Human Smooth Muscle Myosin Heavy Chain Isoforms as Molecular Markers for Vascular Development and Atherosclerosis

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Smooth muscle myosin heavy chains (MHCs) exist in multiple isoforms. Rabbit smooth muscles contain at least three types of MHC isoforms: SM1 (204 kD), SM2 (200 kD), and SMemb (200 kD). SM1 and SM2 are specific to smooth muscles, but SMemb is a nonmuscle-type MHC abundantly expressed in the embryonic aorta. We recently reported that these three MHC isoforms are differentially expressed in rabbit during normal vascular development and in experimental atherosclerosis. The purpose of this study was to clarify whether expression of human smooth muscle MHC isoforms is regulated in developing arteries and in atherosclerotic lesions. To accomplish this, we have isolated and characterized three cDNA clones from human smooth muscle: SMHC94 (SM1), SMHC93 (SM2), and HSME6 (SMemb). The expression of SM2 mRNA in the fetal aorta was significantly lower as compared with SM1 mRNA, but the ratio of SM2 to SM1 mRNA was increased after birth. SMemb mRNA in the aorta was decreased after birth but appeared to be increased in the aged. To further examine the MHC expression at the histological level, we have developed three antibodies against human SM1, SM2, and SMemb using the isoform-specific sequences of the carboxyl terminal end. Immunohistologically, SM1 was constitutively positive from the fetal stage to adulthood in the apparently normal media of the aorta and coronary arteries, whereas SM2 was negative in fetal arteries of the early gestational stage. In human, unlike rabbit, aorta or coronary arteries, SMemb was detected even in the adult. However, smaller-sized arteries, like the vasa vasorum of the aorta or intramyocardial coronary arterioles, were negative for SMemb. Diffuse intimal thickening in the major coronary arteries was found to be composed of smooth muscles, reacting equally to three antibodies for MHC isoforms, but reactivities with anti-SM2 antibody were reduced with aging. With progression of atherosclerosis, intimal smooth muscles diminished the expression of not only SM2 but also SM1, whereas α-smooth muscle actin was well preserved. We conclude from these results that smooth muscle MHC isoforms are important molecular markers for studying human vascular smooth muscle cell differentiation as well as the cellular mechanisms of atherosclerosis. (*Circ Res.* 1993;73:1000-1012.)

**KEY WORDS** • myosin heavy chains • smooth muscle • atherosclerosis • vascular development

The diversity of myosin heavy chain (MHC) molecules, particularly in cardiac and skeletal muscles, has been extensively investigated. MHC isoforms are not only determinants of contractile properties of muscles, such as the velocity of shortening or the economy of force production, but they are also molecular markers for muscle development and muscle diseases.1-8 Compared with cardiac and skeletal muscles, little had been known about smooth muscle and nonmuscle MHC isoforms until their cDNA clones were isolated and specific antibodies were developed.9 We have previously demonstrated that rabbit and rat smooth muscles contain at least three types of MHCs: SM1 (204 kD), SM2 (200 kD), and SMemb (200 kD).10-14 Two smooth muscle MHC isoforms designated as SM1 and SM2 were first identified by Rovner et al15 on porous sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).15 Eddinger and Murphy16 showed by peptide mapping that the structural difference between SM1 and SM2 could lie in light meromyosin. Definite evidence showing that SM1 and SM2 are two MHC isoforms arising from a single gene by alternative RNA splicing was demonstrated by Babij and Periasamy.17 Using cDNA probes and isoform-specific antibodies, we have shown that the expression of SM1 and SM2 in rabbit aorta is developmentally regulated: SM1 is constitutively expressed at all stages, whereas SM2 appears only after birth.13 We have further revealed the presence of a third type of MHC isoform in rabbit embryonic aorta.14 We referred to this novel MHC isoform as SMemb because it is present...
Fig 1. Restriction maps of three cDNA clones from human smooth muscle, SMHC94, SMHC93, and HSM6 (A), nucleotide and amino acid sequences of SMHC94, SMHC93, and HSM6 (B), and amino acid sequence comparison of three types of human myosin heavy chain (MHC) isoforms, SM1, SM2, and SMemb (D). A, The protein coding regions are indicated by a closed box in the restriction map. B and C, Nucleotide and amino acid numbers are indicated. D, Amino acids encoded by SM2 and SMemb are indicated by dashes when identical to those of SM1; when different, substituted amino acid is noted.
Fig 2. Analysis of three types of myosin heavy chain (MHC) mRNA expression using RNase protection assay of RNA samples from aortas of a fetuses at 31 and 33 (fetus 2 on the right) weeks of gestation, a neonate 4 days after birth, an infant of 6 months, a 4-year-old child, adults, 33, 46, 62, and 76 years of age, and a cultured human fibroblast. Total RNA (5 µg) was applied onto each lane. Left, The expression of smooth muscle–specific MHC isoform (SM1 and SM2) mRNA. SM2 mRNA is indicated by the full protection band of 153 nucleotides (nt), and SM1 mRNA as the partially protected band of 81 nt. Right, The expression of nonmuscle-type MHC isoform (SMemb) mRNA. The 157-nt band indicates SMemb mRNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA represented by 68-nt bands was determined as an internal control. RNA was hybridized to both probes for SMemb and GAPDH mRNA in a single reaction.

in greater abundance in embryonic aortas. This MHC appears to be identical with NMHC-B recently characterized in nonmuscle cells.17-19 Kawamoto and Adelstein18 described that nonmuscle cells express two different MHCs: NMHC-A and NMHC-B.

Our recent studies on smooth muscle MHC expression in experimental arteriosclerosis and atherosclerosis have clearly shown the importance of smooth muscle MHC isoforms as molecular markers identifying abnormally proliferating smooth muscle cells during the formation of vascular lesions.14 Furthermore, we demonstrated a marked contrast in MHC expression between the neointimal and medial smooth muscle cells. The neointimal cells are quite similar to the embryonic smooth muscle cells in that both are positive for SM1 and SMemb but negative against SM2, thus indicating that when smooth muscle cells proliferate after vascular injury, they regain the embryonic phenotype.14

The purpose of the present study was to investigate the smooth muscle MHC expression in human arteries at various ages and to examine the pathological aspects of smooth muscle cells in atherosclerosis on the basis of MHC expression. Therefore, we have isolated and characterized three types of cDNA clones encoding human vascular smooth muscle MHC isoforms. We have also produced isomorph-specific antibodies using the deduced amino acid sequences of the carboxyl terminal end and determined MHC protein expression at the histological level. We report the differential expression of smooth muscle MHC isoforms during human vascular development and demonstrate that phenotypically modulated smooth muscle cells in atherosclerotic lesions can be identified by altered MHC isoform expression.

Materials and Methods

cDNA Cloning and Sequence Analysis

cDNA clones for human SM1, SM2, and SMemb were isolated by screening two cDNA libraries constructed from the human umbilical cord (Agt 10, a gift from Chandra Kumar, Schering-Plough, Kenilworth, NJ) and fetal aorta mRNA (Agt 11, Clontech Laboratories, Palo Alto, Calif). The human umbilical cord cDNA library was screened with SMHC40 encoding rabbit SM1, and the human fetal aorta cDNA library was screened with an Sma I–Sma I fragment of FSHHC34 between nucleotides (nt) 491 and 1571 encoding rabbit SMemb.14 cDNA probes were labeled with [α-32P]dCTP using the random-primed DNA labeling kit (Boehringer Mannheim, Germany). Part of the libraries (2.5 x 10^6) was transferred to plaque/colony hybridization transfer membranes (New England Nuclear, Boston, Mass) and hybridized with 32P-labeled cDNA probes in 50% formamide, 5 x SSPE (0.75 mol/L NaCl, 50 mmol/L NaH2PO4, and 5 mmol/L EDTA), 0.1% SDS, 5 x Denhardt’s solution, and denatured salmon sperm DNA (100 µg/mL) at 42°C for 18 hours. Membranes were washed in 2 x standard saline citrate and 0.1% SDS at 42°C. Positive clones were subcloned into pBluescript II SK(−) (Stratagene Inc, La Jolla, Calif), and enzymatic extension reactions were performed using the Taq dye primer cycle sequencing kit (Applied Biosystems, Inc, Foster City, Calif) on DNA thermal cycler 480 (The Perkin-Elmer Corp, Norwalk, Conn; Cetus Corp, Calif). Nucleotide sequences were analyzed by model 373A DNA sequencing systems (Applied Biosystems).

Human Arteries and Fibroblasts

All arteries including those of fetuses were obtained from autopsied patients within 3 hours after death at Tokyo (Japan) University Hospital, Juntendo University Hospital, Tokyo, and Osaka City (Japan) University Hospital. Autopsies were conducted with consent of the bereaved families. Autopsied cases had neither prior clinical history of ischemic heart disease nor major atherogenic factors, like hyperlipidemia, diabetes, or smoking. For RNA and protein analysis, the ascending aorta was excised, and the medial layer was carefully dissected under microscopy and frozen in liquid nitrogen. For immunohis-
ological studies, tissues were fixed in mixed solution of 95% ethanol and 1% acetic acid. Human skin fibroblasts were purchased from Dainippon Pharmaceutical, Osaka, Japan, and cultured at 37°C in a humidified 5% CO₂ atmosphere. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (GIBCO-BRL).

RNA Preparation and RNase Protection Assay

Total RNA was prepared from the human ascending aorta by the acid guanidinium thiocyanate–phenol chloroform method.20 Beforehand, we confirmed in rabbits that aortic RNA within 4 hours after death did not show any significant degradation. A cRNA probe discriminating human SM1 and SM2 mRNA (riboprobe 1) was prepared using a 151-bp Psal-Xbal I fragment from SMHC93 (nt 782 to 932), which includes 112 bp of the common sequence of SM1 and SM2 and 39 bp of the SM2-specific sequence. The cRNA probe for human SMemb (riboprobe 2) was prepared from a 156-bp EcoRI–Psal I fragment of HSM6 (nt 1 to 156). In the assay for SMemb mRNA, we included a cRNA probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) so that SMemb mRNA levels could be standardized by comparing with GAPDH mRNA levels in each specimen. The cRNA probe for GAPDH mRNA (riboprobe 3) was produced by polymerase chain reaction from the sequence of human GAPDH cDNA between nt 481 and 568.21 These fragments were subcloned into pBluescript II SK(−), and antisense RNA probes were synthesized using Riboprobe Gemini System II (Promega Corp, Madison, Wis). Hybridization with RNA samples, digestion with RNase, and analysis on polyacrylamide gels were performed using ribonuclease protection assay RPA II kit (Ambion Inc, Austin, Tex). The full protection of riboprobe 1 with SM2 mRNA is 153 nt, and the partially protected fragments with SM1 mRNA are 81 and 33 nt. The fully protected fragments of riboprobe 2 with SMemb and riboprobe 3 with GAPDH mRNA are 157 and 68 nt, respectively.

Preparation of Isoform-Specific Antibodies

Three short peptides specifying the carboxyl terminal end of SM1 (Arg-Asp-Ala-Asp-Phe-Asn-Gly-Thr-Lys-Ser-Ser-Glu), SM2 (Gly-Pro-Pro-Pro-Gln-Glu-Thr-Ser-Gln), and SMemb (Thr-Ser-Asp-Val-Asn-Glu-Thr-Gln-Pro-Pro-Gln-Ser-Glu) were synthesized and used for immunization. We developed two monoclonal antibodies against SM1 and SM2 as described in our recent report.22 Polyclonal antiserum was used only for SMemb because we have not succeeded in developing monoclonal antibody. The synthesized short peptides were conjugated with bovine serum albumin and injected intraperitoneally into mice at biweekly intervals. Titors of the antisera and supernatant were determined by enzyme-linked immunosorbent assays.

SDS-PAGE and Immunoblotting

Myosin was extracted from human aortas according to Mohammad and Sparrow,23 and MHC isoforms were separated on 4% SDS-PAGE. The gels were stained with Coomassie blue, or the proteins were electrophoretically transblotted onto nitrocellulose membranes. The membranes were immunologically stained by the method of Towbin et al.24

Immunohistochemistry of Aortas and Coronary Arteries

Specimens fixed as described above were embedded with paraffin and sectioned in 3-μm slices. After deparaffinization and rehydration, immunoenzymatic staining was performed using the DAKO LSAB kit (Dako Corp, Calif). Sections were preincubated with 0.3% hydrogen peroxide and normal rabbit serum to reduce nonspecific reactions. Antibody against human SM1, SM2, SMemb, human α-smooth muscle actin (1A4, DAKO A/S, Denmark),25 or human macrophages (HAM-56, Enzo Diagnostics, New York, NY)26 was applied and incubated for 20 minutes at room temperature. Sections were incubated with biotinylated anti-mouse goat immunoglobulin for 10 minutes and then incubated with horse radish peroxidase–labeled streptavidin solution for 10 minutes. The slides were rinsed in Tris-buffered saline with Tween 20 (Wako Pure Chemical Industries, Japan) after each incubation step. Peroxidase activity was revealed by 3,3′-diaminobenzidine tetrahydrochloride (0.2 mg/mL, Sigma Chemical Co, St Louis, Mo) with hydrogen perox-
AORTA

Fetus

18 y.o.

52 y.o.

65 y.o.

SM1

SM2

SMemb

Fig 4. Immunohistochemistry for smooth muscle-specific myosin heavy chain (MHC) isoforms (SM1 and SM2) and nonmuscle-type MHC isoform (SMemb) in the aorta obtained from autopsied patients of various ages. All immunostainings were performed on serial sections. i indicates intima; m, media. Bar=200 μm. In a fetus at 17 weeks of gestation, the aortic wall consists of only the media in which SM2 was undetectable. All MHC isoforms were expressed in the aortic media of the 18-, 52- and 65-year-old patients. However, SM2 expression was decreased in the intima of adults.

Results

cDNA Cloning and Nucleotide Sequencing

The MHC cDNA clones encoding human SM1 (SMHC94), SM2 (SMHC93), and SMemb (HSME6) were isolated by screening human umbilical cord and human fetal aorta cDNA libraries as described in “Materials and Methods.” SMHC94, SMHC93, and HSME6 contained insert sizes of 1.2, 0.9, and 1.0 kb, respectively, and their partial restriction maps are shown in Fig 1A. The nucleotide and the derived amino acid sequences of SM1 (SMHC94) and SM2 (SMHC93) clones are shown in Fig 1B. The two types of MHC cDNA clones encode part of light meromyosin of the myosin molecule and have identical nucleotide and amino acid sequences except at the carboxyl terminus, where SMHC93 has a stretch of 39 extra nucleotides (encodes nine amino acids) that is not found in SMHC94. SMHC94 and SMHC93 are highly homologous with rabbit SM1 and SM2 (92% and 93% identity at the nucleotide level of the translated regions and 96% and 97% identity at the amino acid sequence level, respectively). As shown for rabbit smooth muscle MHC, the SMHC93 clone encodes a shorter carboxyl terminus with nine unique amino acids specific for SM2 myosin. SMHC94 cDNA encodes the SM1 myosin with 43 unique amino acids at the carboxyl end.

The nine unique amino acids of the carboxyl terminus found in rabbit or rat SM2 myosin were the result of alternative RNA splicing of a 39-nt exon in the SM1/2 gene. Therefore, we suggest that the human SM1 and SM2 myosins are produced from a single gene through alternative RNA splicing. Fig 1C shows the nucleotide and amino acid sequences of HSME6 (SMemb). Human SMemb myosin is similar to rabbit SMemb and has 92% nucleotide and 98% amino acid sequence identity. The comparison of all three MHC amino acid sequences is presented in Fig 1D.
**MHC Isoforms in Human Vascular Smooth Muscle**

Aikawa et al

**SM1, SM2, SMemb**

**Fetus**

**t**

**LAD**

**18y.o.**

**33y.o.**

**SM2**

**SMemb**

**Bar=200 μm.** All immunostainings were performed on serial sections. In the fetal coronary artery at 21 weeks of gestation, no intima could be recognized. The SM2 expression in the fetal coronary artery was negative. In the coronary artery of an 18-year-old man, the intimal cells were positive for SM1, SM2, and SMemb. However, in a 33-year-old man, SM2 expression was clearly reduced in the highly thickened intima without atheromatous changes (33y.o., center panel at the bottom), whereas it was maintained in the thin intima of the same case (33y.o., center panel at the top). SMemb expression seemed to be more abundant in the intima than in the media both in the 18- and 33-year-old patients (18y.o., right panel; 33y.o., top right panel).

**Fig 5.** Immunohistochemistry for smooth muscle-specific myosin heavy chain (MHC) isoforms (SM1 and SM2) and nonmuscle-type MHC isoform (SMemb) in the left anterior descending coronary arteries. i indicates intima; m, media.

**mRNA Expression of Smooth Muscle MHC Isoforms**

Using isolated cDNA clones, we designed specific cRNA probes for RNase protection assay to detect three types of MHC mRNA in human aortas. Fig 2, left, demonstrates the expression of SM1 and SM2 mRNA in the ascending aortas of a fetus at 31 weeks of gestation, a neonate 4 days after birth, a 4-year-old infant, and 33- and 46-year-old adults. Fetuses earlier than 31 weeks were not examined because samples were not available. It is shown that both SM1 and SM2 mRNAs were detected at all ages. SM2 mRNA, however, was more abundant in adult than in fetal aortas, which is more evident when compared with the SM1 mRNA in the same sample. Similar changes in SM2 mRNA after birth were also observed in rabbits, although rabbit SM2 mRNA was almost undetectable in fetal aortas. SMemb mRNA was abundant in fetal and neonatal aortas and decreased with growth. It seems likely that SMemb mRNA was increased in the elderly, whereas GAPDH mRNA did not significantly change. Neither SM1 or SM2 mRNA was expressed in cultured fibroblasts, but SMemb mRNA was expressed at high levels (Fig 2, right).

**Immunoblot Analysis of Smooth Muscle MHC Isoforms**

To detect smooth muscle MHC protein expression in the ascending aorta, immunoblot analysis was per-
formed using three isoform-specific antibodies for human SM1, SM2, and SMemb. Fig 3 illustrates the MHC expression in aortas of a fetus at 27 weeks of gestation and a 44-year-old adult. Anti-human SM1 antibody recognized the 204-kD MHC in both the fetal and adult aorta. Anti-human SM2 antibody, however, did not react with MHC of the fetal aorta but recognized the 200-kD MHC in the adult aorta. Anti-SMemb antibody reacted with 200-kD MHC of both fetal and adult aortas.

**Immunohistochemistry of Smooth Muscles in Aortas and Coronary Arteries Without Atherosclerotic Changes**

The ascending aorta and the proximal segment of the left anterior coronary artery were investigated for MHC expression by immunohistochemistry using three types of anti-MHC antibodies (anti-SM1, anti-SM2, and anti-SMemb) and the anti-α-smooth muscle actin antibody. Specimens were collected from each of the following age groups: fetuses (gestational age, 17 to 27 weeks; n=5), younger adults (18 to 33 years old, n=5), and the aged (52 to 76 years old, n=10).

Our immunohistochemical examinations have clearly revealed differential expression of MHC isoforms in various types of arteries at different ages. In fetal aortas, SM1 and SMemb were positive as early as 17 weeks of gestation. On the other hand, SM2 was negative in aortas of four fetuses between 17 and 21 weeks of gestation but weakly positive in a fetus at 27 weeks (data not shown). In the aortic media of all adults, three MHC isoforms were positive. However, in the intima of three younger adults and seven of the aged, the expression of SM2 was reduced, whereas SM1 and α-smooth muscle actin remained positive (Fig 4). SMemb expression was also found to be reduced in two aged adults.

In coronary arteries as well, medial smooth muscles exhibited developmental transition in MHC expression on immunohistochemistry in which SM2 was negative in fetuses before 21 weeks of gestation but positive after birth (Fig 5). Both SM1 and SMemb were positive in the coronary artery through all ages, as seen in the aortas, although the reactivities with anti-SMemb antibody seemed to be decreased with aging. The continued expression of SMemb in human aortas and coronary arteries was different compared with the expression in rabbits, in which SMemb was negative in adults. However, it is noteworthy that SMemb expression depends on the size of arteries, because it was negative in such small-sized arteries as vasa vasorum of the aorta or intramyocardial coronary arterioles in all cases studied (Fig 6).

It is known that human coronary arteries develop the intimal layer from early adolescence, which is several times thicker than the medial layer in the aged. Our immunohistological analyses indicated that the coronary intimal cells were for the most part smooth muscles, because they reacted with both anti-SM1 and anti-α-actin antibodies (Fig 5). The intimal thickening of the major coronary artery was recognized in all of the adults and the aged. In an 18-year-old man (Fig 5), both medial and intimal smooth muscles of the left anterior descending coronary artery reacted equally with all three anti-MHC antibodies. In Fig 5, we also present a 33-year-old patient in whom smooth muscles in the highly thickened intima showed reduced reactivities to
Fig 7. Immunohistochemistry for α-smooth muscle actin, smooth muscle myosin heavy chain isoforms, and macrophages in the shoulder region of atheromatous plaque of the aorta from a 74-year-old man. SM1 and SM2 are smooth muscle–specific isoforms, and SMemb is a nonmuscle-type isoform. Hematoxylin-eosin (HE) and immunostainings were performed on serial sections. i indicates intima; m, media; and MΦ, macrophages. Bar=200 μm. The intimal layer showed atheromatous changes with prominent accumulation of MΦ. Intimal smooth muscles were positive for α-smooth muscle actin and SM1 but negative for SM2. SMemb expression was also reduced in the intima.

anti-SM2 antibody, although smooth muscles in the thin intimal layer maintained SM2 expression. This indicates that intimal smooth muscles undergo phenotypic modulation even before atheromatous changes develop. Reduced expression of SM2 in the nonatherosclerotic thickened intima was more or less observed in all patients after the fourth decade. Accumulation of macrophages was not evident in these specimens (data not shown).

**Immunohistochemistry of Smooth Muscles in Atherosclerotic Aortas and Coronary Arteries**

Atheromatous plaques of the aorta were recognized in 5 of 10 aged patients, showing prominent accumulation of macrophages (Fig 7). Intimal smooth muscles in those areas were positive for both α-smooth muscle actin and SM1 but almost negative for SM2. SMemb expression was reduced in atherosclerotic plaques of two aged patients.

In coronary atherosclerosis, an outstanding pathological feature is the presence of fibrohyaline or fibronectic plaques in the intimal, the smooth muscle–poor, and the ground substance–rich regions. Fig 8 shows the left anterior descending coronary artery with a fibrohyaline region obtained from a 65-year-old patient. Although macrophages were not prominent in this atherosclerotic lesion, both intimal and medial smooth muscles exhibited many varieties of modulated phenotypes in light of the expression of MHC isoforms. Particularly, smooth muscle cells in the fibrohyalineized areas, although small in number, were reduced in the expression not only of SM2 but also of SM1, whereas most of cells were α–actin–positive (Fig 8A). SMemb expression was also reduced but remained positive in intimal smooth
muscles in the thick but nonatheromatous region. Similar changes were observed in 7 of 10 aged patients but in none of the younger adults. However, in three patients with more advanced atherosclerosis, we noticed that α-actin expression beside all MHC isoforms was regionally reduced (data not shown).

In addition to the changes in the intimal layer, we frequently noticed the phenotypic modulation of medial smooth muscles underlying the atherosclerotic plaques because SM1 and SM2 expression was reduced, whereas the expression of α-smooth muscle actin was preserved. These changes in MHC expression in both the intimal and the medial smooth muscles were observed in all patients over 50 years of age.

Also of note in the atherosclerotic coronary intima was the formation of intraplaque neovascularatures composed of SM1-positive but SM2-negative smooth muscles. These neovascularatures in the intima were found in 5 of 10 specimens from the aged. Interestingly, macrophages and lymphoid cells appeared to be recruited to the intima via these vessels (Fig 9). Such intraplaque vessels were more conspicuous in fibrous intima of the elderly than in the fibromuscular intima of younger adults.

**Discussion**

In the present study, we have isolated and characterized cDNA clones for three types of MHC isoforms
expressed in human vascular smooth muscles, which made it possible to raise isoform-specific antibodies. Using these antibodies, we demonstrated that MHC isoforms are differentially expressed during normal development and depending on the types of arteries. We have further provided evidence showing the usefulness of these antibodies in understanding the development and progression of human atherosclerosis.

Proliferation and phenotypic modulation of vascular smooth muscle cells are outstanding phenomena occurring during the development of atherosclerosis. We have recently reported that smooth muscle proliferation in experimental arteriosclerosis and atherosclerosis was associated with dedifferentiation of the smooth muscle cell toward the embryonic phenotype on the basis of the MHC expression. Human arterial systems, however, may present unique profiles of MHC expression at various stages of growth and ages. Furthermore, it is conceivable that human atherosclerosis develops through different processes and by different mechanisms from those in experimental animals because it takes many decades for human atherosclerosis to evolve, whereas only a few weeks or months should be sufficient in animal models. Therefore, in the present study, we tried to reveal specific aspects to the human vascular smooth muscle cell in vivo as well as human atherosclerosis in light of MHC isoform expression. To accomplish this, we have isolated and characterized three types of MHC cDNA clones expressed in human vascular smooth muscles and produced isoform-specific antibodies for immunohistochemistry.

**Human Vascular Smooth Muscles Express At Least Three Types of MHC Isoforms**

We have first isolated three types of cDNA clones from human vascular smooth muscles. SMHC94 and SMHC93 encode human SM1 and SM2, respectively, and were highly homologous to SMHC40 and SMHC29 encoding the rabbit SM1 and SM2 isoforms. HSME6 isolated from the fetal aorta cDNA library was homologous to the rabbit SMemb. Immunoblot analysis using specific antibodies has provided further evidence that these three cDNA clones encode MHC isoforms in human smooth muscle. The nucleotide sequence analysis of the cDNA clones suggests that SM1 and SM2 are generated from a single gene by alternative RNA splicing in a manner identical to that already known in the rat or rabbit, whereas SMemb is encoded by a different MHC gene.

The existence of nonmuscle-type MHC in smooth muscle cells was reported by Rovner et al and by Kawamoto and Adelstein. They demonstrated that, in cultured smooth muscle cells, nonmuscle-type MHC of 196 kD replaced SM1 and SM2. Using cDNA cloning and a specific antibody, we identified another nonmuscle-type MHC of approximately 200 kD (SMemb) in rabbit embryonic aortas as well as in cultured smooth muscle cells. Kawamoto and Adelstein have classified the 196-kD nonmuscle-type MHC as NMHC-A and the 200-kD nonmuscle MHC as NMHC-B (198 kD). We agree with Kawamoto and Adelstein that NMHC-B is identical to our SMemb.

In the rabbit, the expression of SM1, SM2, and SMemb is developmentally regulated at the level of both gene transcription and alternative RNA splicing. SM1 and SM2 are important in the identification of differentiated smooth muscles because so far they have been found to be specific to vascular and nonvascular smooth muscles. SMemb, on the other hand, is most abundantly expressed in embryonic smooth muscle, in proliferating smooth muscle cells of experimental atherosclerotic or arteriosclerotic lesions, and in cultured smooth muscle cells. Therefore, three MHC isoforms expressed in human vascular smooth muscles should provide important molecular tools in the investigation of human vascular development as well as the progression of atherosclerosis.
During Vascular Development

Differential Expression of Human MHC Isoforms During Vascular Development and in Various Types of Arteries

Vascular smooth muscle MHC isoforms are differentially expressed in humans depending on the developmental stage, age, and the type of arteries. In the medial wall of both the aorta and coronary arteries, SM1 mRNA exists in a large amount from early development. However, SM2 mRNA levels were relatively low in fetal aortas at 31 weeks of gestation and increased after birth, which was clearly seen when compared with the SM1 mRNA level in the same sample. On immunohistochemistry, SM2 was not detectable in fetal aortas and coronary arteries at 17 to 21 weeks of gestation. The discrepancy of the expression of SM2 between the RNase protection assay and the immunoblotting could be due to the difference in the gestational age of the fetuses used in these analyses.

A difference between human and rabbit vascular MHC was seen in the expression of SMemb. In rabbits, SMemb quickly disappeared at 4 months after birth, as examined at the mRNA and histological levels. In human aortas, SMemb mRNA seemed to be decreased after birth but increased again in the aged. However, because determination of mRNA levels in the present study was carried out in a limited number of cases, further analysis is necessary to confirm this by using more samples.

Immunohistochemical expression of SMemb differs between various types of arteries. SMemb was positive at all ages in the aortic media but negative in small-sized arteries, like vasa vasorum or intramyocardial coronary arterioles. The physiological significance of continued expression of SMemb in large arteries is not known at the moment. We suggest that it is important to clarify whether vascular smooth muscles expressing SMemb differ from those without SMemb in ultrastructures, contractile properties, and the ability to proliferate.

Intimal as Well as Medial Smooth Muscles in Atherosclerotic Arteries Undergo Phenotypic Modulation in Light of MHC Expression

Immunohistochemical analyses of the present study have revealed several characteristic features in human arteries, especially normal and atherosclerotic coronary arteries. First of all, the major epicardial coronary arteries develop a thickened intimal layer, starting in early adolescence, that is several times thicker than the media in many mature adults. Most of the intimal cells are unequivocally smooth muscles, because they reacted with anti-SM1 and anti-α-smooth muscle actin antibodies. Intimal thickening with smooth muscle cell accumulation may be considered to be physiological in nature, but the present study has demonstrated that smooth muscles in the highly thickened intima modulate their phenotype distinct from those in normal media or in the thin intima.

It has been assumed that coronary intimal thickening from childhood is an integral part of atherosclerosis, i.e., a prerequisite for the development of atherosclerosis. We noticed in this study that with the development of atherosclerosis the number of intimal smooth muscles was decreased and replaced by fibrous tissues. Smooth muscles in such areas were reduced in the expression of all MHC isoforms, first SM2 and then SM1, whereas α-actin expression was well maintained. It is also noteworthy that medial smooth muscles underlying the atherosclerotic lesions were often decreased in the expression of SM1 and SM2. Therefore, immunohistochemistry for MHC isoforms will be useful in elucidating the role of modulated smooth muscle cells in the progression of atherosclerosis.

The aortic or coronary intimal smooth muscles with reduced expression of SM2 compare with the embryonic smooth muscles in the neonontima observed in mechanically injured or high cholesterol–fed rabbit aortas in our previous report. However, phenotypically modulated human intimal cells are definitely different from human embryonic smooth muscles, because the expression of SMemb was not very prominent in the former.

We could not find in the present study the pathological significance of SMemb during the development of atherosclerosis, because the phenotypic modulation of intimal smooth muscle cell with reduced SM2 expres-
sion was not associated with an increase in SMemb expression. One reason would be that, since human atherosclerosis develops over many decades, proliferating smooth muscle cells are few in number at the stable stage of atherosclerosis.32 We suggest that when smooth muscles vigorously proliferate in the restenotic coronary lesion after percutaneous transluminal coronary angioplasty, SMemb expression would be pronounced, as demonstrated by Simons et al33 using in situ hybridization for NMHC-B (SMemb). However, in the present study, we pointed out that SMemb could be expressed in the nonatheromatous intima at all ages. Therefore, we suggest that SMemb expression is not necessarily a phenomenon specific to restenosis after angioplasty.

Finally, we have demonstrated the presence of tiny vessels in the atherosclerotic plaques. These vessels are composed of SM1-positive but SM2-negative cells. Interestingly, large numbers of macrophages and lymphoid cells were found to accumulate around these vessels, much more than on the luminal side of the plaque. Therefore, in understanding the progression of atherosclerosis, it is important to clarify the relation between the formation of these intraplaque vessels and the accumulation of macrophages.

Phenotypic modulation of arterial smooth muscles is an important feature characterizing the development of atherosclerosis. Our present study has revealed the importance of MHC isoforms as new molecular markers in the study of the process of human atherosclerosis. However, functional aspects of the phenotypically modulated smooth muscle cell are not known from the MHC isoform expression. We believe that future studies should be directed to correlate the altered MHC isoform profiles with the activation of the smooth muscle cell, like cytokine or growth factor production.34–38 At the same time, understanding the regulatory mechanisms of smooth muscle MHC gene expression in normal and diseased arteries will provide insight into the pathogenesis of atherosclerosis as well as smooth muscle cell differentiation.39

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