Developmentally Regulated Expression of Vascular Smooth Muscle Myosin Heavy Chain Isoforms*

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Two types of smooth muscle myosin heavy chain (MHC) isoforms, SM1 and SM2, were recently identified to have different carboxyl termini (Nagai, R., Kuro-o, M., Babij, P., and Periasamy, M. (1989) J. Biol. Chem. 264, 9734-9737). SM1 and SM2 are considered to be generated from a single gene through alternative RNA splicing. In this study we investigated expression of vascular MHC isoforms during development in rabbits at the mRNA, protein, and histological levels. In adults, all smooth muscle cells reacted with both anti-SM1 and anti-SM2 antibodies on immunofluorescence, suggesting the coexpression of SM1 and SM2 in a single cell. In fetal and perinatal rabbits, however, only anti-SM1 antibody consistently reacted with smooth muscles. Reactivity with anti-SM2 antibody was negative in the fetal and neonatal blood vessels and gradually increased during 30 days after birth. These developmental changes in SM1 and SM2 expression at the histological level coincided with mRNA expression of each MHC isoform as determined by S1 nuclease mapping, indicating that expression of SM1 and SM2 is controlled at the level of RNA splicing. However, sodium dodecyl sulfate-polyacrylamide gel electrophoresis of myosin from fetal and perinatal aortas revealed the presence of large amount of SM2. Interestingly, fetal SM2 did not cross-react with our anti-SM2 antibody on immunoblotting. We conclude that expression of SM1 and SM2 are differentially regulated during development and that a third type of MHC isoform may exist in embryonic and perinatal vascular smooth muscles.

Recent investigations have revealed polymorphism of many contractile proteins. Especially, myosin heavy chain (MHC) isomers of the cardiac and the skeletal muscles have been extensively studied. These MHC isomers are encoded by a multigene family, whose expression is regulated developmentally, hormonally, in a tissue-specific manner, and by mechanical stress (1-8). In smooth muscles, however, MHC heterogeneity has not been well defined until quite recently. Two putative MHC isoforms, designated as SM1 (204 kDa) and SM2 (200 kDa), were separated by Rovner et al. (9) on a porous SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Eddinger and Murphy (10) showed that the structural difference between SM1 and SM2 could lie in the light meromyosin by peptide mapping. Recently, Nagai et al. (11, 12) succeeded in isolating two types of smooth muscle MHC cDNA clones, SMHC40 and SMHC29, and suggested that SMHC40 and SMHC29 mRNAs originate from a single smooth muscle MHC gene through an alternative splicing mechanism at the 3' end of the coding region. Nagai et al. (12), furthermore, raised antibodies against two synthetic peptides deduced from the unique carboxyl termini of SMHC40- and SMHC29-type MHC and demonstrated by immunoblotting that SMHC40 mRNA is translated into SM1 and SMHC29 mRNA into SM2.

In order better to understand smooth muscle MHC gene expression, we tried in this study to determine whether or not expression of smooth muscle MHC isoforms is subject to developmental regulation as known for the cardiac and the skeletal muscle MHCs. Our results clearly demonstrate that expression of SM1 and SM2 is developmentally regulated in the vascular system. We further demonstrate evidence for the existence of a third type MHC isoform in the embryonic and perinatal vascular smooth muscles.

MATERIALS AND METHODS

Tissue Preparation—White rabbits were killed within 24 h and at 10, 20, 30 days, and 4 months after birth. Aortas were excised and frozen immediately after death. Aortas from fetuses of 23-28 days gestation were also obtained. Various vascular tissues, such as aorta, renal artery, pulmonary artery, and peripheral veins, were also prepared for immunofluorescence histochemistry.

Immunological Analysis of Smooth Muscle MHC Isoforms—Previously we reported anti-SM1 and anti-SM2 antibodies, C1 and C2, raised in rats using short peptides which are specific to the carboxyl termini of SM1 and SM2 (12). However, since these antisera were found inadequate for immunohistological studies, we reimmunized mice with the same synthetic peptides and obtained two new polyclonal antisera, 1-Se and 2-Si, specific to SM1 and SM2, respectively. Immunoblotting and indirect immunofluorescence were performed using 1-Se and 2-Si antisera as described (12-14).

Electrophoresis of Smooth Muscle Myosin—Myosin was extracted (15) from aortas of various developmental stages and subjected to SDS-PAGE (9). The gels were either stained with Coomassie Blue or the proteins were electrophoretically transferred to nitrocellulose membranes (14).

RNA Isolation and S1 Nuclease Mapping—Total RNA was isolated from aortas by the modified hot phenol procedure (16). S1 nuclease mapping was performed as described previously (11, 17). The single-stranded probe used in this study was the PstI fragment from the 3'-coding region and the 5'-untranslated region of SMHC29. This DNA probe is 206 nucleotides long and able to distinguish SMHC29 and SMHC40 mRNAs (11). It is expected that full protection of the probe by SMHC29 (SM2) mRNA is 224 nucleotides and the partially protected fragment which represents SMHC40 (SM1) mRNA is 79 nucleotides. Originally we estimated that the expected full length protection of this probe was 220 nucleotides and the partially pro-
Expression of SM1 and SM2 at the Histological Level—
Anti-SM1 (1-Se) and anti-SM2 (2-Si) antisera exhibited specific reaction on immunoblotting with each MHC isoform as shown in our previous report on rat antisera (12) (data not shown). When applied to frozen sections, 1-Se and 2-Si antisera stained all smooth muscle cells in the adult vascular system, suggesting that the two types of smooth muscle MHC isoforms are coexpressed in a single cell (data not shown). Those antibodies did not stain adult cardiac and skeletal muscles. Fetal and perinatal blood vessels showed a striking difference from adults in that the reactivity with anti-SM2 antibody was negative or very weak (Fig. 1). By 30 days after birth the intensity of staining was as strong as adults. On the other hand, the reactivity with anti-SM1 antibody was positive in all vascular smooth muscles at any stage from the fetus to the adult. This characteristic pattern of reactivity with anti-SM1 and anti-SM2 antibodies during development was similarly seen in other blood vessels so far examined such as pulmonary artery, renal artery, and systemic veins (data not shown).

Expression of SM1 and SM2 at the mRNA Level—
S1 nuclease protection analysis was performed to study SM1 and SM2 mRNA expression. To determine whether the partially protected fragment of the cDNA probe should correctly match the length of the common sequence between SM1 and SM2 mRNA, the S1 nuclease-digested hybrids were run next to a Maxam-Gilbert sequencing ladder of the same end-labeled DNA fragment. As shown in Fig. 2, the partially protected probe maps exactly the point of divergence between SM1 and SM2 mRNA. This indicates that the fully protected fragment of 224 nucleotides in this S1 nuclease assay represents SM2 mRNA and the partially protected fragment of 79 nucleotides represents SM1 mRNA. Expression of SM1 and SM2 mRNAs in aortas during development is demonstrated in Fig. 3. The fully protected S1 nuclease probe of 224 nucleotides was apparently undetectable in the fetal aortas and barely detectable in neonates. The intensity of the fully protected band was increased after birth, although even in 30-day-old rabbits the intensity seemed still weaker than in 4-month-old animals. To the contrary, the partially protected fragment of 79 nucleotides was demonstrated during all developmental stages. These results on SM1 and SM2 mRNA expression in aortas matched well with the histological expression of both MHC isoforms in the vascular system, suggesting that SM1 and SM2 expression is developmentally regulated by the amount of each mRNA which is generated through alternative RNA splicing.

Expression of SM1 and SM2 at the Protein Level—
To further test whether the observed expression patterns of SM1 and SM2 at the mRNA and histological levels are also seen at the protein level, we ran SDS-PAGE using myosin from aortas of all developmental stages (Fig. 4). To our surprise, in fetal and perinatal aortas SM2 was predominantly expressed at the protein level on SDS-PAGE. Only the aortas of 30-day-old rabbits showed a similar SM1 and SM2 expression pattern as adults. To examine the molecular characteristics of MHC isoforms expressed in the fetal and perinatal aortas, we carried out an immunoblotting analysis. Fig. 5 shows the reactivity of SM1 and SM2 of the fetal and neonatal aortas against three kinds of anti-smooth muscle MHC antibodies: nonspecific anti-smooth muscle MHC antibody (18), anti-SM1, and anti-SM2 antibodies. In fetal aortas, SM2 exhibited no cross-reactivity with our anti-SM2 antibody, while the same SM2 reacted with nonspecific anti-MHC antibody. Fetal SM1, on the other hand, was recognized by anti-SM1 antibody. In neonatal aortas, both SM1 and SM2 were recognized by individual isoform-specific antibody as in adult aortas. The same pattern was also obtained for SM1 and SM2 from rabbits of older than 10 days (data not shown). These data suggest that fetal SM2, at least, is a structurally different MHC molecule from adult SM2.

DISCUSSION

In the present study, we have demonstrated developmentally regulated expression of MHC isoforms, SM1 and SM2, in vascular smooth muscles. Taken mRNA expression together with the histological distribution of SM1 and SM2, we conclude that SM1 is more selectively expressed in aortas over SM2 during early development by differential alternative splicing of SM1 mRNA from the smooth muscle MHC gene transcripts.

MHC isoforms of the vertebrate striated muscle are encoded by a multigene family (1–7) and their expression is developmentally regulated at the level of gene transcription (7, 17, 19); that is, different MHC genes are transcribed at the specific stage of development. On the other hand, our present study has shown that expression of smooth muscle MHC isoforms are regulated at the level of RNA splicing. Similar example of alternative RNA splicing which generates diverse

**Fig. 1.** Indirect immunofluorescence on the aortas from various developmental stages. Frozen sections were reacted with anti-SM1 (upper row) and anti-SM2 antibodies (lower row).
Smooth Muscle MHC Isoform Expression

Fig. 2. Determination of the size of the partially protected probe of S1 nuclease mapping. S1 nuclease mapping was performed using a 3'-end-labeled PstI fragment which contains the coding region and the untranslated region of SMHC29. The single-stranded DNA probe was hybridized to RNA from adult aortas. S1 nuclease-resistant fragments were run next to a Maxam-Gilbert sequencing ladder of the same DNA probe. In this figure, the nucleotide sequence of the SMHC29 mRNA instead of the DNA probe is shown. Note that the position of the partially protected fragment coincided with the last common nucleotide between SMHC29 and SMHC40 mRNA (shown as $G^\ast$).

Fig. 3. S1 nuclease mapping of smooth muscle MHC mRNA from aortas of various stages of development. The band at 224 indicates full protection of the probe and the band at 79 indicates partially protected probe.

Fig. 4. Electrophoresis of SM1 and SM2 of aortas during various stages of development.

Fig. 5. Immunoblotting of SM1 and SM2 of aortas from fetal (left) and neonatal (right) rabbits. The 3.5% polyacrylamide gel was blotted onto a nitrocellulose membrane and immunostained with nonspecific anti-smooth muscle MHC (anti-SMHC), anti-SM1, and anti-SM2 antibodies or stained with Amido Black.

isoforms of contractile proteins during development has been known in two troponin T isoforms of rat fast skeletal muscles (20). However, Drosophila melanogaster is the only example so far to demonstrate alternative RNA splicing of an MHC gene (21–23). In Drosophila, there exists only a single MHC gene whose RNA expression is regulated through alternative splicing in 16 ways at three splicing sites (24). Studies on the MHC gene organization and the manner of splicing may clarify the molecular evolution of the MHC multigene family.

Another important result in this communication is the MHC isoform profiles on SDS-PAGE during early development. SDS-PAGE of myosin from adult aortas revealed more predominant expression of SM1 than SM2, corresponding to mRNA levels of SM1 and SM2. In fetal and perinatal aortas, however, the protein content of SM2 was found much more than SM1 despite the negligible amount of SM2 mRNA as compared to SM1 mRNA. However, the lack of cross-reactivity of fetal SM2 with our anti-SM2 antibody suggests the presence of a third type of MHC isoform in the fetal aortas, which has the same mobility as adult SM2 on SDS-PAGE. In neonatal and postnatal aortas, SM2 reacted with anti-SM2 antibody on immunoblotting, although the same anti-SM2 antibody was negative or weakly positive against smooth muscles in situ on immunofluorescence. The level of SM2 mRNA expression was also low in those arteries. Based on these results, we suggest that the major proportion of SM2 in neonatal and postnatal aortas could be a fetal-type SM2 and that the adult-type SM2, present in a small amount in those arteries, was detected by anti-SM2 antibody on immunoblotting. It is not clear at this moment whether this putative fetal SM2 is another smooth muscle MHC isoform or one of nonmuscle MHCs such as the one expressed in the cultured smooth muscle cells (25). In order to answer this question, characterization of MHC cDNA clones from a fetal aorta cDNA library is currently in progress in our laboratory.

In summary, our present study demonstrates that expression of vascular smooth muscle MHC isoforms undergoes developmental regulation. Further studies on the MHC isoform expression in smooth muscles seem important in understanding on regulation of vascular contraction and on smooth muscle cell differentiation.

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