanism previously described in the dermatologic and osteoarticular

Table 1. Echocardiographic data for young and old mice

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>Old</th>
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<tr>
<td>HR, beats/min</td>
<td>563 ± 72</td>
<td>548 ± 57</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>4.25 ± 0.37</td>
<td>4.12 ± 0.28</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>2.20 ± 0.26</td>
<td>2.20 ± 0.19</td>
</tr>
<tr>
<td>SW, mm</td>
<td>0.57 ± 0.10</td>
<td>0.73 ± 0.11*</td>
</tr>
<tr>
<td>PW, mm</td>
<td>0.66 ± 0.11</td>
<td>0.83 ± 0.13*</td>
</tr>
<tr>
<td>RWT</td>
<td>0.29 ± 0.06</td>
<td>0.38 ± 0.05*</td>
</tr>
<tr>
<td>FS, %</td>
<td>48.3 ± 3.9</td>
<td>46.3 ± 3.8*</td>
</tr>
<tr>
<td>LVMI, mg</td>
<td>93 ± 16</td>
<td>121 ± 19*</td>
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Values are means ± SE. HR, heart rate; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; SW, septal wall thickness; PW, posterior wall thickness; RWT, relative wall thickness, calculated as (PW + SW)/LVEDD; FS, fractional shortening, calculated as [(LVEDD – LVESD)/LVEDD] × 100; LVM, left ventricular mass, calculated as 1.055 [(PW + SW + LVESD)³/(LVEDD)³] × 100. *P < 0.05, young vs. old.

Fig. 1. Microscopic inspection of the hematoxylin and eosin-stained sections in young (A) and old (B) mice did not reveal any gross pathology in the cardiac tissues. The number of cardiomyocytes per high-power fields was greater in young mice compared with old mice, whereas the cell size was similar. Lamin A and C (A/C) expression in young (C) and old (E) cardiomyocytes and lamin B1 expression in young (D) and old (F) cardiomyocytes are shown. Note that the expression of lamin A/C but not B1 is decreased in old cardiomyocytes. The scatter plot (G) shows the proportion of nuclei positive for lamin A/C in each of the 10 high power fields analyzed per mouse (9% vs. 62%, P < 0.001).

Fig. 2. Immunofluorescence staining of lamin A/C in young (A) vs. old (C) cardiomyocytes. A and C: overlap between propidium iodine (PI) and green fluorescence. Young cardiomyocytes show bright fluorescence staining at the nuclei (A, white arrows) by lamin A/C antibody. Lamin A/C labeling is reduced in old cardiomyocytes (C, white arrows). B and D: PI counterstaining to determine the total number of nuclei in the field. E and F: lamin A/C distribution in young (E) and old (F) cardiomyocytes. Note that the distribution of lamin A/C as shown by the red arrows is peripherally scattered and perinuclear in old vs. homogeneous and intranuclear in young cardiomyocytes.
systems (9, 30) and are now discovered in the cardiovascular system.

Our finding of reduced myocardial lamin A/C expression with aging is preceded by the well-documented finding of reduced myocardial lamin A/C expression with inherited LMNA mutations (1, 39). LMNA mutations are among the most common causes of familial autosomal-dominant cardiomyopathy (18). Individuals with these mutations often have heart failure with increased LV wall thickness (37). Up to 88% of affected individuals have electrophysiological disturbances such as sick sinus syndrome, atrioventricular block, and atrial fibrillation or flutter (10, 16). This constellation of findings shares several features with the physiopathological changes seen in the aging heart (22, 23). We speculate that LMNA-related familial cardiomyopathy and age-related senile cardiomyopathy may represent two entities in a spectrum of lamin A/C-deficient heart disease.

The newly discovered association of aging and myocardial lamin A/C expression is a fundamental first step in lamin-cardiology research. It opens the door for further characterization of the age-related changes in myocardial lamin A/C expression and distribution. More importantly, it opens the door for mechanistic research to test whether there exists a causal link between the observed decline in lamin A/C and the parallel abnormalities in myocardial structure and function. One potential link between lamin A/C and aging hearts may be that both are epitomized by an impaired cellular and nuclear response to stressors. The structural model of age-related changes in lamin A/C suggests that loss of lamin function causes nuclear fragility which leads to permanent damage or death in the face of mechanical or environmental stressors (33). Similarly, age-related changes in the heart are described as a state of fragility or reduced adaptation to acute and chronic stressors such as exercise or myocardial ischemia (11, 13–15, 21, 27, 29).

In agreement with prior studies conducted in animal models and in human subjects (2, 3, 6, 31, 40), old mice demonstrated increased LV wall thickness with preserved cavity size resulting in increased relative wall thickness (concentric remodeling) and mass. Systolic function was slightly, yet significantly, reduced as previously demonstrated by Yang et al. (40). Although diastolic function was difficult to assess given the rapid heart rates of our mice under physiological conditions (mean, 548–563) and the challenge of transducer positioning for reliable parallel mitral inflow, our finding of concentric hypertrophy is consistent with the finding of a relaxation abnormality in senescent mice demonstrated by Taffet et al. (36). We do not intend for the echocardiographic data to be causally explanatory; however, we believe that these data add an important dimension to our study by showing that the morphological and functional correlation of our histopathological findings is consistent with the expected changes of myocardial aging.
Clinically, lamin A/C has the potential to be a prognostic marker and a therapeutic target. Among 15 patients with nonischemic cardiomyopathy requiring LV assist device support, lamin A/C expression over time was a strong predictor of myocardial recovery leading to the explantation of the device (4). In presymptomatic LMNA mutation carriers, lamin A/C expression may be used as a screening tool to identify high-risk subjects who may benefit from more aggressive therapy (28). Therapeutic agents such as farnesylation modulators have been shown to prevent or reverse some of the nuclear defects in Hutchinson-Gilford progeria syndrome (7, 8, 12, 25, 38). These agents modulate posttranslational conversion of precursor prelamin A to the active lamin A, correcting the nuclear defect associated with lamin A depletion or prelamin A accumulation. To our knowledge, the effect of farnesylation modulators on normal aging or on the cardiovascular system has not been evaluated.

In conclusion, the expression of lamin A/C is substantially reduced and the distribution is scattered peripherally in nuclei of cardiomyocytes isolated from a validated mouse model of aging. Further research in this field may attempt to clarify the causal link between lamin A/C and aging hearts and to explore the value of farnesylation modulators as novel therapeutic agents to counter the potentially negative effects of lamin A/C depletion on the heart.

ACKNOWLEDGMENTS

We thank Dr. Ernesto Schiffрин (Chief, Department of Medicine, Sir Mortimer B. Davis Jewish General Hospital) for a diligent review and Dr. Alexandre Marcus (Department of Pathology, St. Mary’s Hospital) for assistance in examining the histological sections. This project was presented at the American Geriatrics Society Annual Meeting (Chicago, IL, 2006).

GRANTS

This work was supported by operating grants from the Canadian Institutes for Health Research and the Heart and Stroke Foundation of Quebec. G. Duque holds a bursary from the Fonds de la Recherche en Santé du Québec.

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