Expression of N-cadherin in myocardial tissues during the development of a rat heart

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ABSTRACT. We investigated the expression and distribution of N-cadherin during the development of a rat heart. Immunohistochemistry (IHC) was performed to detect the expression and distribution of N-cadherin in the myocardial tissues of rats at embryonic day 18 (E18d), postnatal day 5 (P5d), postnatal day 19 (P19d), postnatal day 40 (P40d), and postnatal year 1 (P1y). Reverse transcription polymerase chain reaction was used to determine mRNA expression levels of N-cadherin in the myocardial tissues at E18d, P5d, P19d, P40d, and P1y. The IHC results showed that at E18d N-cadherin was dispersedly distributed both on the cell surface and in the cytoplasm of the myocardial cells, and gradually became concentrated at the end-to-end intercalated discs of the cardiomyocytes from birth through immaturity. In the young, middle-aged, and old rats, N-cadherin was typically distributed at the

intercalated discs at the end of the myocardial cells. No significant differences in the mRNA expression levels of N-cadherin were detected in the myocardial tissue of rats at E18d, P5d, P19d, P40d, and P1y. During the development of the rat heart, observable changes in the distribution of N-cadherin occurred in the myocardial tissues, but there were no detectable changes in the expression of N-cadherin, indicating that N-cadherin is indispensable to maintaining the physical structure and function of the heart.

Key words: N-cadherin; Intercalated disc; Cardiomyocyte; Rat; Heart development

INTRODUCTION

Cadherins are a family of calcium-dependent cell-surface glycoproteins that mainly mediate cell-cell adhesion. More than 100 members of the cadherin family have been identified (de Simone et al., 2000; Vakili et al., 2001). Cadherins are ubiquitously expressed in the embryonic and adult tissues of a variety of organisms, from the lower animals, such as various insects and nematodes, to higher animals, such as vertebrates. Cells expressing the same cadherins are detected by those expressing other cadherins by cadherin-cadherin homologous interactions, resulting in the formation of different cell compartments with different adhesion properties, which establishes a basis for organ formation during embryogenesis (Ashrafian et al., 2007; Swynghedauw et al., 2010). In addition, in early embryonic development, cadherins are also involved in cell sorting, mediating cell aggregation, and maintaining cell polarity, all of which are important for the genesis and development of organs and tissues (Ashrafian et al., 2007; Swynghedauw et al., 2010; Yan et al., 2011). Cadherins mainly consist of extracellular, transmembrane, and cytoplasmic domains. According to the characteristics of tissue distribution of cadherins and the tissues from which cadherins were initially isolated, cadherins are classified into three types: epithelial cadherin (E-cadherin), neural cadherin (N-cadherin), and placental cadherin (P-cadherin) (Levy et al., 1990; Frey et al., 2004; Satoh et al., 2006). E-cadherin is predominantly distributed in adult epithelial cells. P-cadherin is mostly found in the placenta and epithelial cells, and is also transiently expressed in multiple organs and tissues during their development. N-cadherin is found primarily in the nerve and muscle tissues. The first cadherin gene was identified by Takeichi (1991). more than 30 years ago (Gumbiner, 1996; Molkentin and Dorn, 2001; Olson and Schneider, 2003). As research continued, it was discovered that cadherin function is not limited to mechanical adhesion between cells, but extends to cell localization, proliferation, and differentiation; cadherin relays extracellular stimuli to intracellular signaling molecules through cadherin-catenin-cytoskeleton interactions (Gumbiner, 1996; Tepass, 1999; Yagi and Takeichi, 2000; Ivanov et al., 2001). Cell-cell adherens junctions (AJs) have been shown to play important roles in cell recognition, adhesion, and tissue morphogenesis. In the myocardial cells, the muscle cells are joined mechanically together by N-cadherin-mediated AJs, which also provide the anchor points for the cytoskeleton within the cytoplasm, thus maintaining the structural integrity and polarity of the tissue in the adult organism (Zhong et al., 1999; Ivanov et al., 2001). In adult rat heart tissues, AJs, gap junctions (GJs), and desmosomes are co-localized at the intercalated discs in specific areas of the plasma membrane of cardiomyocytes in different ways. The

changes in AJ dynamics and the expression levels of AJ-related proteins always occur during the development of the intercalated discs (Yagi and Takeichi, 2000). However, few studies have focused on the mechanisms by which AJs are concentrated at the intercalated discs. Therefore, in the present study, we investigated the distribution and age-related changes of N-cadherin during the development of the intercalated discs in cardiomyocytes. Our objective was to provide morphological data to clarify the mechanisms of the development of the intercalated discs, and the construction and physiological functions of the myocardial cells.

MATERIAL AND METHODS

Animals

A total of 25 adult nulliparous female Kunming rats (each weighing 180-220 g) obtained from the Laboratory Animal Centre of Xinxiang Medical University were used in this study. Rats were fed with pelleted rat chow and water provided *ad libitum*. At 8:00 p.m. to 10:00 p.m., the female rats were caged with the males (one female and one male/cage), and checked for the presence of vaginal plugs the next morning. The day on which the vaginal plug was detected was considered day 1 of pregnancy. The rats were randomly assigned to five groups (each comprising five rats): embryonic day 18 (E18d), postnatal day 5 (P5d), postnatal day 19 (P19d), postnatal day 40 (P40d), and postnatal year 1 (P1y). For the groups E18d and P5d, all ventricles were taken from each rat. Part of the left ventricle was taken from each rat in the groups P19d, P40d, and P1y.

Immunohistochemistry assay

Myocardial tissues were routinely embedded in paraffin and sliced, and then an immunohistochemistry assay was performed according to the manufacturer protocol. Briefly, the sections were incubated with an anti-N-cadherin primary antibody (1:100, Wuhan Boster Biological Engineering Company, Wuhan, Hubei Province, China) overnight at 4°C. After washing with phosphate-buffered saline (PBS), a second incubation with biotin-conjugated rabbit anti-goat IgG was performed at 37°C for 30 min, and this was followed by further washing with PBS. Sections were then incubated with a streptavidin-avidin-biotin complex (Wuhan Boster Biological Engineering Company, Wuhan, Hubei Province, China) solution at 37°C for 30 min. After incubation with a 3,3'-diaminobenzidine solution to detect immunostaining, the sections were stained with hematoxylin and examined under a light microscope. Brown cells indicated positive expression. For negative controls, the primary antibody was replaced with PBS. Five myocardial specimens were selected from each group, five sections were selected from each specimen, and four visual fields with uniform distribution and staining of myocardial tissue were randomly selected from each section. A total of 100 visual fields were selected from each group. The quantitative analysis for the grayscale values from the selected visual fields was performed at 400X magnification using the Motic BA400 pathological image analysis system.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from the myocardial tissues according to the instructions

provided with an AxyPrep total RNA Miniprep kit. Ultraviolet (UV) absorbance at 260 and 280 nm was determined using a UVmini-1240 UV spectrophotometer to evaluate the concentration and purity of each RNA sample. RNA integrity was detected by 1% agarose gel electrophoresis. The following primers were used for PCR amplification.

The forward and reverse primers corresponding to rat N-cadherin were as follows: 5'-TGTTGCTGCAGAAAACCAAG-3' (sense), 5'-TTTCACAAGTCTCGGCCTCT-3' (antisense), generating a 460-bp cDNA fragment. The sequences of the primers for rat β-actin, yielding a 206-bp cDNA product, were as follows: 5'-CACCCGCGAGTACAACCTTC-3' (sense) and 5'-CCCATACCCACCATCACACC-3' (antisense). These primers were designed by the Primer Premier 5.0 software, synthesized by the Shanghai Biological Engineering Technology Co., Ltd., and purified by polyacrylamide gel electrophoresis. The primers were dissolved in RNase-free water to a final concentration of 100 μM. The primer solutions were preserved at -70°C until required. The first strand of cDNA was synthesized according to the instructions provided with an M-MLV reverse transcriptase first-strand cDNA synthesis kit. The cDNA fragment of N-cadherin was then amplified by PCR using β-actin mRNA as an internal standard. The PCR program was as follows: 95°C for 5 min; 35 cycles of 94°C for 30 s, 55°C for 30 s, and an extension at 72°C for 10 min. The PCR products obtained were electrophoresed on a 1.5 % agarose gel and stained with ethidium bromide at the final concentration of 0.5 mg/mL. The results of the electrophoresis were scanned and recorded using the Gelworks software from the Syngene Gene Genius gel imaging analysis system. The semi-quantitative analysis of N-cadherin mRNA expression was carried out by establishing the ratio of the grayscale values of the target fragment to the β-actin fragment.

Statistical analysis

All experiments were repeated in triplicate, and all data are reported as means ± standard deviation. Statistical analyses were performed with the SPSS17.0 software. The difference of means between multiple groups was assessed by one-way analysis of variance. Statistical significance was established between two groups using the least-significant difference test. Differences were considered to be statistically significant at $P < 0.05$.

RESULTS

Expression levels of N-cadherin mRNA in myocardial tissue during rat heart development

The 460-bp N-cadherin-specific DNA fragments were successfully amplified in the groups E18d, P5d, P19d, P40d, and P1y, but no significant differences in N-cadherin mRNA expression levels were detected between the groups E18d, P5d, P19d, P40d, and P1y ($P > 0.05$) (Figure 1, Table 1).

Developmental changes in the distribution and expression of N-cadherin protein in myocardial tissues

In the myocardial cells at E18d, N-cadherin was distributed both on the cell surface and in the cytoplasm in a punctuated and scattered pattern. N-cadherin was then gradually

concentrated at the end-to-end intercalated discs of rat cardiomyocytes from P5d to P19d. At P40d and P1y, N-cadherin was found to be typically located at the intercalated discs at the end of the myocardial cells. However, no significant differences in the protein expression levels of N-cadherin were detected in the myocardial tissues of rats at E18d, P5d, P19d, P40d, and P1y ($P > 0.05$) (Figure 2).
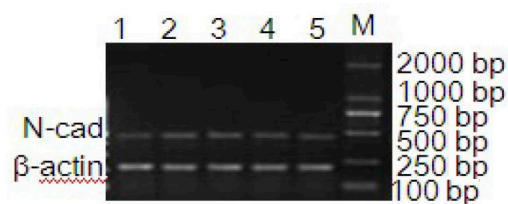

Figure 1. Expressions of N-cadherin mRNA in myocardial tissue during the development of the rat heart. *Lane M* = marker, *lane 1* = E18d; *lane 2* = P5d; *lane 3* = P19d; *lane 4* = P40d; *lane 5* = P1y.

Table 1. Average optical densities (means ± SD, N = 5) of N-cadherin revealed by immunohistochemistry at the different developmental stages of the rat heart tissue.

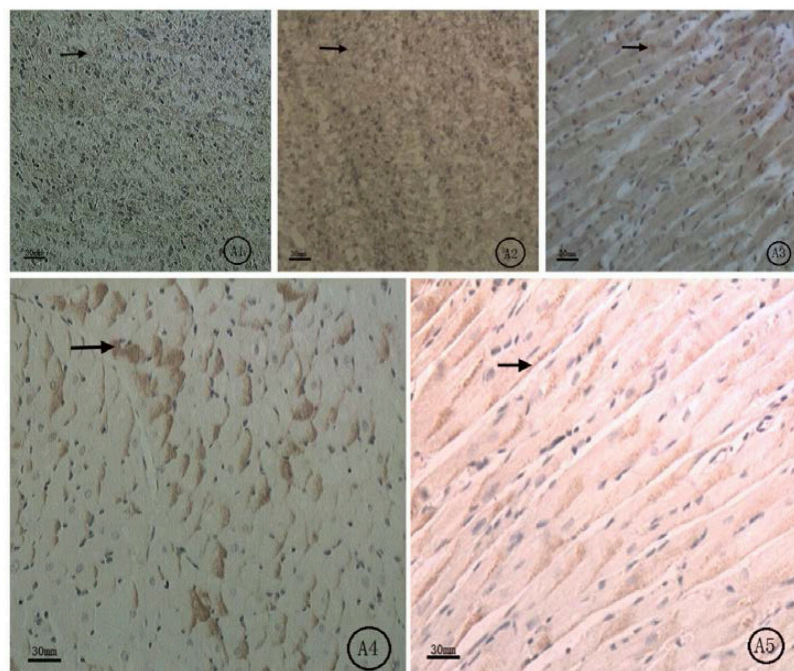| Developmental stage	E18d	P5d	P19d	P40d	P1y
Optical density of N-cadherin	0.65 ± 0.05*	0.71 ± 0.06*	0.59 ± 0.09*	0.61 ± 0.08*	0.56 ± 0.05*

*$P > 0.05$.

Figure 2. Expression of N-cadherin in myocardial tissue revealed using immunohistochemistry at magnification 400X; Bar = 10 μm. A1: E18d; A2: P5d; A3: P19d; A4: P40d; A5: P1y.

DISCUSSION

Despite studies on the development of cardiac intercalated discs in small mammals by electron microscopy, the integral distributions of GJ and AJ on the cell surface and throughout a single cardiomyocyte, and the changes in the distribution pattern of the junctions after birth, are still not well defined (Gumbiner, 1996; Ivanov et al., 2001; Molkentin and Dorn, 2001). In the early stage of rapid heart growth, changes in the junction structure occur with maturation of intercalated discs to maintain electrical and mechanical coupling between myocardial cells. The ability of myocardial cells to adapt to physiological changes may involve the formation of appropriate junctions by which cells adhere to, and then communicate with, each other (de Carvalho and Taboga, 1996; Sommers, 1996; Langeggen et al., 2002). N-cadherin, a Ca^{2+}-dependent cell adhesion molecule, is predominantly expressed in myocardial cells, and changes in the expression and distribution of N-cadherin occur during heart development. N-cadherin-mediated AJs are located in the mature intercalated discs of myocardial cells, and play crucial roles in cell recognition, migration, and the regulation of the responses to cell growth and differentiation signals (Tepass et al., 2000; Chaklader et al., 2013).

Linask (Rosales and Juliano, 1995; Suzuki, 1996) found that the disordered expression of N-cadherin inhibited early development of the heart and the occurrence of myocardial cells in a study on chicken embryos. Our results showed that, at E18d, P5d, P19d, P40d, and P1y, there were no significant differences in the mRNA expression levels of N-cadherin in the myocardial cells between the different time points.

Our results showed that the cell adhesion molecule N-cadherin was expressed in the myocardium in all rat cardiac chambers. In the myocardial cells at E18d, N-cadherin, usually granular in shape, was evenly distributed both on the cell surface and in the cytoplasm. Then, at P5d, it was easy to distinguish the regions adjoining the end of the myocardial cell, those at the end-to-end of the myocardial cell, or those at the sides of the myocardial cell because the ventricular myocytes elongated and then the contracted linear myofilaments began to form. At P19d, the ends of the myocardial cells gradually became clear, but the number of ends decreased, and the ends could be identified by their morphology. In the neonatal rat myocardial cells, N-cadherin was distributed not only in the cytoplasm, but also at the lateral junctions of the cells and the transverse junctions at the end-to-ends of the myocardial cells. In addition, from P5d to P19d, the expression levels of N-cadherin at the transverse junctions located at the end-to-ends of the myocardial cells rapidly increased. At the same time, N-cadherin rapidly developed to the adult form, which not only better fulfills the requirements of the adult ventricle, but also reflects the ability of the cardiac muscle to adapt to hemodynamic changes. N-cadherin was typically located at the mature intercalated discs of the rat myocardial cells in a zipper-like pattern. When the typical rat intercalated discs formed at P40d, the expression of N-cadherin at the end of the myocardial cell reached a maximum, suggesting dynamic changes in the distribution of N-cadherin and N-cadherin-mediated cell-cell junction structures in the development of the rat heart. These results indicate that the spatial and temporal distribution pattern of N-cadherin is consistent with that of the development of the intercalated disc, which adapts to the functions of the cardiac muscle at the different stages of development. Our data also showed that N-cadherin is distributed at the intercalated discs and forms a zipper-like structure, revealing that the transmembrane molecule N-cadherin enhances cell-cell contact and provides enough membrane area (Takeichi, 1991; Uemura, 1998) for cell-cell junctions

in establishing the contacts between myocardial cells. The results suggest that the expression pattern of N-cadherin plays a key role in the early development of the rat heart and in the reconstruction of myocardial cells, and the change in the distribution of N-cadherin mediated-AJ at the intercalated disc may be an important factor for the maturation of cardiac electromechanical function in mammals.

The transmembrane protein N-cadherin may enhance cell-cell contact and provide enough membrane area for cell-cell junctions, which adapt to maintain the physiological structure and mechanical function of the heart, indicating that N-cadherin protein might be required for heart survival.

Conflicts of interest

The authors declare no conflict of interest.

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