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Myosin-Activating Protein Kinases are Possible Regulators of Nonmuscle Myosin in Developing Human Heart

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We studied the localization of myosin-activating protein kinases in cardiomyocytes obtained from fetal human heart at 8-9 weeks gestation. It was found that at this developmental stage, smooth muscle/nonmuscle myosin light chain kinase (MLCK, 108 kDa) and its high-molecular weight isoform (MLCK, 210 kDa), skeletal MLCK and death-associated protein kinase (DAPK) are co-localized with nonmuscle myosin IIB in the premyofibrils. The data obtained suggest that cardiac nonmuscle myosin at 8-9 weeks gestation may serve as the substrate of the studied myosin-activating protein kinases that are likely to cooperatively regulate the formation of myofibrils. We revealed high-molecular weight isoform of smooth muscle/nonmuscle kinase MLCK-210 in developing human heart and determined the ratios of MLCK-108 and MLCK-210 at different gestational stages. In this case, the approximate time period of changes in these isoforms ratio was revealed (between 8-9 and 13 weeks), that can be associated with functional changes in the developing myocardium.

Key Words: *myosin-activating protein kinases; sarcomerogenesis; premyofibril; fetal cardiomyocytes*

Myofibrils of mature human cardiomyocytes consist of many basic contractile units, sarcomeres. Transformation of premyofibrils to sarcomere-containing myofibrils and replacement of constituting nonmuscle/smooth muscle proteins with sarcomeric ones are the key stages of sarcomerogenesis (*i.e.* formation of myofibrils; myofibrillogenesis). There is evidence that premyofibrils stability is an important prerequisite of the sarcomerogenesis in embryonic cardiomyocytes [3]. However, the regulatory mechanisms of sarcomerogenesis underlying stabilization of premyofibrils are poorly understood. Nonmuscle myosin IIB (NMIIB) is a basic protein of premyofibrils. Its phosphorylation is a mandatory condition of the formation of stable filaments. It was demonstrated *in vitro* that NMIIB can be phosphorylated by smooth muscle/nonmuscle myosin light chain kinase (MLCK) [7]. There is experimental evidence that skeletal MLCK and MLCK-108 can be actively involved in the assembly of new myofibrils. For example, experiments on rat cardiomyocyte culture showed that inhibition of the enzyme activity of MLCK decelerates sarcomerogenesis, while overexpression of skeletal MLCK in cardiomyocytes enhances it [3]. These data support the fact that MLCK

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is a natural stabilizer of nonmuscle myosin. In addition to MLCK, several protein kinases with a broader specificity were revealed in the heart, which were capable to phosphorylate regulatory myosin light chains *in vitro* including DAPK and integrin-associated kinase (ILK) [4,6]. There is no consensus as to which of the myosin activating kinases are involved in the mechanisms of sarcomerogenesis regulation, providing the stability of premyofibrils in the developing human cardiomyocytes.

Here we studied the localization and content of myosin-activating protein kinases in developing human myocardium.

MATERIALS AND METHODS

We used cardiomyocytes of human fetuses (8-9 and 13 weeks gestation) provided by V. I. Kulakov Research Center of Obstetrics, Gynecology, and Perinatology, which is licensed to obtain this material. To identify the localization of myosin-activated protein kinases, the method of double indirect immunofluorescence with computer processing of digital images was used. Fetal cardiomyocytes at 8-9 weeks gestation were seeded on slides and after 3-fold washout from the growth medium in phosphate-buffered saline (FBS) were fixed in 3.7% formaldehyde for 2 min at room temperature. Then the cells were washed in FBS, permeabilized with 1% Triton X-100 for 2 min at room temperature, and washed again. After that the cells were incubated with primary antibodies in appropriate dilutions, washed in FBS, and incubated with secondary antibodies conjugated to fluorophore. Immunofluorescence images were obtained using a Axiovert 200M microscope (Carl Zeiss) and shot with a high-definition digital camera AxioCam.

The content and ratio of protein kinases were investigated by quantitative immunoblotting. Electrophoresis was performed by the method of Laemmli. Immunoblotting was carried out using PVDF membrane (Millipore) in Tris-glycine/ethanol buffer. Filters were washed in FBS and blocked with 5% skim milk in FBS with 0.2% Tween-20. Specific primary antibodies (1-4 μg/ml), secondary horseradish peroxidase-conjugated antibodies and chemiluminescent substrate for signal detection (Amersham) were used. The film with the signals was scanned using Epson Scan II software and processed using Scion Image software. The obtained data were processed with Microsoft Excel, standardizing intensities of protein kinase signals to that of glyceraldehyde-3-phosphate dehydrogenase (standardization of sample load). The final statistical analysis of the results was carried out using Graph Pad Prism 4 software.

RESULTS

Using the immunofluorescence approach, we determined the localization of myosin-activated protein kinases in developing human heart. In cultured cardiomyocytes from human fetal heart at 8-9 weeks gestation, skeletal MLCK is co-localized with nonmuscle myosin IIB along the entire length of premyofibrils (Fig. 1). MLCK-108 and its high-molecular-weight isoform MLCK-210 (embryonic myosin light chain kinase, 210 kDa) detected by us by immunoblotting are also localized along fibrillar structures positive for NMIIB (Fig. 1). Colocalization of studied MLCK with nonmuscle myosin suggests that nonmuscle premyofibrilar myosin is their possible substrate in the developing heart. It should be noted that skeletal MLCK is co-localized with nonmuscle myosin only at the early stages of cardiogenesis. Later its location becomes diffuse in the majority of cardiomyocytes (data not shown). Skeletal MLCK may be apparently involved in premyofibril stabilizing only on the early stages of myocardial development. High content of skeletal MLCK in embryonic human heart in comparison with the heart of adults revealed by us confirms the importance of skeletal MLCK in heart development [1]. It is possible that at later terms, the function of skeletal MLCK is performed by other enzymes and its expression decreases to complete shutdown of gene activity in the heart. Thus, in our previous studies we demonstrated the lack of MLCK in the myocardium of adult rats [2].

Recently, several new protein kinases phosphoryling myosin *in vitro* were found, including ILK and DAPK. We have found that DAPK is localized along NMIIB-positive fibrillar structures in cardiomyocytes of fetal human heart at 8-9 weeks gestation (Fig. 1). At the same period, ILK is detected in focal contacts of cardiomyocytes, where it is co-localized with vinculin (data not shown). It is known that this protein kinase is a structural protein that maintains the integrity of multiprotein complexes, plays a central role in transduction of biochemical signals initiated by cell–matrix interaction [6]. ILK apparently does not contribute to stabilization of NMIIB.

Thus, co-localization of MLCK-108, MLCK-210, skeletal MLCK, and DAPK with nonmuscle myosin of premyofibrils at the early stages of human cardiogenesis (8-9 weeks) revealed by us suggests that nonmuscle myosin IIB can serve as their possible substrate. Its phosphorylation by these protein kinases may be relevant when stabilizing premyofibrils in the developing heart.

In the course of the study, we identified high molecular weight isoform of smooth muscle/nonmuscle myosin light chain kinase, MLCK-210, in cardiomyocytes, which is also called embryonic [5]. To locali-

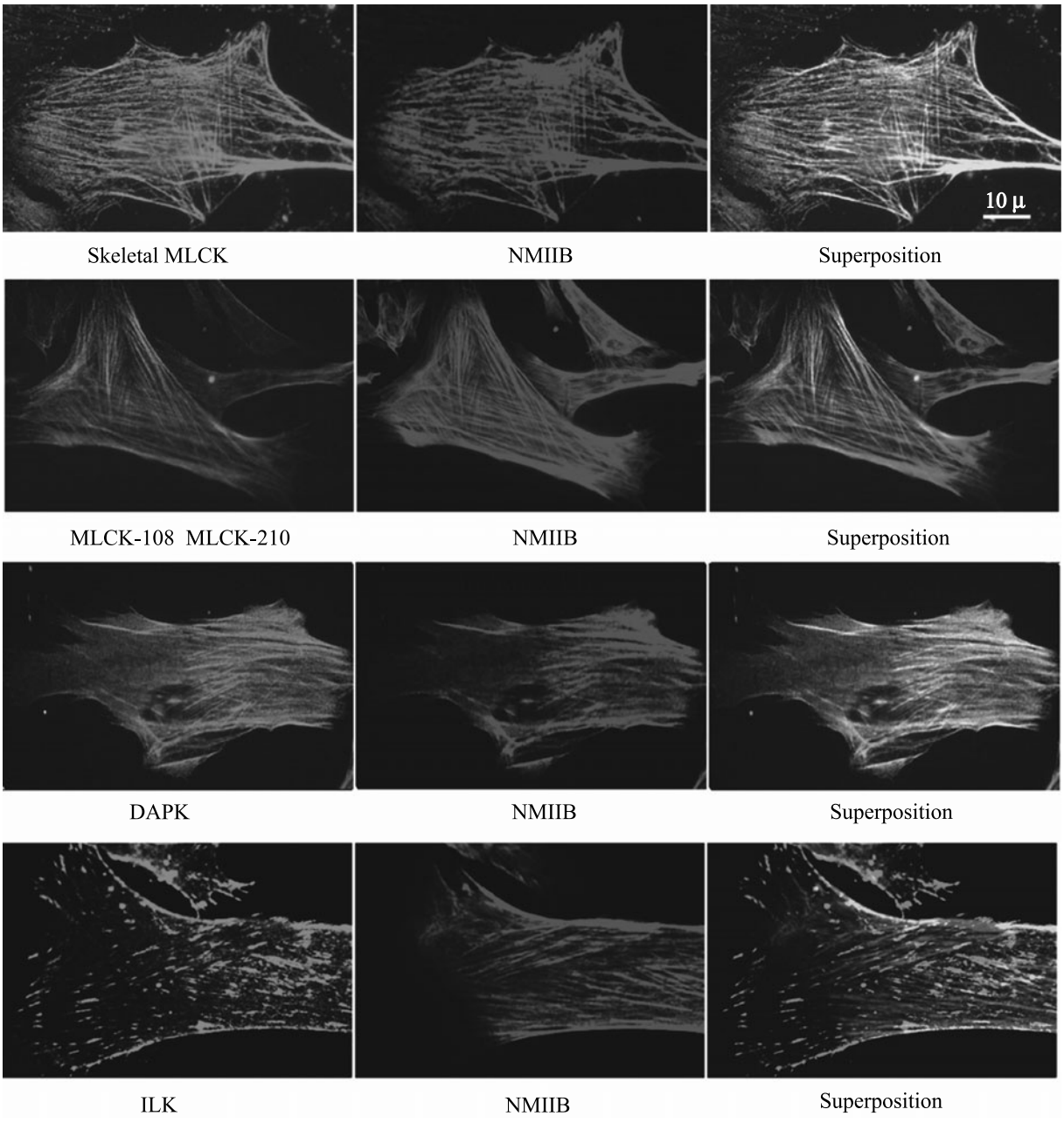

Fig. 1. The localization of nonmuscle myosin and myosin-activating protein kinases in human fetal cardiomyocytes. Skeletal MLCK: skeletal myosin light chain kinase; MLCK-108: smooth muscle/nonmuscle myosin light chain kinase; MLCK-210: embryonic myosin light chain kinase; DAPK: death-associated protein kinase; ILK: integrin-linked kinase; NMIIB: nonmuscle myosin IIB.

ze these isoforms by immunofluorescence, we used monoclonal antibodies (clone K-36, Sigma) that detect both isoforms. Thus, we revealed that their localization was the same (Fig. 1). To assess possible involvement of each isoform in stabilization of nonmuscle myosin, we used a method of quantitative immunoblotting. We studied the content of MLCK-108 and MLCK-210 and assessed their ratio in the cultured cardiomyocytes of fetal human heart at 8-9 and 13 weeks gestation. The levels of MLCK-108 were lower in cardiomyocytes at 8-9 weeks gestation and increased at 13 weeks gestation. On the contrary, the levels of MLCK-210 were higher in cardiomyocytes at earlier gestational age and decreased in cardiomyocytes at 13 weeks gestation. Their ratio was 60% MLCK-210 to 40% MLCK-108 in cardiomyocytes at 8-9 weeks gestation; 87% MLCK-108 to 13% MLCK-210 at 13 weeks gestation (Fig. 2). Thus, at earlier stages of development, both isoforms may stabilize premiofibrills. Later MLCK-108 apparently plays a key role in the formation of premyofibrils. Similar changes in MLCK-108 and MLCK-210 expression in developing

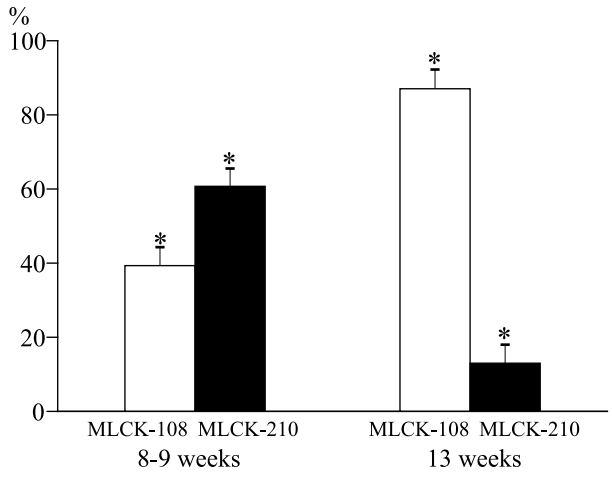

Fig. 2. Relative content of MLCK-108 and MLCK-210 in human fetal cardiomyocytes. *$p<0.05$.

human heart were revealed in the study of embryonic, neonatal, and adult mouse hearts [5]. In this work, the authors compared the expression levels of two MLCK isoforms. In neonatal heart, decreased 6.3-fold content of embryonic MLCK-210 compared with prenatal heart was shown, and this isoform was not expressed in the adult mouse heart. On the contrary, expression of MLCK-108 increased 5-fold in the adult heart as compared to embryonic. These data also suggest the modification of the functional value of smooth muscle/nonmuscle myosin light chain kinase isoforms at the early stages of cardiogenesis.

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