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Argatroban, specific thrombin inhibitor, induced phenotype change of cultured rabbit vascular smooth muscle cells

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Abstract

To investigate whether Argatroban ((2R, 4R)-4-methyl-1-[N2-((RS)-3-methyl-1,2,3,4-tetrahydro-8-quinolinesulfonyl)-L-arginyl]-2-piperidinecarboxylic acid hydrate, a selective thrombin inhibitor, exerts a direct action on phenotype conversion of vascular smooth muscle cells, cultured rabbit aortic vascular smooth muscle cells were employed. Myosin heavy chain isoforms (SM1, SM2, and SMemb) mRNA expressions were evaluated by in situ hybridization and reverse transcription-polymerase chain reaction (RT-PCR). After the cells were incubated in serum-free medium containing argatroban (10 and 50 μg/ml) and platelet-derived growth factor (PDGF)-BB (10 and 50 ng/ml) for 3 h, total RNA was extracted. In situ hybridization demonstrated that myosin heavy-chain isoform mRNAs were homogenously expressed in argatroban- and PDGF-BB-treated cells. RT-PCR revealed that SM1/SM2 mRNA expressions were not changed with argatroban, while SMemb mRNA expression was increased to 1.6-fold with a statistical significance ($P < 0.05$). Treatment with argatroban (10 and 50 μg/ml) at 24 h did not change SM1/SM2 mRNA expressions. Although SMemb mRNA expression was slightly increased, there was no statistical significance. Other phenotype markers including plasminogen activator inhibitor-1 (PAI-1) and β-actin mRNAs were also significantly increased by argatroban. In conclusion, argatroban can directly induce phenotype conversion of vascular smooth muscle cells with the resultant up-regulation of SMemb, PAI-1, and β-actin mRNAs.

Keywords: Argatroban; PDGF, (platelet-derived growth factor); Smooth muscle cell, vascular; RT-PCR, (reverse transcription-polymerase chain reaction); In situ hybridization

1. Introduction

Argatroban ((2R, 4R)-4-methyl-1-[N2-((RS)-3-methyl-1,2,3,4-tetrahydro-8-quinolinesulfonyl)-L-arginyl]-2-piperidinecarboxylic acid hydrate is a potent and selective synthetic thrombin inhibitor derived from L-arginine (Okamoto et al., 1981). In many experimental animal models including cerebral thrombosis and peripheral arterial occlusion, argatroban exhibits antithrombotic effects. It has been utilized clinically as an antithrombotic agent for the treatment of heparin-induced thrombocytopenia, acute myocardial infarction, cardiac procedures, percutaneous coronary intervention, coronary artery bypass graft, and cerebral thrombosis (Moledina et al., 2001).

Although percutaneous transluminal coronary angioplasty and other vascular reconstructive procedures have increased immediate success for restoring patency of narrowing vessels, vascular restenosis occurs in 20–50% of patients within 6 months. Coagulation factors including thrombin and tissue factor are involved in the development of vascular restenosis after interventional treatment (Arno et al., 2002; Ip et al., 1991). Many clinical trials demonstrated that anti-coagulant and anti-platelet drugs prevented the restenosis (Bauters et al., 1996; Topol and Serruys, 1998). Argatroban was also tested whether it prevented the restenosis after balloon angioplasty in experimental and clinical study (Imanishi et al., 1997). They demonstrated that locally administered argatroban using hydrogel-coated balloon catheter prevented post-percutaneous transluminal coronary angioplasty restenosis. However, another specific thrombin inhibitor, bivalirudin, failed to prevent the restenosis (Burchenal et al., 1998). Therefore, argatroban

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might have pleiotropic action. That is, argatroban could exert its inhibitory effect on the restenosis through an independent pathway of thrombin. Furthermore, Zhang et al. (2001) demonstrated that in experimental animal model of subarachnoid hemorrhage, argatroban suppressed the expression of platelet-derived growth factor (PDGF)-BB in the basilar artery. However, there are few reports on the direct effects of argatroban on vascular wall cells. To understand the mechanism by which argatroban inhibits the vascular restenosis, the direct effect of argatroban on vascular endothelial cells and smooth muscle cells needs to be tested.

There are many marker proteins for differentiation/dedifferentiation of vascular smooth muscle cells, such as α-actin (Blank et al., 1995), myosin heavy chain isoform (Borrione et al., 1989; Miano et al., 1994), caldesmon (Blank et al., 1995), elastin (Hayashi et al., 1995), whose expressions are highly dependent on smooth muscle phenotype: contractile and synthetic type (Babij et al., 1992). Although most populations of vascular smooth muscle cells in vivo are of the contractile type, they can be transformed into synthetic type in response to vascular injury (Okamoto et al., 1992). Myosin heavy chain isoform is one marker for differentiation/dedifferentiation of smooth muscle cells and have several phenotypes including SM1, SM2, and SMemb (Aikawa et al., 1993). It was demonstrated that SM1/SM2 were expressed in contractile cell type, while SMemb was expressed in synthetic cell type (Kuro-o et al., 1991).

In the present study, we aimed to test whether argatroban has a direct effect on phenotype conversion of vascular smooth muscle cells. Therefore, it is essential to determine the phenotype of vascular smooth muscle cells in in vitro and in vivo studies. In the present study, to detect the expression and localization of myosin heavy-chain isoform mRNAs in cultured rabbit vascular smooth muscle cells, in situ hybridization and reverse transcription-polymerase chain reaction (RT-PCR) were employed.

2. Materials and methods

2.1. Materials

Argatroban was obtained from Daiichi Pharmaceutical (Tokyo, Japan). Recombinant human PDGF-BB was purchased from PeproTech EC (London, England). Unless otherwise stated, the reagent grade chemicals were used.

2.2. Animals, animal care, and cell culture

All animal studies were reviewed and approved by the Animal Care Committee at the University of the Ryukyus according to the Management of Experimental Animals (Notification No. 6, March 27, 1980, from the Prime Minister's Office, Tokyo, Japan) for the care and use of the animals, together with the guide for animal experiments issued by the University of the Ryukyus. Vascular smooth muscle cells were isolated and cultured by a modification of the procedures by Yoshida et al. (1988). Thoracic aorta was excised from male Japanese white rabbits (body weight of 2.5 kg) and cut into small pieces (about 2 × 2 mm) after the endothelium was mechanically removed. The pieces were planted on cell culture dishes with the luminal side down and maintained under 5% CO_2 at 37 °C in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY, USA) containing 10% fetal bovine serum plus penicillin and streptomycin. After 7–10 days in culture, the vascular smooth muscle cells migrated out of the tissue and began to proliferate. When primary cultures reached confluence, the cells were trypsinized and were passaged with a ratio of 1:2.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from 4×10^5 cultured vascular smooth muscle cells using RNA extraction kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturers' instruction, based on the guanidine thiocyanate method (Chirgwin et al., 1979; Okayama et al., 1987). After reverse transcription with 1.25 U/ml of moloney murine leukemia virus reverse transcriptase (Life Technologies, MD, USA) using an oligo $(dT)_{31}$ primer for 2 h at 37 °C, the first strand cDNA templates (1 μl) were amplified by the PCR method in 30 cycles with 0.5 U of *Taq* DNA polymerase (Promega, Madison, WI, USA) in 25 μl of 10 mM Tris buffer (pH 9.0) containing 50 mM KCl, 0.2 μM each of dNTPs, 5 mM $MgCl_2$, and 5 μM each primer. Each cycle consisted of denaturing at 95 °C for 1 min, followed by annealing for 1 min at 60 °C for SM1, 64 °C for SM2, and 54 °C for SMemb, and extension at 74 °C for 0.5 min. Isoform specific primers for myosin heavy chain (SM1, SM2 and SMemb), plasminogen activator inhibitor (PAI)-1, β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used were designed based on the published sequences of cDNAs as follows: SM1 (GenBank accession number M77812, 5883–6456), 5′-GGCGAGGAAACGAGACCTCGT-3′ (forward), 5′-AGGTGTGGTAGCCAGGAGAGG-3′ (reverse); SM2 (J03614, 1439–1571), 5′-CTCTGCGTCGCGAGC GTC-3′ (forward), 5′-ACAGGAAACTTCGCAGTG-3′ (reverse); SMemb (D90237, 1372–1559), 5′-CCAATTAGCTTCT-CTTCCAGC-3′ (forward), 5′-CGGCGGAGTTCCTCTGT GT-3′ (reverse); β-actin (X03672, 794–1198), 5′-GAGCTA-TGAGCTGCCTGACGG-3′ (forward), 5′-TTGCGGTG-CACGATGGAGGG-3′ (reverse); PAI-1 (M24067, 881–1177), 5′-ACCCTCAGCATGTTCATTGC-3′ (forward); 5′-CTCGTTCACCTCGATCTTGAC-3′ (reverse); GAPDH (M33197, 591–1034), 5′-AGTCCATGCCATCACTGCC-3′ (forward), 5′-ACCACCCTGTTGCTGTAGCC-3′ (reverse). Schematic cDNA sequences of myosin heavy chain are shown in Fig. 1A. The PCR products were separated on

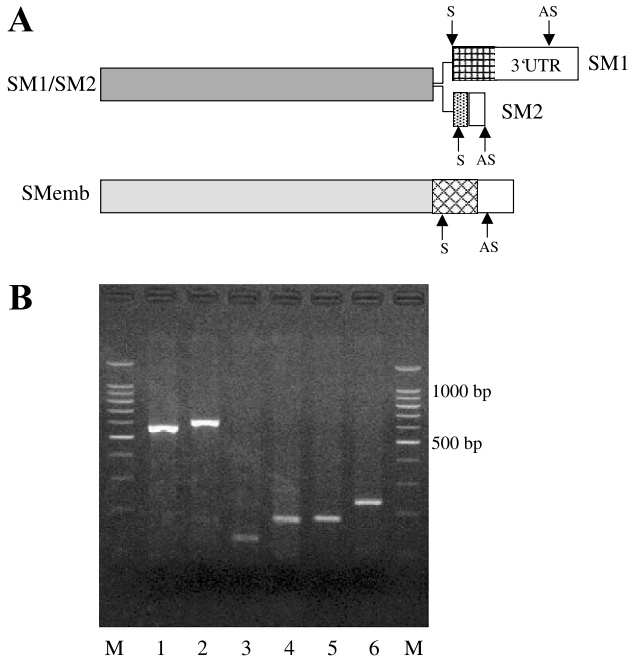

Fig. 1. RT-PCR amplification of cDNAs of myosin heavy chain isoforms in cultured vascular smooth muscle cells. (A) Schematic diagram of cDNAs of SM1/SM2 (upper) and SMemb (lower). SM1 and SM2 are splicing variants and coding region having different amino acid sequences at C-terminal region are indicated as patterned boxes. SMemb has about 70% of identity to SM1/SM2 (gray box) and has a distinct nucleotide sequence (patterned). White box indicates 3′ untranslated region (3′UTR). S and AS with arrows represent the position of sense primer and antisense primer, respectively. (B) Agarose electrophoresis of RT-PCR products by isoform specific primers and by those having RNA polymerase promoter sequence primers. Lane M: 100-bp DNA ladder marker. Lanes 1 and 2: SM1 and SM1with RNA promoter sequence. Lanes 3 and 4: SM2 and SM2 with RNA promoter sequence. Lanes 5 and 6: SMemb and SMemb with RNA promoter sequence. RT-PCR, reverse transcription-polymerase chain reaction; UTR, untranslated region.

2.5% agarose (NuSieve™ GTG agarose, FMC Bioproducts, ME, USA) containing ethidium bromide with 0.5× Tris–borate EDTA (pH 7.5). For quantitative RT-PCR, the number of PCR cycle was increased from 15 to 35 cycles. The fluorescent intensity of amplified cDNA bands of myosin heavy chain isoform was valued by NIH image®.

2.4. Preparation of RNA probe by in vitro transcription

SM1, SM2, and SMemb cDNA templates having RNA polymerase (T7 and Sp6) promoter sites were prepared by PCR. To synthesize biotin-labeled sense RNA probes for in situ hybridization, the cDNA templates were incubated at 37 °C for 2 h with 50 U of T7 RNA polymerase (Takara Shuzo, Japan) in 20 μl of 40 mM Tris buffer (pH 8.0) containing 8 mM $MgCl_2$, 2 mM spermidine, 5 mM dithiothreitol, 4 mM NTPs/biotin-16-UTP (Roche Diagnostics, Germany) mixture, and 20 U RNase inhibitor (Takara Shuzo). In the same way, biotin-labeled antisense RNA probes were synthesized with Sp6 RNA polymerase (Takara Shuzo) with the exception of adding 0.01% of bovine serum albumin.

2.5. In situ hybridization

In situ hybridization was carried out according to the method by Raap et al. (1991). Cultured rabbit vascular smooth muscle cells on cover glasses were washed twice with phosphate-buffered saline (PBS) at 37 °C, then fixed at room temperature for 30 min in a solution of 4% formaldehyde, 5% acetic acid and 0.9% NaCl. The fixed cells were rinsed with PBS and stored at 4 °C in 70% ethanol. Before in situ hybridization, the fixed cells were dehydrated by incubating successively in 70%, 80%, 90%, 100% ethanol and washed in 100% xylene to remove residual lipids. For rehydration, the cells are incubated successively in 100%, 90%, 80%, 70% ethanol and PBS. The fixed cells were treated at 37 °C with 0.1% pepsin in 0.1 N HCl to increase permeability to RNA probes. After wash with PBS for 5 min, the cells were post-fixed with 1% formaldehyde for 10 min. The cells were hybridized for 16 h at 50 °C for SM1 and SM2, at 42 °C for SMemb with 1 μg of sense/antisense biotin-labeled RNA probes in hybridization buffer consisting of 60% deionized formamide, 300 mM NaCl, 30 mM sodium citrate, 10 mM EDTA, 25 mM NaH_2PO_4 (pH 7.4) and salmon sperm DNA (250 ng/μl). After hybridization, cells were washed three times by 0.5× wash buffer consisting of 0.5× sodium citrate–sodium chloride and 0.1% sodium dodecyl sulfate at room temperature and washed once at 37 °C. For immunohistochemistry detection, the cells were incubated for 1 h at room temperature with 1: 3000 diluted alkaline phosphatase-conjugated streptavidin (Roche Diagnostics) in 100 mM Tris–HCl (pH 7.5) containing 150 mM NaCl and 0.1% Tween 20 after blocking non-specific binding sites on the cells with 0.1% blocking reagent. After the cells were equilibrated with 100 mM Tris–HCl (pH 9.5) containing 100 mM NaCl and 50 mM $MgCl_2$, nitroblue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indoyl-phosphate, 4-toluidine salt (BCIP) substrate (Roche Diagnostics) solution was added to develop color.

2.6. Effect of argatroban on the expression of myosin heavy-chain isoform mRNA

The effect of specific thrombin inhibitor, argatroban, on phenotype conversion of cultured vascular smooth muscle cells was tested. The cultured vascular smooth muscle cells were detached by incubating with 0.01% trypsin–EDTA solution for 5 min and replated on 25-cm^2 flask with 1.5×10^5 cells. After the cells were cultured in 10% fetal bovine serum–DMEM for 9 h, the medium was changed to serum-free DMEM containing PDGF-BB (10 and 50 ng/ml) or argatroban (10 and 50 μg/ml), and then incubated for 3 or 24 h. After incubation, total RNA was extracted to perform RT-PCR and the cells were fixed with 10% formalin–PBS for in

situ hybridization to detect mRNA expressions of myosin heavy chain isoform.

2.7. Effect of argatroban on the expression of SMemb protein

To measure the protein level of SMemb after stimulation of the cultured vascular smooth muscle cells by argatroban, the Western blots were performed according to the method by Towbin et al. (1979). Three and twenty-four hours after stimulations, crude myosin extracts were prepared according to the method by Rovner et al. (1986) with a minor modification. In brief, cultured vascular smooth muscle cells (1.5×10^5 cells) were removed from flasks by Rubber Policeman, rinsed with PBS, and were collected by centrifugation (1600 rpm, 4 °C) for 5 min. 50 μl of extraction buffer (50 mM NaH_2PO_4, 1 mM EGTA, 0.125 mM α-phenylmethanesulfonyl fluoride, pH 7.0) was added to cell pellet and homogenized by disposable pestles with Mini Cordless Grinder (Funakoshi, Tokyo, Japan). After centrifugation (10,000 rpm, 4 °C) for 10 min, the pellets were dissolved in Gubba–Straub solution (150 mM NaH_2PO_4, 300 mM NaCl, 1 mM EGTA, 0.125 mM α-phenylmethanesulfonyl fluoride, 1 mM 2-mercaptoethanol, 10 mM ATP, pH 6.7), and then gently mixed at 4 °C for 1 h. After centrifugation (10,000 rpm, 4 °C) for 10 min, the supernatants were colleted as crude myosin extracts. To separate the proteins in the crude myosin extracts, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 8–25% gradient gel was performed using a Phast System (Amersham Biosciences, NJ, USA). The proteins in the gel were electrically blotted onto polyvinylidene difluoride (PVDF) membrane (Immune-Blot™ PVDF Membrane, Bio-Rad Laboratories, CA, USA) by Phast-Transfer (Amersham Biosciences) at 15 °C, 20 V, and 25 mA for 90 min. After blotting, the membrane was blocked with 3% non-fat milk in PBS for 2 h at room temperature, and then the membrane was incubated at 4 °C overnight with monoclonal anti-nonmuscle myosin heavy chain (SMemb) (Yamasa, Tokyo, Japan), which was diluted to 1:1000 by 3% non-fat milk in PBS. After washing five times with 0.1% Tween 20 in PBS (PBST) for 10 min each, the membrane was incubated with 1:3000 diluted sheep anti-mouse IgG_{2b} (The Binding Site, Birmingham, UK) in 1% non-fat milk in PBST at room temperature for 1 h. After washing four times by PBST and two times by PBS, substrate solution (0.1 mg/ml 3-amino-9-ethylcarbazole in 0.1 M sodium phosphate buffer) was added. The intensity of colored SMemb protein bands were analyzed by NIH image.

2.8. Data analysis

Relative mRNA expressions of myosin heavy chain isoform were calculated by dividing the intensity of PCR products of argatroban and PDGF-BB by the intensity of PCR products of PBS. Transformation index (TI) represents the ratio of relative SMemb mRNA expression to the sum of relative mRNA expressions of SM1 and SM2. Thus, when TI is 0.5, there is no phenotype change of vascular smooth muscle cells. If TI is greater than 0.5, phenotypic conversion into synthetic type was occurred. Data are represented as means ± S.E. Statistical analyses were performed by one-way or two-way analysis of variance (ANOVA), followed by multiple comparison (post-hoc test). $P < 0.05$ was defined as statistical significance.

3. Results

3.1. Expression of myosin heavy-chain isoform mRNAs in cultured rabbit vascular smooth muscle cells

RT-PCR using specific primers for myosin heavy chain isoforms (SM1, SM2, and SMemb) amplified cDNA fragments, the size of which were 574, 146, and 188 base pairs (bp), respectively (Fig. 1B). The sequencing analysis revealed that the PCR products have 100% homology with the published cDNA sequences of myosin heavy chain isoforms (M77812, J03614, and D90237), respectively (data not shown). Since the isoform-specific primers could amplify cDNA of myosin heavy chain isoforms, myosin heavy chain isoform cDNA fragments containing RNA polymerase promoter sites (T7 and Sp6) were produced by PCR using primers, which had the sequences of the specific primers plus RNA polymerase promoter sequences. Con-

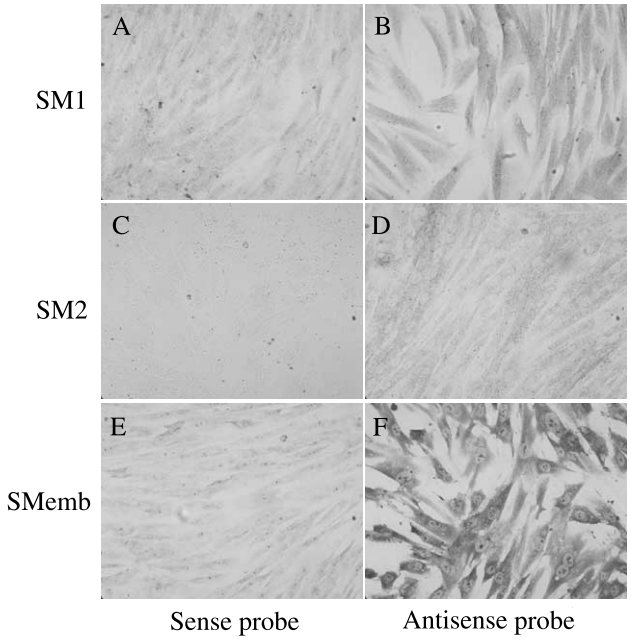

Fig. 2. In situ hybridization to detect mRNAs of SM1 (A and B, upper), SM2 (C and D, middle), and SMemb (E and F, lower) in cultured rabbit aortic vascular smooth muscle cells. Sense RNA probes (S probe) were used in left panels (A, C and E), and sense RNA probes (S probe) were used in right panels (B, D and F). Magnification of × 400.

sequently, these primers could amplify cDNA fragments, the size of which was longer than the original PCR products by the size of RNA polymerase promoters: SM1, 613 bp; SM2, 184 bp; SMemb, 214 bp (Fig. 1B). Thus, RNA polymerase promoter sites were actually added to cDNA fragments of myosin heavy chain isoforms.

3.2. In situ hybridization

As shown in Fig. 2, in situ hybridization revealed that mRNAs of myosin heavy chain isoforms were expressed in cultured vascular smooth muscle cells. The vascular smooth muscle cells, especially perinuclear cytoplasm, were stained dark purple. In addition, the intensity of the color with antisense SM1 and SMemb was much stronger than that with SM2. Sense RNA probes that were expected not to hybridize with any mRNAs were used to show background staining. Consequently, there was almost no color seen in left panels. Therefore, antisense RNA probes used were specific for each target mRNA.

3.3. Effect of argatroban and PDGF-BB on the mRNA expressions of myosin heavy-chain isoform

In situ hybridization demonstrated that SM1 mRNA was homogenously expressed in almost all cells of PBS (pre), PBS (3 h), argatroban- and PDGF-BB-treated cells. In addition, SM1 mRNA was rather expressed in nuclear than in cytoplasm, whereas SM2 mRNA was rather expressed in cytoplasm. The development of staining for SM1 was stronger in argatroban and PDGF as compared with PBS (3 h) (Fig. 3, left column). With regard to the expression of SM2 mRNA, there were no significant changes (Fig. 3, middle column). SMemb mRNA was expressed in both

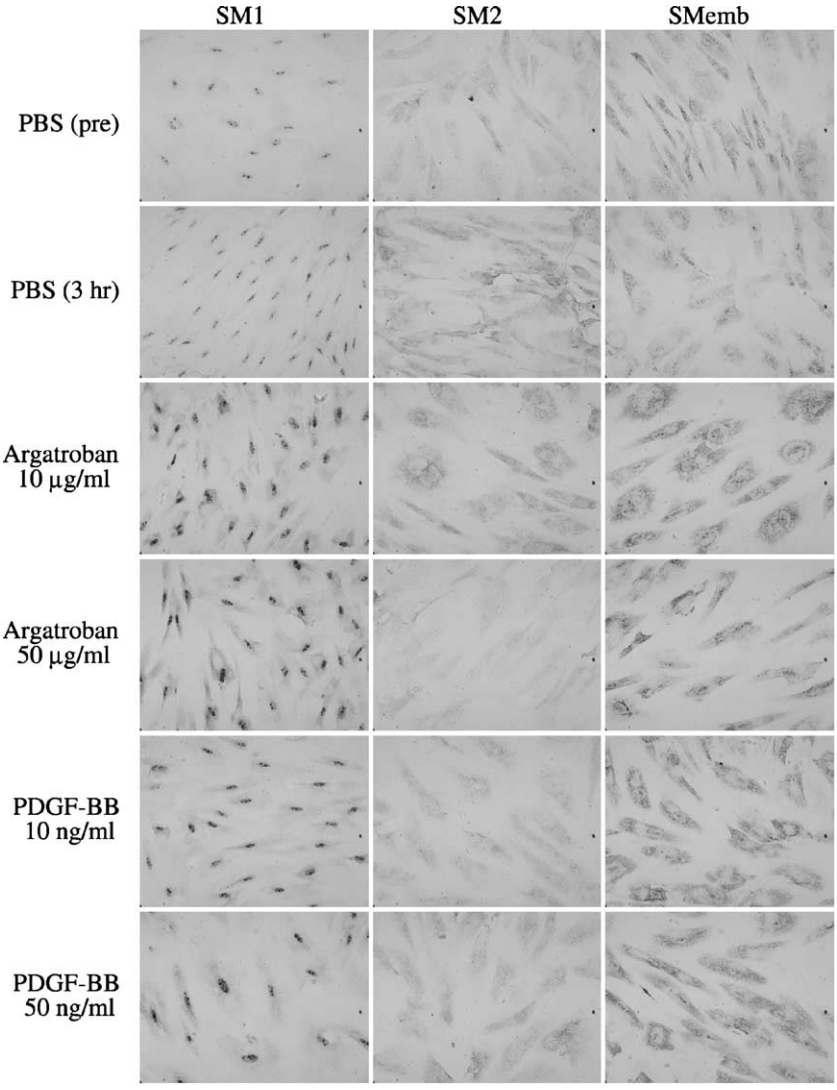

Fig. 3. Effect of argatroban on the expression of myosin heavy chain isoform mRNAs by in situ hybridization. The cultured vascular smooth muscle cells on coverslips were incubated in serum-free medium containing phosphate buffered saline (PBS), argatroban (10 and 50 μg/ml) and PDGF-BB (10 and 50 ng/ml) for 3 h. See the details in text. Magnification of × 400.

cytoplasm and nuclear (especially nucleolus). The developing color was strong in argatroban- and PDGF-treated cells (Fig. 3, right column).

As shown in Fig. 4, RT-PCR revealed that PDGF-BB (10 and 50 ng/ml) significantly increased the expressions of SM1 mRNA to 1.2-fold ($P<0.05$) and 1.6-fold ($P<0.01$), respectively. The expression of SM2 mRNA was also increased to 1.6-fold with statistical significance ($P<0.05$), whereas SMemb mRNA expression was slightly increased (Fig. 4). When the ratio of SMemb mRNA expression to SM1 plus SM2 mRNA expressions (transformation index, TI) was calculated, PDGF-BB (50 ng/ml) significantly decreased the TI ($P<0.05$).

Fig. 5 shows that the effect of two doses (10 and 50 μg/ml) of argatroban on the expressions of myosin heavy-chain isoform mRNA. Cultured vascular smooth muscle cells were incubated with argatroban for 3 and 24 h. Short-term treatment with argatroban revealed that SM1 and SM2 mRNA

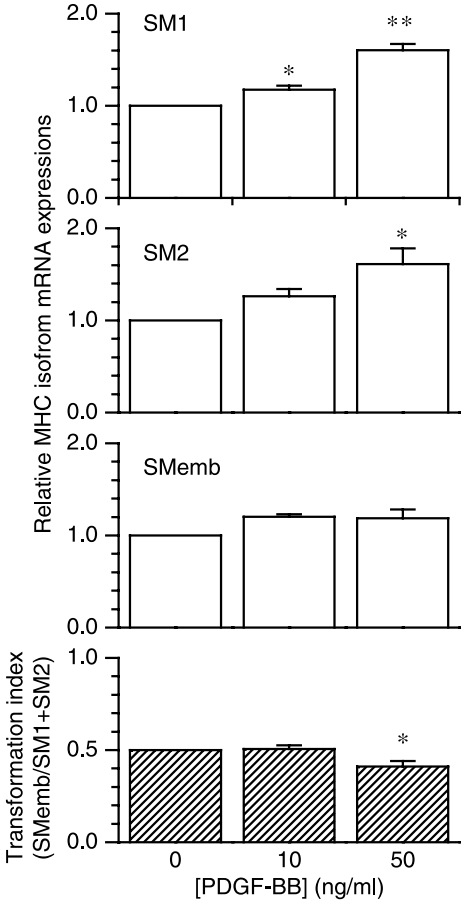

Fig. 4. RT-PCR quantitation of relative myosin heavy chain (MHC) isoform mRNA expressions after 3-h stimulation by PDGF-BB (10 and 50 ng/ml, $n=5$). PCR cycles for SM1 (top panel), SM2 (second panel), and SMemb (third panel) were 28, 30, and 26. Intensity of the specific bands were valued by NIH image and normalized by the value of PBS. Transformation index (bottom panel) was calculated by dividing relative SMemb mRNA by the sum of relative SM1 and SM2 mRNA expression. Data are represented as means ± S.E. *, **: Significantly different from 0 ng/ml of PDGF-BB at $P<0.05$ and $P<0.01$, respectively (one-way ANOVA).

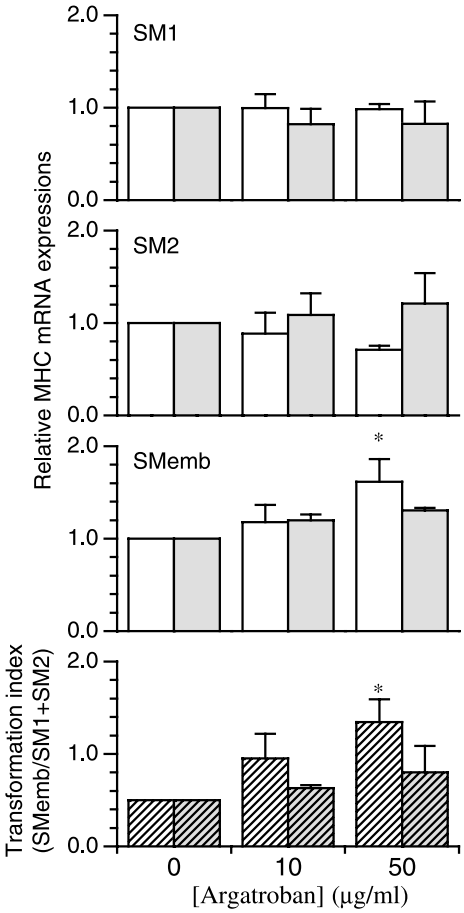

Fig. 5. RT-PCR quantitation of relative myosin heavy chain (MHC) isoform mRNA expressions after 3-h (open bars) and 24-h (gray bars) treatments by argatroban (0, 10 and 50 μg/ml, $n=5$). PCR cycles for SM1, SM2, and SMemb were 28, 30, and 26. Intensity of the specific bands were valued by NIH image and normalized by the value of PBS (3 or 24 h). Transformation index (bottom panel) of 3-h (hatched bars) and 24-h (hatched gray bars) treatment with argatroban were calculated by dividing relative SMemb mRNA by the sum of relative SM1 and SM2 mRNA expression. Data are represented as means ± S.E. *: Significantly different from 3-h stimulation with 0 μg/ml of argatroban at $P<0.05$ (one-way ANOVA).

expressions were not changed, while SMemb mRNA expression was increased to 1.6-fold with a statistical significance ($P<0.05$). TI was significantly increased to 2.4-fold by argatroban (50 μg/ml), as expected. Long-term treatment with argatroban (10 and 50 μg/ml) did not change SM1/SM2 mRNA expressions. Although SMemb mRNA expression was slightly increased, there was no statistical significance. In addition, TI was slightly increased; however, there was no statistical significance.

3.4. Effect of argatroban on the expression of SMemb protein

We further explored if the increase in SMemb mRNA was accompanied by changes in SMemb protein. To do this, we extracted myosin heavy chain proteins from cultured vascular smooth muscle cells and rabbit aorta tissues to determine

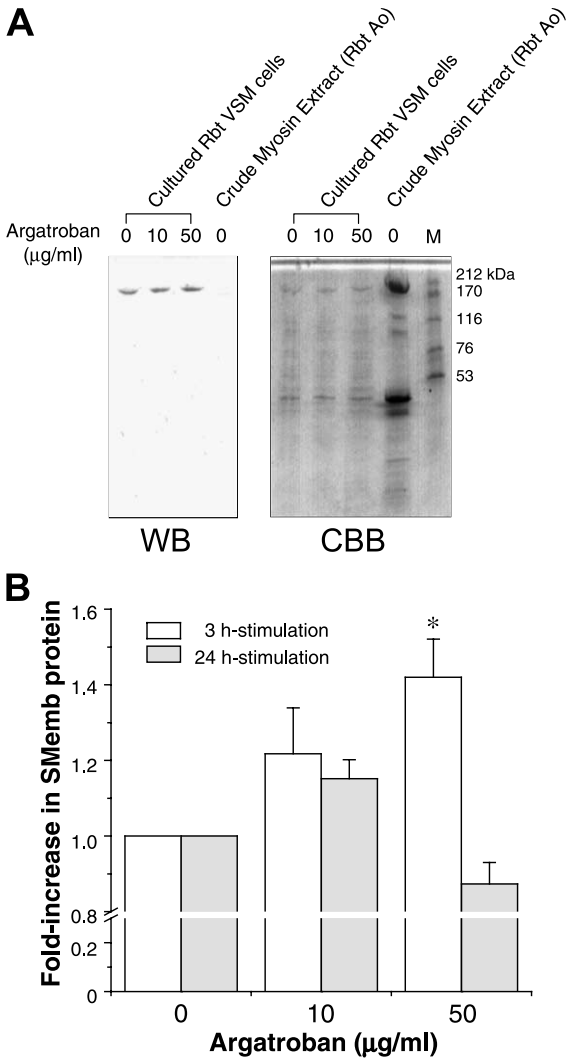

Fig. 6. Western blot analysis on the relative SMemb protein expressions. (A) Proteins in crude myosin extracts prepared from cultured rabbit aortic vascular smooth muscle cells 3 h after treatment by argatroban (cultured Rbt VSM cells) and from rabbit aortic tissue (Crude Myosin Extract (Rbt Ao)) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Coomassie brilliant blue staining of the gel is shown in right panel (CBB). M represents molecular markers: myosin (212 kDa), α_2-macroglobuin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa), and glutamic dehydrogenase (53 kDa). In the left panel (WB), transferred SMemb proteins on the polyvinylidene difluoride (PVDF) membrane were detected by monoclonal anti-nonmuscle myosin heavy chain (SMemb). (B) Intensity of the SMemb protein bands were valued by NIH image and normalized by the value of argatroban (0 μg/ml) (3 or 24 h). Fold increases in SMemb protein after 3-h (open bars) and 24-h (gray bars) treatments with argatroban (0, 10 and 50 μg/ml, $n=3$) are shown. Data are represented as means ± S.E. *: Significantly different from 3-h stimulation by 0 μg/ml of argatroban at $P<0.05$ (one-way ANOVA).

the level of SMemb protein expression by Western blots using a monoclonal antibody against SMemb. In Fig. 6A, SDS-PAGE demonstrated that crude myosin extracts from rabbit aortic tissue contained myosin heavy chain (ca. 200 kDa), actin (ca. 42 kDa), and tropomyosin (ca. 30 kDa). The crude myosin extracts from cultured vascular smooth muscle cells after treatment by argatroban (0, 10, 50 μg/ml) mainly consist of 200- and 42 kDa protein bands, which likely corresponds to myosin heavy chain and actin, respectively, based on their molecular weights. Anti-SMemb monoclonal antibody-labeled single 200-kDa protein bands in lanes of cultured vascular smooth muscle cells, but not in crude myosin extract from rabbit aortic tissue (Fig. 6A, left panel termed as WB). As shown in Fig. 6B, the averaged data demonstrated SMemb protein level was significantly increased to 1.4-fold by 3-h stimulation by argatroban (50 μg/ml). However, SMemb protein level was not increased by 24-h stimulation with argatroban.

3.5. Effect of argatroban on mRNA expressions of other phenotype markers

To further test whether other phenotype markers such as PAI-1 and β-actin mRNA expressions were up-regulated by argatroban, RT-PCR was carried out. As shown in Fig. 7 (middle), β-actin mRNA expression by 3- and 24-h treatments with argatroban (50 μg/ml) was increased with a statistical significance. PAI-1 mRNA expression was increased by 3- and 24-h treatments of argatroban (50 μg/ml) (Fig. 7, top). Although there was no statistical significance by one-way ANOVA, there was a statistically signifi-

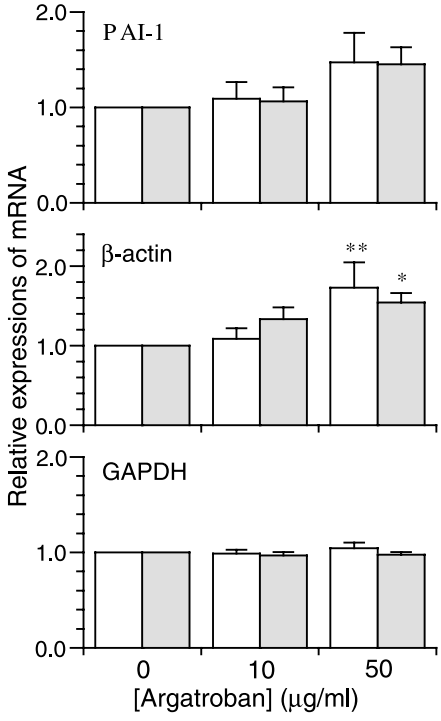

Fig. 7. RT-PCR quantitation of relative plasminogen activator inhibitor (PAI)-1, β-actin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expressions after 3-h (open bars) and 24-h (gray bars) treatments with argatroban (0, 10, and 50 μg/ml, $n=5$). PCR cycles for PAI-1, β-actin, and GAPDH were 28, 17, and 24, respectively. Intensity of the specific bands were valued by NIH image and normalized by the value of PBS (3 or 24 h). Data are represented as means ± S.E. *, **: Significantly different from 24- and 3-h stimulation by 0 μg/ml of argatroban, respectively. *$P<0.05$, **$P<0.01$, by one-way ANOVA.

cant increase in the expression PAI-1 mRNA by two-way ANOVA ($P=0.0144$, 50 vs. 0 μg/ml argatroban; $P=0.0437$, 50 vs. 10 μg/ml argatroban). When GAPDH mRNA expression was measured as an internal control, argatroban had no effect on its expression (Fig. 7, bottom). In addition, the increases in mRNA expression of β-actin and PAI-1 were sustained up to 24 h.

4. Discussion

Our data demonstrated that PDGF-BB up-regulated SM1/SM2 mRNA expressions, while argatroban up-regulated SMemb mRNA expression in rabbit cultured vascular smooth muscle cells. Thus, argatroban may induce phenotype conversion of cultured vascular smooth muscle cells from contractile to synthetic type. This finding could not support the fact that locally administered argatroban prevented vascular restenosis after balloon angioplasty (Imanishi et al., 1997). The expressions of myosin heavy-chain isoform mRNA in cultured vascular smooth muscle cells, however, depend on cellular density, serum concentration, and substrate for attachment (Thyberg, 1996). Thus, the discrepancy between our in vitro data and the in vivo data must be considered very carefully.

Even though the vascular smooth muscle cells were cultured up to 12th passage, RT-PCR revealed that the vascular smooth muscle cells expressed SM1/SM2 mRNAs as well as SMemb mRNA (data not shown). Thus, vascular smooth muscle cells appear to have transitional differentiated/undifferentiated states between synthetic and contractile phenotypes. This finding opposes the previous report that rabbit cultured vascular smooth muscle cells highly expressed SMemb mRNA but not SM1/SM2 (Kuro-o et al., 1991).

Interestingly, PDGF-BB unexpectedly stimulated SM1/SM2 mRNA expressions; thereby leading vascular smooth muscle cells to differentiated state (Fig. 4). It was demonstrated that PDGF-BB can induce the phenotypic modulation of vascular smooth muscle cells by the balance and cross-regulation between the phosphatidylinositol (PI) 3-kinase/Akt and Ras/Raf/mitogen-activated protein (MAP) kinase-extracellular signal-regulated kinase (ERK) (MEK) signaling cascades, which determines the temporal pattern of ERK1/2 phosphorylation (Hayashi et al., 1999; Hu et al., 1996; Pyles et al., 1997; Reusch et al., 2001). In our preparation of cultured vascular smooth muscle cells, PDGF-BB enhanced the expression of SM1/SM2 mRNA, suggesting that PDGF-BB might activate the PI-3kinase/Akt pathway, which may lead vascular smooth muscle cells to differentiate.

Argatroban significantly increased the expression of SMemb mRNA, but not SM1 and SM2 (Fig. 5). In addition, the increase in SMemb mRNA was accompanied by the increase in SMemb protein (Fig. 6). Since argatroban have a guanidine chain, it is possible for argatroban to serve as nitric oxide donor like L-arginine. It was reported that there was a progressive and significant increase in the mean concentration of plasma nitric oxide in peripheral arterial obstructive disease patients after commencement of argatroban infusion with an unknown mechanism (Ueki et al., 1999). Our preliminary data demonstrated that 24 h treatment with L-arginine had no effect on the expression of SMemb mRNA (data not shown). Thus, argatroban-induced phenotype conversion might not be involved in nitric oxide production.

It has been recently clarified that SMemb gene transcription is transactivated by basic transcriptional element binding protein-2 (BTEB2) and a homeobox gene, Hex (Nagai et al., 2001). The transcription of PAI-1 gene is also transactivated by BTEB2. PAI-1 expression is known to be increased in synthetic vascular smooth muscle cells (Nagai et al., 2001). β-actin is another phenotype marker, which expression is also increased in synthetic vascular smooth muscle cells (Kocher et al., 1984). In agreement, RT-PCR revealed that PAI-1 and β-actin mRNA was increased by argatroban treatment (Fig. 7). Therefore, the activation of BTEB2 may be involved in argatroban-induced increases in mRNA expressions of SMemb and PAI-1. However, the transactivation of β-actin gene is not regulated by BTEB-2. Thus, there might be a common factor that involves in the transactivation of these three genes. Argatroban may increase mRNAs that were associated with phenotype conversion to synthetic.

However, it remains to be clarified how argatroban is involved in regulation of SMemb, PAI-1, and β-actin gene transcriptions. In future experiments, this will be elucidated by constructing reporter genes expressed under the control of SMemb, PAI-1, and β-actin promoters. Furthermore, it has not been demonstrated whether there is a cross-talk between SM1/2 and SMemb genes. Simons and Rosenberg (1992) demonstrated that antisense SMemb oligonucleotides suppressed the proliferation of vascular smooth muscle cells, implicating that SMemb mRNA and/or SMemb protein might participate in regulation of vascular smooth muscle cell proliferation. We will test whether SM1/2 and SMemb function as coactivators or repressors to each other.

In the present study, we demonstrated that argatroban, a selective synthetic thrombin inhibitor, could directly induce phenotype conversion of cultured vascular smooth muscle cells. The clarification of the mechanism by which the expression of SM1/2 and SMemb mRNA of vascular smooth muscle cells will provide us possible therapeutics to prevent restenosis after angioplasty.

References

Aikawa, M., Sivam, P.N., Kuro-o, M., Kimura, K., Nakahara, K., Takewaki, S., Ueda, M., Yamaguchi, H., Yazaki, Y., Periasamy, M., 1993. Human smooth muscle myosin heavy chain isoforms as molecular markers for vascular development and atherosclerosis. Circ. Res. 73, 1000–1012.

Arno, H.M., Levi, M., Peters, R.J.G., 2002. Tissue factor and coronary restenosis. Cardiovasc. Res. 53, 313–325.

Babij, P., Kawamoto, S., White, S., Adelstein, R.S., Periasamy, M., 1992. Differential expression of SM1 and SM2 myosin isoforms in cultured vascular smooth muscle. Am. J. Physiol. 262, C607–C613.

Bauters, C., Meurice, T., Hamon, M., McFadden, E., Lablanche, J.-M., Bertrand, M.E., 1996. Mechanisms and prevention of restenosis: from experimental models to clinical practice. Cardiovasc. Res. 31, 835–836.

Blank, R.S., Swartz, E.A., Thompson, M.M., Olson, E.N., Owens, G.K., 1995. A retinoic acid-induced clonal cell line derived from multipotential P19 embryonal carcinoma cells expresses smooth muscle characteristics. Circ. Res. 76, 742–749.

Borrione, A.C., Zanellato, A.M., Scannapieco, G., Pauletto, P., Sartore, S., 1989. Myosin heavy-chain isoforms in adult and developing rabbit vascular smooth muscle. Eur. J. Biochem. 183, 413–417.

Burchenal, J.E.B., Marks, D.S., Mann, J.T., Schweiger, M.J., Rothman, M.T., Ganz, P., Adelman, B., Bittl, J.A., 1998. Effect of direct thrombin inhibition with bivalirudin (Hirulog) on restenosis after coronary angioplasty. Am. J. Cardiol. 82, 511–515.

Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J., Rutter, W.J., 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18, 5294–5299.

Hayashi, A., Suzuki, T., Tajima, S., 1995. Modulations of elastin expression and cell proliferation by retinoids in cultured vascular smooth muscle cells. J. Biochem. (Tokyo) 117, 132–136.

Hayashi, K., Takahashi, M., Kimura, K., Nishida, W., Saga, H., Sobue, K., 1999. Changes in the balance of phosphoinositide 3-kinase/protein kinase B (Akt) and the mitogens-activated protein kinases (ERK/p38MAPK) determine a phenotype of visceral and vascular smooth muscle cells. J. Cell. Biol. 145, 740–772.

Hu, Y., Cheng, L., Hochleitner, B.W., Xu, Q., 1996. Activation of mitogen-activated protein kinases (ERK/JNK) and AP-1 transcription factor in rat carotid arteries after balloon injury. Arterioscler. Thromb. Vasc. Biol. 17, 2808–2816.

Imanishi, T., Arita, M., Tomobuchi, Y., Hamada, M., Hano, T., Nishio, I., 1997. Effects of locally administered argatroban on restenosis after balloon angioplasty: experimental and clinical study. Clin. Exp. Pharmacol. Physiol. 24, 800–806.

Ip, J.H., Fuster, V., Israel, D., Badimon, L., Badimon, J., Chesebro, J.H., 1991. The role of platelets, thrombin and hyperplasia in restenosis after coronary angioplasty. J. Am. Coll. Cardiol. 17, 77B–88B.

Kocher, O., Skalli, O., Bloom, W.S., Gabbiani, G., 1984. Cytoskeleton of rat aortic smooth muscle cells. Normal conditions and experimental intimal thickening. Lab. Invest. 50, 645–652.

Kuro-o, M., Nagai, R., Nakahara, K., Katoh, H., Tsai, R.C., Tsuchimochi, H., Yazaki, Y., Ohkubo, A., Takaku, F., 1991. cDNA cloning of a myosin heavy chain isoform in embryonic smooth muscle and its expression during vascular development and in arteriosclerosis. J. Biol. Chem. 266, 3768–3773.

Miano, J.M., Cserjesi, P., Ligon, K.L., Periasamy, M., Olson, E.N., 1994. Smooth muscle myosin heavy chain exclusively marks the smooth muscle lineage during mouse embryogenesis. Circ. Res. 75, 803–812.

Moledina, M., Chakir, M., Gandhi, P.J., 2001. A synopsis of the clinical uses of argatroban. J. Thromb. Thrombolysis 12, 141–149.

Nagai, R., Suzuki, T., Aizawa, K., Miyamoto, S., Amaki, T., Kawai-Kowase, K., Sekiguchi, K.I., Kurabayashi, M., 2001. Phenotypic modulation of vascular smooth muscle cells: dissection of transcriptional regulatory mechanisms. Ann. N.Y. Acad. Sci. 947, 56–66.

Okamoto, S., Hijikata, A., Kikumoto, R., Tonomara, S., Hara, H., Ninomiya, K., Maruyama, A., Sugano, M., Tamao, Y., 1981. Potent inhibition of thrombin by the newly synthesized arginine derivative no. 805. The importance of stereostructure of its hydrophobic carboxamide portion. Biochem. Biophys. Res. Commun. 101, 440–446.

Okamoto, E., Imataka, K., Fujii, J., Kuro-o, M., Nakahara, K., Nishimura, H., Yazaki, Y., Nagai, R., 1992. Heterogeneity in smooth muscle cell population accumulating in the neointimas and the media of poststenotic dilatation of the rabbit carotid artery. Biochem. Biophys. Res. Commun. 185, 459–464.

Okayama, H., Kawaischi, M., Brownstein, M., Lee, F., Yokota, T., Arai, K., 1987. High-efficiency cloning of full-length cDNA; Construction and screening of cDNA expression libraries for mammalian cells. In: Wu, R., Grossmann, L.Methods Enzymol., vol. 154. Academic Press, San Diego, pp. 3–28.

Pyles, J.M., March, K.L., Franklin, M., Mehdi, K., Wilenski, R.L., Adam, L.P., 1997. Activation of MAP kinase in vivo follows balloon overstretch injury of porcine coronary and carotid arteries. Circ. Res. 81, 904–910.

Raap, A.K., Van de Rijke, F.M., Dirks, R.W., Sol, C.J., 1991. Bicolor fluorescence in situ hybridization to intron- and exon mRNA sequences. Exp. Cell Res. 197, 319–322.

Reusch, H.P., Zimmermann, S., Schaefer, M., Paul, M., Moelling, K., 2001. Regulation of Raf by Akt controls growth and differentiation in vascular smooth muscle cells. J. Biol. Chem. 276, 33630–33637.

Rovner, A.S., Thompson, M.M., Murphy, R.A., 1986. Two different heavy chains are found in smooth muscle myosin. Am. J. Physiol. 250, C861–C870.

Simons, M., Rosenberg, R.D., 1992. Antisense nonmuscle myosin heavy chain and c-myb oligonucleotides suppress smooth muscle cell proliferation in vitro. Circ Res. 70, 835–843.

Thyberg, J., 1996. Differentiated properties and proliferation of arterial smooth muscle cells in culture. Int. Rev. Cytol. 169, 183–265.

Topol, E.J., Serruys, P.W., 1998. Frontiers in interventional cardiology. Circulation 98, 1802–1820.

Towbin, H., Staehelin, T., Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354.

Ueki, Y., Matsumoto, K., Kizaki, Y., Yoshida, K., Matsunaga, Y., Yano, M., Miyake, S., Tominaga, Y., Eguchi, K., 1999. Argatroban increases nitric oxide levels in patients with peripheral arterial obstructive disease: placebo-controlled study. J. Thromb. Thrombolysis 8, 131–137.

Yoshida, Y., Mitsumata, M., Yamane, T., Tomikawa, M., Nishida, K., 1988. Morphology and increased growth rate of atherosclerotic intimal smooth-muscle cells. Arch. Pathol. Lab. Med. 112, 987.

Zhang, Z., Nagata, I., Kikuchi, H., Xue, J.-H., Sakai, N., Sakai, H., Yanamoto, H., 2001. Broad-spectrum and selective serine protease inhibitors prevent expression of platelet-derived growth factor-BB and cerebral vasospasm after subarachnoid hemorrhage: vasospasm caused by cisternal injection of recombinant platelet-derived growth factor-BB. Stroke 32, 1665–1672.