Unique Expression Patterns of Myosin Heavy Chain Genes in the Ductus Arteriosus and Uterus of Rabbits

Hisanao SAKURAI, Shin-ichiro IMAMURA, Yoshiyuki FURUTANI, Atsuyoshi TAKAO, Kazuo MOMMA and Rumiko MATSUOKA*

Department of Pediatric Cardiology and 1) Research Division, The Heart Institute of Japan, Tokyo Women’s Medical University, 8–1 Kawada-cho, Shinjuku-ku, Tokyo 162–8666, Japan

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ABSTRACT. In smooth muscle tissue, two smooth muscle myosin heavy chain (MHC) isoforms (SM1, SM2) and two non-muscle MHC isoforms (NMA, NMB) have been identified. The purpose of our study was to clarify whether smooth muscle MHC mRNA expression reflects the physiological and functional state of the muscle. We studied the expression pattern of MHC mRNAs, using the S1-nuclease mapping procedure, in functionally and morphologically changeable organs; the ductus arteriosus (DA) during development (25 and 29 days of gestation, and from 3-day-old neonates) and uteri from virgin, day-10 pregnant (P10) and day-29 pregnant (P29) rabbits. The results demonstrated that SM2 expression was greater in the fetal DA than in the fetal aortic and pulmonary arteries, but that it decreased significantly following closure of DA. In the gravid uterus, SM1 expression was significantly (P<0.05) strong compared to other MHC mRNAs from virgin to P10 rabbits. During pregnancy, NMB expression showed a tendency to increase until P10, and after P10, SM2 expression increased dramatically and NMB expression decreased to give almost a mirror image of the SM2 expression. Smooth muscle type (SM1, SM2) was significantly (P<0.05) strong compared to non-muscle type expression (NMA, NMB) at P29. These data suggest that smooth muscle MHC mRNA, especially SM2 expression reflects the physiological and functional state of the smooth muscle.—KEY WORDS: ductus arteriosus, myosin heavy chain, non-muscle, smooth muscle, uterus.


Myosin is a hexamer composed of two heavy chains of about 200 kDa and four light chains of about 16–27 kDa. It is a major cytoskeletal protein, which is found in the contractile apparatus in muscle cells as well as in non-muscle cells. In smooth muscle tissue, protein and molecular data provide evidence for two smooth muscle myosin heavy chains (MHCs), designated as SM1 and SM2, approximately 204 kDa and 200 kDa, respectively, which are the products of alternate mRNA splicing differing in their carboxy termini [2, 21, 23, 30], and two non-muscle MHCs, designated as NMA and NMB, approximately 196 kDa and 198 kDa, respectively [13, 14, 16, 27].

Expression of smooth muscle MHCs and/or non-muscle MHCs are unique between species [9], organs [4, 5, 10, 24] and developmental stages [7, 10, 24]. The exact function of the four MHCs (SM1, SM2, NMA and NMB) is still under study, but they appear to play a role in cytokinesis, cell motility and cell shape change [29] and specialized cellular functions [18, 20].

Based on the results of previous studies, the purpose of our study was to clarify whether smooth muscle MHC mRNAs expression reflects the physiological and functional state of the muscle. Therefore, we examined the expression of two MHC mRNAs in the ductus arteriosus (DA) during development, and the expression of four MHC mRNAs in the uterus during pregnancy as unique functions. The DA is a unique vessel, consisting largely of dense layers of smooth muscle, that closes after birth, whereas the aortic artery (Ao) and pulmonary artery (PA) are composed mainly of elastic fibers. On the other hand, the uterus follows a hypertrophic and hyperplastic process as a result of chronic stretching induced by the ingrowing fetus. Therefore, the DA and the uterus offer an excellent model system to study MHC gene expression.

MATERIALS AND METHODS

Tissue samples: Female adult Japanese white rabbits and their fetuses were used for these studies. DA, Ao and PA were obtained from fetuses at 25 and 29 days of gestation (G25 and G29) and from 3-day-old neonates. Uteri from virgin rabbits and from day 10 (P10) and day 29 (P29) pregnant rabbits were also obtained. The outer layer and the endothelium were mechanically removed from the blood vessels and only the medial layer (smooth muscle) was subjected to our analysis. These samples were immediately frozen in liquid nitrogen and stored at -80°C until use.

Total RNA extraction: Total RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction [6] was performed on the tissue samples. The tissues were minced on ice and homogenized in denaturing solution [solution D (1 ml/100 mg tissue)]. Solution D contained 4 M guanidinium thiocyanate (Fluka Chemika Biochemika, Buchs, Switzerland), 25 mM sodium citrate (Kanto Chemical Co., Inc., Tokyo, Japan), pH 7, 0.5% sarcosyl.

* Correspondence to: Dr. MATSUOKA, R., Department of Pediatric Cardiology, The Heart Institute of Japan, Tokyo Women’s Medical University, 8–1 Kawada-cho, Shinjuku-ku, Tokyo 162–8666, Japan.
(Sigma Chemical Company, St. Louis, MO, U.S.A.), and 0.1 M 2-mercapto ethanol (Sigma Chemical Company, St. Louis, MO, U.S.A.). Sequentially, 0.1 ml of 2 M sodium acetate, pH 4, 1 ml of phenol (water-saturated), and 0.2 ml of chloroform-isoamyl alcohol mixture (49:1) were added to the homogenate, with thorough mixing by inversion. The final suspension was shaken vigorously for 10 sec and cooled on ice for 15 min. Samples were centrifuged at 10,000 g for 20 min at 4°C. The aqueous phase was transferred to a fresh tube, mixed with an equal volume of isopropanol, and then stored at -20°C for at least 1 hr. Sedimentation at 10,000 g for 20 min was again performed, and the resulting RNA pellet was dissolved in 0.3 ml of solution D and precipitated with 0.3 ml of isopropanol at -20°C for 1 hr. After centrifugation for 10 min at 4°C, the RNA pellet was washed with 75% ethanol, sedimented, vacuum-dried (2 min) and dissolved in TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). The RNA suspension was stored at -20°C after 1/10 volume of 2 M sodium acetate (pH 4) and 2.5 volume of 100% ethanol were added.

Probes for S1 nuclease mapping: Single-strand DNA to yield 5’ phosphoryl mono- or oligonucleotides is degraded by S1 nuclease, while double-stranded RNA and DNA-RNA hybrids are resistant to the enzyme. Using this principle, we prepared two types of S1 probes (SM70 and RANMB) and performed S1 nuclease mapping analysis.

SM70 (Fig. 1a) is an oligonucleotide of 70 nucleotides (nt) (antisense sequence of SM2), including a 4 mers extra sequence, which was synthesized using a DNA synthesizer (Cyclone plus DNA synthesizer, PerSeptive BioSystems, Inc., Cambridge, Mass, U.S.A.). The 5’ end of this probe was labeled with γ-32P-ATP (Amersham International plc, Buckinghamshire, UK).

The 771 bp rabbit non-muscle type A (RANMMHCA) and B (RANMMHB) were produced by the polymerase chain reaction (PCR) using rabbit thymus and brain total RNA, respectively, as templates. Two pairs of primers taken from the sequence of the human non-muscle type A cDNA (nt 1164 and 1935) and type B cDNA (nt 980 and 1751), one pair from each [27], were synthesized using a DNA synthesizer. The nucleotide sequences of RANMMHCA and RANMMHB were determined by the dyeoxy-chain termination method [25] and are available through DDBJ (GenBank/EMBL) Access Number D63693 and D63694. Then RANMMHCB was subcloned into the pBluescript II SK(-) plasmid (Stratagene, La Jolla, CA, U.S.A.), and a pair of primers was synthesized as complementary 25 mers (TTCAACCAACACATGTTATCCTCCTTG). PCR was performed, using RANMMHCB as a template, and a 166 bp fragment was obtained, which included an extra 10 bp sequence (RANMB: Fig. 1b). The RANMB probe was labeled with α-32P-ddATP (Amersham International plc, Buckinghamshire, UK) at the 3’-end and strand-separated on a 5% acrylamide gel.

In order to prepare radioactively equivalent SM70 and

Fig. 1. a: The top schematic representation shows rabbit smooth muscle cDNA. The 39 nucleotides (nt) from SM2 were inserted at the nucleotide position 5884 in SM1 (6644 in total size [2]). The bottom sequence indicates the SM70 probe (70 nt) used in this study. The probe contains a 66 nt sequence of SM2 and a 4 nt extra sequence. In the S1 nuclease mapping analysis, SM70 was recognized as a fully protected 66 nt fragment (SM2) and also as a partially protected 36 nt fragment (SM1). b: The sequence indicates the RANMB probe, consisting of 166 nt, including an extra 10 nt sequence. RANMMHCB is a 771 bp sequence located at the end of the head portion of the non-muscle type B MHC. The RANMB was amplified using RANMMHCB as a template. In the S1 nuclease mapping analysis, RANMB was recognized as a fully protected 156 nt fragment (NMB) and a partially protected 112 nt fragment (NMA).

RANMB probes, γ-32P-ATP [5,000 Ci/mmol] and α-32P-ddATP [5,000 Ci/mmol] with the same examination dates were used on the same day to label the probes. The labeled DNA probes were counted and diluted to 3 × 10^4 cpm per μl with hybridization buffer (85% deionized formamide, 0.05% SDS, 0.4 mM EDTA, 10 mM PIPES, pH 6.4).

S1 nuclease mapping: RNA-DNA hybridization, using 3 × 10^4 cpm of DNA probes and 15 μg of the total tissue RNA extract was carried out for 22 hr at 42°C in 85% formamide. S1 nuclease (Takara Shuzo Co., Ltd., Kyoto, Japan, 170 units) digestion was performed for 1 hr at 25°C and the digestion products were run on 8% polyacrylamide/8.3 M urea sequencing gels [3].

Densitometry: To equalize the total RNA in each sample, a part (1.8 μg) of the total RNA (15 μg) in each sample was run on a Northern blot gel (1% agarose) at 4°C. Photographs of the ethidium bromide-stained gels were scanned, using
RESULTS

The expression pattern of MHC genes in DA during development: Figure 2 shows the gene expression ratio of SM2/(SM1+SM2) in Ao, PA and DA at G25, G29 and in 3-day-old neonates. Percent values of SM2 expressed in DA were 18.8 ± 0.2% at G25, 19.0 ± 3.6% at G29, and 9.9 ± 0.1% at the neonatal stage. The data showed that in the fetal DA, the gene expression ratio of SM2/(SM1+SM2) was significantly (p<0.05) large compared to Ao and PA, and that it decreased significantly (p<0.05) after birth.

Our previous study showed that in Ao and PA, although the gene expression ratio of SM2/(SM1+SM2) was not significantly different between the fetal stage and after birth, it increased dramatically [24]. These data indicate that the SM2 expression pattern of DA is different from that of Ao and PA.

Expression pattern of MHC genes in the uterus during pregnancy: To clarify whether the smooth muscle MHC mRNAs expression reflects the physiological and functional state of the muscle and whether a particular SM2 expression pattern relates to cell contractility, we used the uterus during pregnancy for further studies.

The expression of MHC genes in the uterus obtained from virgin, P10, and P29 rabbits was analyzed (Figs. 3a-c). Figure 3a shows an actual gel used to generate quantitative data. The analysis of radioactive densities at each stage is shown in Fig. 3b. The gene expression ratio plots of the 4 types of MHC mRNAs (Fig. 3c) were similar to their radioactive density plots (Fig. 3b). SM1 expression was significantly (p<0.05) strong compared to other MHC mRNAs from the virgin to P10 stages. Although SM1 was not significantly different during pregnancy, it showed a tendency to decrease at P10. NMB expression during pregnancy was not significantly different from virgin to P29, but it tended to increase at P10 to almost a mirror image of SM2 expression. Furthermore, SM2 expression increased significantly (p<0.05) after P10 (Fig. 3c), and smooth muscle type expression (SM1, SM2) was significantly (p<0.05) strong compared to the non-muscle type (NMA, NMB) at P29.

DISCUSSION

The microscopic structure of DA which consists largely of dense layers of smooth muscles is quite different from that of the adjacent Ao or PA which are composed mainly of elastic fibers [11]. Our previous study showed that the gene expression of four MHC mRNAs in the fetal DA was apparently strong compared to other large vessels before birth [24], and this result is consistent with the microscopic structure of DA. In this study, we also found that SM2 expression in the fetal DA was strong compared to Ao and PA, which decreased significantly (p<0.05) after birth. Since 1) the expression of the four MHC mRNAs in the rabbit vascular system was developmentally regulated [24] and 2) SM2 expression appears clearly before birth in DA and umbilical artery [15, 24], it may be reasonable to assume that DA is also regulated developmentally and differentiates precociously compared to Ao and PA. Therefore, preparation for ductal closure may require to increase in SM2 expression before closure of DA.

Using the uterus during pregnancy, we also studied the correlation between the expression of the four MHC mRNAs. During the early days of pregnancy, only NMB expression appeared to increase, conversely, SM1 expression appeared to decrease until P10. Subsequently, SM2 expression increased significantly. The uterus at P29 was thought to be more differentiated than at virgin, since increased SM2 expression should be a useful molecular
marker to detect the smooth muscle differentiation in the development [15, 24]. Furthermore, the temporal changes in NMB expression were roughly a mirror image of SM2 expression. In order to understand this interesting phenomenon, it may be important to understand changes from smooth muscle to non-muscle MHC isoforms in cultured smooth muscle cells of pregnant rabbit uterus which have been suggested to be due to a shift of the cells from the contractile to the mitotic state [22, 26]. Furthermore, SMemb (NMB) appears in adult rabbit aortic tissue after induced smooth muscle cell proliferation in experimental atherosclerotic and arteriosclerotic lesions [17]. Thus, the initial increase in NMB expression in the rabbit uterus may be necessary for the organ to proliferate smooth muscle cells, and it also may provide an explanation for the mirror image pattern between NMB and SM2 expression and for the increased SM2 expression after P10. These changes seem to be important as a preparative process for giving birth. The SM2 expression pattern in DA before birth and the MHC expression pattern in the uterus during pregnancy, indicates that the MHC expression pattern may reflect functional and physiological states. Based on above results, it is interesting to note that a correlation exists between SM2 gene expression and smooth muscle contractility.

The primary trigger for smooth muscle contraction is an increase in sarcoplasmic free Ca\(^{2+}\) concentration. This activates calmodulin-dependent myosin light chain kinase, which catalyses myosin phosphorylation, thereby activating crossbridge cycling and the development of force or contraction of the muscle cell [1]. However, the exact mechanisms regulating contractile response of smooth muscle remain unsolved. Some investigators have attempted to define the relationship between the contractile properties of smooth muscles and their MHC components [8, 19, 20, 28]. Meer and Eddinger [19] reported that increased SM2 expression appears to impart to the MHC the ability to attain a shorter final length following maximal contraction. This result certainly appears to explain the change in SM2 expression in this study. In an in vitro study, we have previously shown that SM2 expression in cultured rabbit aortic smooth muscle cells increases when the cells are mechanically stressed [12], suggesting a correlation between the SM2 expression and mechanical stress in vivo. Furthermore, our previous study showed that relatively large amounts of SM2 were present in the esophagus, stomach, small intestine and urinary bladder of adult rabbits, and that the smooth muscle of these organs is often stretched and exhibits vigorous contractile activity [24]. From these results, it may be tempting to speculate that SM2 expression

Fig. 3. a: Expression of smooth muscle (SM1, SM2) and non-muscle (NMA, NMB) myosin heavy chain mRNAs in rabbit uterus during pregnancy, shown by S1 nuclease mapping analysis (15 \(\mu\)g of total RNA was used). P: probe, 1: non-pregnant uterus (virgin), 2: 10-day-old pregnant uterus, 3: 29-day-old pregnant uterus. b: Radioactive densities of SM1, SM2, NMA and NMB expression in the uterus (n=4). c: The relative amounts of SM1, SM2, NMA and NMB expression in the uterus. For each stage, percent values (means ± SD) were calculated from radioactive densities of S1 nuclease mapping gel bands. ⧫: SM1, ⧫: SM2, ⧫: NMA, ⧫: NMB. * Significant at \(p<0.05\), ** Significant (vs all data at same stage) at \(p<0.05\) (** at P10 show that there is a significant difference between smooth muscle MHCs and non-muscle MHCs.), NS: not significant.
is related to the development of force or contraction of the muscle cell. The importance of the correlation between SM2 gene expression and smooth muscle contractility in rabbit awaits further experimental tests.

In conclusion, we suggest that smooth muscle MHC mRNA expression in rabbit reflects the physiological and functional state of the muscle.

In addition, it should be kept in mind that detailed studies on changes in MHC expression, such as in organs during development or the uterus during pregnancy, are not only needed for each organ and animal species, but also are important for elucidation of the correlation between the component of MHCs and the functional state of the muscle.

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