Remodeling of the intercalated disc related to aging in the mouse heart

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ARTICLE INFO

Article history:
Received 24 April 2015
Received in revised form 10 August 2015
Accepted 8 September 2015
Available online 14 November 2015

Keywords:
Intercalated disc
Heart
Mouse
Aging
Connexin 43

ABSTRACT

Background: Aging is related to declined cardiac hemodynamic function. As pumping performance may be significantly related to slowed ventricular depolarization and non-synchronous contraction, we hypothesized that aging may cause dysfunction of intercalated disc (ID), which is the structure responsible for intercellular electrical communication between cardiomyocytes.

Methods: Male C57BL/6j mice were used for the study at two ages: 4 and 24 months. Electrocardiographic recording was made to analyze the time of ventricular depolarization. Then mice were killed, and the hearts were harvested for examination in transmission electron microscopy (TEM) and immunofluorescence imaging. The expression of connexin 43 (Cx43), N-cadherin, and β-catenin in the myocardium of the left ventricle was evaluated using Western blotting.

Results: In senescent mice, analysis of averaged QRS complex showed its significant prolongation. At the ultrastructural level, we found frequent disruptions of the ID (affecting 29 ± 5% of them), mainly at the site of adherens junction, with relatively preserved desmosomal intercellular connections and diminished number of gap junctions. Western blotting revealed significantly decreased abundance of Cx43 protein in aged animals, which may cause slowed impulse propagation through the gap junctions and contribute to the observed electrocardiographic alterations. The level of RNA for Cx43 is similar between young and old animals, which suggests a post-transcriptional mechanism of Cx43 protein downregulation.

Conclusions: Our study shows age-related disorganization of ID, which may be responsible for slowed conduction of the depolarization wave within the heart, and supports the hypothesis of cardiac dysfunction in senescence.

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Introduction

Aging is related to morphological changes of myocardium and alteration in the cardiac performance. Left ventricular hypertrophy, fibrosis, and depressed systolic and diastolic function have been reported in senescent human and animal hearts [1]. These changes, not necessarily leading to overt heart failure, decrease the cardiac reserve and make the heart more susceptible to injury by common factors, such as pressure or volume overload or by ischemia. Age-related changes in myocardial morphology affecting hemodynamics have been described in laboratory animals [2,3]. In addition to changes in the intrinsic properties of the force development in the single cardiomyocyte, impaired cardiac hemodynamics may result at least in part from less simultaneous excitation of the heart [4]. In an exaggerated presentation, it is seen in patients with injured heart and intraventricular conduction defect, in whom significant improvement in cardiac performance after resynchronization of the left ventricular excitation with the pacemaker is frequently observed [5]. Human data provided by large-scale electrocardiographic (ECG) analysis reveal age-related prolongation of the QRS, suggesting decline in the velocity of the excitation wave spreading throughout the myocardium [6]. However, in animal models, much less is known about ventricular depolarization disturbances in senescence. Proper propagation of
the electrical impulse throughout the myocardium is possible with preserved structure and function of the intercalated discs (ID). They connect adjacent cells with the mechanical joint, formed by adherens junction (AJs) and desmosomes (Des). The first bridges actin cytoskeleton of the adjacent cells and the second, being reinforcement to the AJs, is anchored to the intermediate filaments. The third component of the junctional complex is the gap junction (GJ), which contains clusters of low-resistance ionic channels, each formed by a pair of connexin hemichannels. Each of these hemichannels is built into the sarcolemma at the GJ site and forms a stable noncovalent complex with its counterpart in the adjacent cell. In the ventricular myocardium, connexons are mainly formed by connexin 43 (Cx43). Close proximity of the neighboring plasma membranes, that depends mainly on preserved AJs and Des, is crucial for maintenance of the GJ channels [7]. Rapid ion transfer between cells permits coordinated depolarization of the cardiac cells and allows these cells to work as a syncytium and ordered excitation allows for the most efficient external work to eject the stroke volume.

The aim of the study was to assess the ultrastructure of ID in senescent mice and to relate its morphology to the functional, electrocardiographic index of the impulse conduction velocity within the ventricular myocardium.

Material and methods

Animals

Sixteen, male, 4-month-old (M group) and sixteen, 24-month-old (24M group) C57BL/6j mice were used in the study. Animals were obtained from the Center of Experimental Medicine of the Medical University of Bialystok and were originally purchased from Jackson Laboratories (Bar Harbor, ME, USA). The weight of the 4 M animals was 31.54 ± 2.06 g and for 24 M it was 33.03 ± 2.76 g. Animals were kept in constant temperature of 22 ± 1 °C in 12:12 dark–light cycle with constant access to standard chow and water.

The experimental procedures were carried out according to the European Council Directive of 24 November 1986 (6/609/EEC) and were approved by the Local Animal Ethics Committee at the Medical University of Bialystok.

Electrocardiography

Ten mice from each group were placed into the anesthesia induction chamber flushed with 2% isoflurane for about 30–40 s to achieve brief anesthesia. Precise anesthetic delivery was provided by rodent Halovet Vaporizer (Hugo Sachs Elektronik-Harvard Apparatus, March-Hugstetten, Germany). Then animals were quickly placed on the ECG acquisition table. The gel pad electrodes were placed on the front limbs. The signal was acquired using the Animal BioAmp (ADInstruments, Bella Vista, New South Wales, Australia) and PowerLab (ADInstruments) analog-to-digital interface connected to the computer and recorded with LabChart 7 Pro software (ADInstruments) for the period of sedation that lasted for at least 20 s. Analysis was performed using the ECG analysis LabChart module (ADInstruments). The width of the QRS complex was measured on the QRS averaged from at least 30 evolutions using the method proposed previously [8]. Briefly, the beginning of the depolarization was the first detectable deflection of the QRS, while the end was settled at the isoelectric line just at the beginning of the second main wave which in previous papers was referred to as the J wave or T wave, and which was reported to evince the early repolarization [9]. The markers of the beginning and the end of the QRS complex were adjusted manually.

Histology and immunofluorescence

Mice were killed by cervical dislocation and hearts were immediately excised, rinsed with ice-cold phosphate-buffered saline (PBS), and samples were collected for histological examinations, ultrastructural examination, and molecular analyses. Left ventricular myocardium was split into samples. One of these samples was immersed in the freezing mounting medium and frozen in liquid nitrogen immediately after harvesting and another was put into the phosphate-buffered formalin and then processed into paraffin blocks. Paraffin-embedded tissues were sectioned into 4-μm thick sections and stained with picro-sirius red [10]. From each section, 5 random view areas were photographed under 20× magnification. Collagen content was estimated using an automated quantification performed with Image J software (National Health Institute, Bethesda, MD, USA). Frozen samples were sectioned into 5 μm-thick sections and used for immunolabeling. After blocking with 10% donkey serum/PBS, sections were incubated with anti-Cx43 (#3512, Cell Signaling, Danvers, MA, USA; 1:500). Beta-catenin immunolabeling was performed to delineate the ID using anti-β-catenin antibody (sc-1496, SantaCruz Biotechnology, Santa Cruz, CA, USA; 1:200); as in cardiomyocytes, it is expressed solely at the IDs. In addition to individual staining procedures, the mixture of the above antibodies was used to co-localize both antigens in ID. After washing, the secondary antibodies (anti-rabbit IgG conjugated with biotin: #711-065-152, JacksonImmuno, West Grove, PA, USA, and anti-goat IgG conjugated with cyanine Cy3 – #705-165-147, JacksonImmuno) were then applied for 1 hour. Subsequently, slides were incubated with streptavidin-Alexa Fluor® 488 conjugate (#ab150089, Abcam, Cambridge, UK) and nuclei of cells were counterstained with Hoechst 33258 (Sigma-Aldrich, St. Louis, MO, USA; 1:5000). Finally, slides were coverslipped using Dako Fluorescence Mounting Medium (#S3023, Dako, Glostrup, Denmark) and evaluated using Olympus BX41 microscope equipped with the epifluorescence module and Olympus XC30 digital camera and the confocal imaging system Fluoview FV10i (Olympus, Tokyo, Japan). Low magnification confocal images were acquired at 1.8 μm and high magnification images at minimum achievable thickness of the confocal layer – 0.8 μm.

Transmission electron microscopy

Approximately, 1 mm² of the left ventricular myocardium was taken from 4 animals of both young and aged groups. After brief rinsing in ice-cold PBS, samples were fixed in a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer (CB) for 3 h at 4 °C. Then samples were washed in CB at 4 °C and postfixed in 1% osmium tetroxide in CB for 1 h at 4 °C and next dehydrated through a graded series of ethanol and embedded in Glycid ether 100 (Serva, Heidelberg, Germany). Ultrathin sections were contrasted with uranyl acetate and lead citrate and mounted on nickel grids and evaluated in a transmission electron microscope OPTON 900 PC (Zeiss, Oberkochen, Germany). Electronograms were acquired with the frame transfer CCD camera and evaluated using the Image5P software (Zeiss, Germany). In each animal, 50–100 IDs were inspected and the width of the intercellular space was measured.

Western blot

Western blotting procedure was performed as described previously [11]. Briefly, eight samples of protein extracts from each group (50 μg of protein per lane) were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes (BioRad, Hercules, CA, USA). We
used Ponceau Red (Sigma, St. Louis, MO, USA) staining to control equal loading of gels. Primary antibodies against mouse Cx43 (CellSignaling, #3512), β-catenin (SantaCruz Biotechnology, #sc-1496), N-cadherin (Dako, #M3613), and α-tubulin (SantaCruz Biotechnology, #sc-5286) were used. Secondary antibodies were conjugated with horseradish peroxidase (anti-rabbit Star54, Serotec, Oxford, UK or anti-mouse, Sigma #A9309 as appropriate). Blots were visualized using enhanced chemiluminescence reaction (Pierce, Rockford, IL, USA), and exposed on the X-ray film (X-Omat Blue, Kodak, Rochester, NY). Then blots were incubated with the Restore™ Western Blot Stripping Buffer (Thermo Scientific, Waltham, MA, USA). Films were scanned and quantified using the Image J software (National Institutes of Health, Bethesda, MD, USA). The abundance of Cx43, N-cadherin, and β-catenin proteins were normalized to the level of α-tubulin. The results of particular experiments were related to the expression of proteins in the control group, which was set as 1.0.

**Real-time quantitative polymerase chain reaction**

Left ventricular samples (ca. 30 mg in weight) were crushed in liquid nitrogen and RNA was extracted using commercially available RNaseasy Fibrous Tissue Mini Kit (Qiagen, Venlo, The Netherlands), according to the supplied protocol. Reverse transcription was performed using the TaqMan® Reverse Transcription Reagents (Life Technologies, Gaithersburg, MD, USA) and the quantitative real-time polymerase chain reaction (RT-PCR) was performed using the SYBR® Green PCR Master Mix (Life Technologies) according to the manufacturer’s manual. The sequence of primers used for Cx43 analysis was: 5’GAACACGGCAGGCTGAAGCTGAACAGAGTAAGTTAGCCAGAAGCTGAG 3’ and 5’GACCCGAGAGACACGGCAAGGTGAAGATCTGAAGATGAGCAGAAGCTGAG 3’. GAPDH mRNA was quantitated using commercially available probes (Mm99999915.1; Applied Biosystems, Foster City, CA, USA). The expression of Cx43 RNA (the number of cycles necessary to obtain the cut-off point – Ct value) was related to the expression of the GPDH. Then the 2−ΔΔct method with GPDH gene as internal control and one of the young animals as internal calibrator was used to present changes in gene expression as described previously [12].

**Statistical analysis**

Statistical analysis was performed using Statistica 10 (StatSoft, Tulsa, OK, USA) with non-parametric Mann–Whitney test. A value of p < 0.05 was considered significant. The numerical data are presented as mean value ± standard deviation.

**Results**

**Histology**

Sirius red staining revealed higher content of collagen in the myocardium of aged animals (2.1 ± 0.9% vs 0.7 ± 0.3% of the view area; p < 0.005). Deposition of the fibrotic tissue occurred mainly between the muscle fibers along the long axis of cardiomyocytes, while sites of IDs contained no visible collagen-specific staining. Representative microphotographs are presented in Fig. 1.

**Electrocardiography**

Heart rate at which the electrocardiographic signal was recorded was not significantly different between aged and young groups (respectively, 474 ± 72 beats per minute vs 523 ± 92 beats per minute, p = NS). The mean QRS complex width was significantly wider in old mice (12.4 ± 1.3 ms vs 10.3 ± 0.9 ms in the young; p = 0.003). In addition, we noticed significant prolongation of the PR interval in older mice (48.2 ± 7.9 ms vs 38.3 ± 3.4 ms in the young; p < 0.01). Representative averaged ECG tracings from young and aged animals are presented in Fig. 2.

**Connexin 43, β-catenin, and N-cadherin expression**

Western blotting revealed significantly diminished expression of Cx43 in aged mice. In this group, the mean density of the Cx43 band in western blots was reduced by 24.3% as compared to the young group (p = 0.018, Fig. 3a). In parallel, the level of Cx43 mRNA was not changed in the old mice as compared to the young, suggesting post-transcriptional mechanism of Cx43 downregulation (Fig. 3b). Immunofluorescence microscopy showed location of Cx43 mainly at the IDs (Fig. 4c, d). The distribution of Cx43 within ID was focal, interchanging with β-catenin, which demarcates AJ segments (Fig. 4e). As compared to young animals, the Cx43 fluorescence in the 24-month-old animals was slightly less intensive and confined to only short segments of the IDs (Fig. 4c, d). Some degree of lateralization of the Cx43 expression on the cardiomyocytes of aged animals was also noticed (Fig. 4c, d), but we did not evaluate this issue in detail. The expression of β-catenin protein was increased in the myocardium of old mice by 35% (p < 0.05; Fig. 5a) and the expression of N-cadherin was comparable between young and aged groups (Fig. 5b).

**Transmission electron microscopy**

The ultrastructural examination of the IDs in old animals revealed their frequent disruption, which was not present in the young animals at all. Widening of the intercellular space was present at the AJ regions in 29 ± 5% of IDs, and plasma membranes
In normal AJ regions in young animals, and in preserved normal AJ in aged animals, the distance between plasma membranes was 17 ± 3 nm. The ultrastructure of the subsarcolemmal protein complex, linking integrins of the plasma membrane with cytoskeleton, was not changed in disrupted IDs as compared to normal IDs. β-Catenin, which is one of the proteins building this electron dense band, was not downregulated in aged animals (and even upregulated), suggesting preservation of the intracellular portion of the protein complex of the ID.

It was difficult to find the GJ within disrupted ID, while Des were relatively well preserved, focally holding the adjacent plasma membranes in close proximity. We also noticed less foldings of the AJ in ID of aged mice, which was especially evident at regions of ID disruption, where plasma membrane was completely flattened.

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**Fig. 2.** Example of averaged electrocardiogram tracing from young mouse (upper panel) and from the aged mouse (lower panel). The QRS beginning and end are marked with vertical lines (QRS Start and QRS End, respectively). QRS widening is present in the old mouse and PR interval is also prolonged.

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**Fig. 3.** (a) The relative expression of the Cx43 protein in young group (WT 4m) and in aged group (WT 24m). Representative blot with Cx43 and the housekeeping protein (tubulin) are shown above the graph. (b) The relative Cx43 mRNA expression in both groups. Bars represent the mean value, and whiskers represent the standard deviation. Cx43, connexin 43.
ID had low electron density, occasionally contained myelin-like figures, and never contained collagen fibers. Representative ultrastructural photographs are presented in Fig. 6.

**Discussion**

Previous studies described the development and maintenance of the ID in fetal, early postnatal, and mature age [13–17]. To our best knowledge, this is the first report describing remodeling of the ID in senescent mice not subjected to any experimental manipulation. In addition to age-related degenerative changes of ID described above, we show parallel prolongation of the ventricular depolarization time. In contrast to previous publications, our model does not transcend the physiological aging. The hypothesis of interplay between structural proteins building the AJ and Des and the connexin 43, forming the gap junctions, was recently postulated [18]. Preserved mechanical coupling of cardiomyocytes was shown to be crucial for the proper electrical

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**Fig. 4.** Immunoconfocal imaging of β-catenin in the young (a) and aged (b) animals: red – β-catenin, nuclei – blue (Hoechst 33258). Cx43 in young (c) and aged (d): green – Cx43, nuclei – blue; red-autofluorescence; lateralization of the Cx43 in aged animals is marked with arrowheads. Confocal image of merged staining for β-catenin (red) and Cx43 (green) is presented in the lower panel (e); magnification of the intercalated disc marked with the box is shown on the right and present the two adjacent confocal planes and the overlap of these two confocal planes on the last picture. Cx43, connexin 43.
cell–cell interconnection [7,19]. On the other hand, the structure of adhesion complexes and their protein composition appear to be independent of the presence and function of the gap junctions [20]. Widening of the intercellular space, separating plasma membranes of the ID, requires loss of proper organization of the adherens junction and/or Des. The mechanism of ID disruption with age is not clear. It is important to recall the immunolabeling ultrastructural studies that have shown co-localization of proteins characteristic for Des and AJ within the same ID regions and lack of clear distinction between AJ and Des [21]. These mixed-type junctions are estimated to constitute the majority of the ID in mature mammalian heart [7]. Thus widening of the ID in aged mice described in the present paper may result from disturbed regulation of one or more of the group of proteins forming the mixed-type junction, with secondary downregulation of Cx43. N-cadherin is the main adhesion molecule of the ID, present typically at the AJ. Proper adhesive function of the cadherin requires the catenins that link the cadherin to the actin cytoskeleton [7]. As shown by the studies on transgenic animals, N-cadherin is essential for cardiac development and function maintenance.

**Fig. 5.** The relative expression of (a) β-catenin and (b) N-cadherin in young (WT 4m) and old groups (WT 24m). Bars represent the mean value, and whiskers represent the standard deviation. Representative western blots are shown above the graphs.

![Western blots for β-catenin and N-cadherin](image)

**Fig. 6.** Ultrastructure of the intercalated disc in young mice (a, b). Widening of the intercellular space in aged animals with the presence of the myelin-like figures (in the insert, marked with arrowhead) and focally present desmosomes is shown in pictures c and d. M, mitochondrion; arrows, desmosome; asterisk, gap junction. Bars = 2 μm.
Complete deletion of N-cadherin disturbs the primitive heart tube formation and causes embryonic lethality [22]. Cardiac-specific N-cadherin deletion in adult mice resulted in disassembly of the ID structure, including AJs and Des as well as downregulation of Cx43. In consequence, a modest dilated cardiomyopathy developed, leading to impairment of cardiac function and high mortality within two months from the genetic engineering intervention [23]. The ultrastructural description of the ID provided by these authors emphasizes dissolution of the ID and difficulties in finding the intercellular space between the myocytes [23]. This description is not similar to the picture we have noticed in aged animals. In our data, a clear electron-dense material adjacent to the plasma membrane was present, even at the disrupted IDs, and intercellular space was well demarcated. In addition, the expression of N-cadherin in the hearts of old mice was not changed in relation to young. Cx43 is the main gap junction channel-forming protein in the ventricular myocardium. Mice lacking Cx43 gene develop cardiac malfunction – right ventricular outflow tract obstruction – and die at birth [24]. Moreover, deletion of the Cx43 causes slowing of the impulse propagation in ventricular myocardium during embryonic development [25]. Postnatal cardiac-restricted inactivation of Cx43 also results in significant reduction of ventricular depolarization dynamics and development of spontaneous ventricular arrhythmia causing sudden cardiac death [26]. Different stimuli may affect Cx43 expression and integrity of the gap junction. Chronic and acute heart failure, chronic ischemia, oxidative stress, and pressure overload are all characterized by downregulation and remodeling of the gap junction proteins [27–32]. Aging-related reduction of the Cx43 expression was previously described in rats [33] and was attributed to delayed ventricular activation time; however, the concomitant ultrastructure of the ID was not examined [34]. In our observation, aged mice present with significant widening of the QRS complex in ECG, that is paralleled by reduced Cx43 protein but the level of Cx43 mRNA remained the same as in young animals, suggesting modification of the post-transcriptional portion of protein expression pathway. The link between prolonged timing of the QRS and reduced Cx43 expression was previously provided by studies on transgenic mice with either conditional Cx43 knockout [35] or heterozygous for a Cx43 null mutation [36]. Disruption of the ID described in our study provides ultrastructural background for functional and proteomic evidence for affected impulse conduction in the myocardium of aged mouse.

β-Catenin interacts with N-cadherin at the AJ sites and the complex of these proteins is an important regulator of the function of the ID [15]. It was previously shown that β-catenin expression in the cardiovascular system may change with age and is the subject for wide regulation [37,38]. There is cross-regulation between β-catenin and Cx43. In vitro studies showed that it is involved in Cx43 expression, being its positive transcriptional regulator [39]. Our observations reveal elevation of β-catenin protein level in the heart with concomitant decreased expression of the Cx43 protein and preserved Cx43 mRNA abundance. This suggests post-transcriptional defect in Cx43 expression, possibly linked to ID disruption. Increase of β-catenin level could be a secondary, compensatory reaction to decreased Cx43 expression. This hypothesis is supported by similar inter-relation between both proteins that was observed in the in vitro study, where genetic manipulation producing knockdown of Cx43 increased significantly β-catenin signaling [40].

Another possibility for ID disruption lies in the dysfunction of Des. This ultrastructural pathology is characteristic for arrhythmogenic right ventricular cardiomyopathy (ARVC) and is often characterized by disruption of ID and widening of the intercellular space. Specific desmosomal cadherins responsible for formation of the firm adhesion between cells are desmocollin 2 and desmoglein 2. Transgenic mice with cardiac overexpression of the mutant desmoglein 2 develop ID remodeling with separation of the opposed membranes and widening of the intercellular spaces at the level of Des/AJs and these changes affected IDs between otherwise morphologically normal cardiomyocytes [41]. The above-mentioned description is similar to the one we have seen in this study; however, our electron microscopy observations suggest rather proper desmosomal ultrastructure and separation of the opposing plasma membranes not at the sides of Des, but at the sites of AJs. In the previous literature, there are few data describing changes of the ID related to age. The majority of information, where disruption of the ID was noticed, has been devoted to ARVC. Finally, it is worth mentioning that widening of the ID was observed in anthracycline-induced cardiac injury and in diabetic rats [42,43]; however, the influence of these changes for impulse conduction or for the Cx43 expression in the myocardium was not examined.

Conclusion

Aging causes morphological alterations of the ID in mice. Ultrastructural changes, of which the most evident is widening of the intercellular space within the ID, is paralleled by reduced expression of Cx43 and prolonged ventricular depolarization time. Deciphering mechanisms responsible for these changes require further investigations.

Funding

This research was supported by the Polish National Science Centre (NCN) [grant number: DEC-2011/01/B/NZ4/04862].

Disclosures

The authors declare that there is no conflict of interest.

Acknowledgments

We are indebted to Krystyna Filończuk and Ewa Waszkiewicz for expert technical assistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jjcc.2015.10.001.

References
