RNAi Targeting Embryonic Myosin Heavy Chain Isoform Inhibited Bound Thrombin-Induced Migration of Vascular Smooth Muscle Cells

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Key Words

Bound thrombin · SMemb gene · siRNA · Cultured vascular smooth muscle cells · Migration

Abstract

To investigate the effect of bound thrombin, a complex of α -thrombin with fibrin fragments derived from clots, on proliferation and migration of cultured rabbit vascular smooth muscle cells, cell proliferation was measured by WST-1 reagent and migration was evaluated by counting migrated cells through pores of cell culture insert (8 μ m size) after 48-hour treatment with bound thrombin (10 U/ml). To examine the role of an embryonic myosin heavy chain isoform (SMemb) in these effects by bound thrombin, the cells were subsequently treated for 48 h with an siRNA expression vector (ORF-2/*pSilencer*) directed against the open reading frame of SMemb mRNA. SMemb and plasminogen activator inhibitor-1 mRNA expressions were measured by Northern blot analysis. Bound thrombin significantly increased SMemb mRNA expression by 1.4 ± 0.01 -fold and significantly increased plasminogen activator inhibitor-1 mRNA expression by 2.65 ± 0.69 -fold ($p < 0.01$ vs. PBS treatment for each), which were abolished by treatment with ORF-2/*pSilencer*. Although bound thrombin had no effect on cell proliferation, bound thrombin significantly increased migration by 1.93 ± 0.20 -fold ($p < 0.05$). ORF-2/*pSilencer* treatment significantly reduced the bound thrombin-stimulated migra-

tion activity by 1.28 ± 0.15 -fold ($p < 0.05$). Thus, SMemb plays an important role in bound thrombin-induced cell migration activity of cultured vascular smooth muscle cells.

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Introduction

The proliferation of vascular smooth muscle (VSM) cells plays a major role in vascular restenosis following coronary interventions [1–3]. The highly proliferative VSM cells consist of synthetic type, which can produce growth factors and extracellular matrices, thereby developing intimal hyperplasia. It was demonstrated that SMemb, an embryonic myosin heavy chain (MHC) isoform, was highly expressed in synthetic cell type, while other MHC isoforms (SM1 and SM2) were expressed in contractile type [4]. Therefore, a switch of MHC isoform expression seemed to closely link to proliferation and migration of VSM cells.

It was demonstrated by in vitro study that thrombin activity in the presence of fibrin clot was not inhibited by heparin. Francis et al. [5] revealed that the activity of thrombin remained in the clot. The residual thrombin in clot has been called postclotting thrombin (bound thrombin). After percutaneous transluminal coronary angioplasty, levels of fibrinopeptide A, thrombin-antithrombin III complex and prothrombin fragments 1+2 were

significantly increased in many patients, indicating coronary thrombosis by procoagulant activity [6, 7]. It was reported that the coronary thrombosis by procoagulant activity was resistant to the treatment by high doses of heparin [8, 9]. In vitro studies demonstrated that thrombus-associated thrombin and factor Xa were resistant to heparin treatment [10, 11]. Therefore, heparin-resistant procoagulant activity might be caused by bound thrombin. In addition, coagulation factors including thrombin and tissue factor are involved in the development of vascular stenosis after interventional treatment [12, 13]. We hypothesized that bound thrombin in the thrombus plays an important role in the development of restenosis.

We have reported some unique biological and physicochemical characteristics of bound thrombin [14–16], including platelet aggregation, complex with fibrin fragments. We also demonstrated that bound thrombin enhanced the phenotype conversion of VSM cells towards a synthetic type by upregulating mRNA expressions of SMemb and plasminogen activator inhibitor type 1 (PAI-1) by the comparative reverse transcription polymerase chain reaction (RT-PCR) method [17]. Therefore, bound thrombin might contribute to the pathogenesis of restenosis after vascular injury. We also clarified the role of SMemb gene expression in phenotypic change in cultured rabbit VSM cells using siRNA-targeting SMemb mRNA. Consequently, it was demonstrated by the comparative RT-PCR method that PAI-1 and β -actin mRNA expressions were downregulated in the treated cells with SMemb siRNA, indicating the inhibition of phenotypic change toward the synthetic type by the knockdown of SMemb gene expression [18].

We constructed siRNA expression plasmid vector-targeting SMemb mRNA. In order to more accurately test whether the bound thrombin-induced upregulation of SMemb and PAI-1 mRNA could be inhibited by the SMemb-specific siRNA expression vectors, the expression of mRNAs was measured by Northern blot analysis in this study. We also tested whether the SMemb-specific siRNA expression vector inhibited bound thrombin-induced changes in cell proliferation and migration activities.

Materials and Methods

Animals, Animal Care and Cell Culture

All animals were cared for according to the Guidelines for Animal Experiments in Research Institutes (Notice No. 71 of the Ministry of Education, Culture, Sports, Science and Technology,

2006) and the Guidelines for Animal Experiments issued by the University of the Ryukyus. VSM tissues were isolated and cultured as previously reported [19]. In brief, thoracic aorta was excised from male Japanese white rabbits weighing 2.5 kg (Kyudo Co., Kumamoto, Japan) and cut into small pieces (about 4×4 mm) after the endothelium was mechanically removed. The tissues were plated on cell culture dishes with the luminal side down, and cultured under 5% CO₂ at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) plus penicillin and streptomycin. The 6th passage of the cultured VSM cells was employed for the experiments.

Preparation of Bound Thrombin

Preparation of bound thrombin was carried out as previously described [15, 20]. In brief, a mixture of 0.5 ml of bovine α -thrombin (500 U/ml) and 2.5 ml of rabbit fibrinogen (1.5 mg/ml in serum-free DMEM) was incubated for 15 min at 37°C to allow a clot to form. After removal of the supernatant, the clot was rinsed twice with 2 ml of serum-free DMEM. The clot was then crushed mechanically using a glass rod (approx. 500 strokes). The insoluble materials were removed by centrifugation at 3,000 rpm for 15 min at 4°C. The clotting activity of the bound thrombin was measured by BBL™ FibroSystem® (Becton Dickinson, Cockeysville, Md., USA). The prepared bound thrombin was further diluted with 10% FBS in DMEM to a final concentration of 10 U/ml. For comparison, purified rabbit fibrinogen (10 μ g/ml) and bovine α -thrombin (10 U/ml) were used.

Construction of Plasmid Vectors for Expression of SMemb mRNA-Specific siRNAs

To search candidates for siRNA-targeting nucleotide sequences which have 21 nucleotides, start with double adenine and have guanine/cytosine contents from 40 to 50%, we used an siRNA insert design on-line tool provided on the homepage of Ambion Inc. (Austin, Tex., USA). Consequently, 3 target sites of rabbit SMemb mRNA (DDBJ accession No. D10280) were chosen: 5'-aaggaccttgaagcgcaaatc-3' 454–474 (ORF-1), 5'-aacaaggatctgaaggccaag-3' 955–975 (ORF-2) and 5'-aacctgtcttaagactctgc-3' 1630–1650 (3'UTR). These sequences did not have any homology to other MHC isoforms and any mammalian cDNA sequences based on the BLAST search. After the sense and antisense hairpin siRNA templates were annealed, the siRNA expression vectors were constructed using pSilencer™ 2.1-U6 neo (Ambion) according to the manufacturer's procedures. The 3 kinds of annealed template were ligated into pSilencer, which were digested with *Bam*HI and *Hind*III.

Transfection of SMemb-siRNA Expression Vector into Cultured VSM Cells

The constructed siRNAs were introduced intracellularly into the cultured VSM cells with Lipofectamine™ and Plus™ reagents (Invitrogen, Carlsbad, Calif., USA) according to the manufacturer's procedures. First, precomplex of each SMemb/pSilencer with 30 μ g Plus reagent in 200 μ l OPTI-MEM® medium (Invitrogen) was prepared, and then incubated for 15 min at room temperature. Following this, 20 μ g Lipofectamine reagent was diluted with 200 μ l OPTI-MEM. To form the siRNA-Plus-Lipofectamine reagent complexes, 200 μ l of precomplexed siRNA and 200 μ l of the diluted Lipofectamine reagent were combined and incubated for 30 min at room temperature. After that, the 400 μ l of com-

plexes was diluted into 1.6 ml of OPTI-MEM and added to the VSM cells in 25-mm² culture flasks (Becton Dickinson). VSM cells were incubated for the first 4 h at 37°C, and then 2 ml of DMEM containing 10% FBS was added and incubated for another 44 h. We tested if cell viability was affected by transfection after 48 h. Cell viability was evaluated as the relative number of cells to nontreatment (without Lipofectamine and pSilencer expression vector). Consequently, cell viability was neither changed by treatment with Lipofectamine nor by pSilencer expression vector: nontreatment, 0.53 ± 0.018 ; Lipofectamine alone, 0.49 ± 0.015 ; (-)/pSilencer (1 µg/ml), 0.52 ± 0.041 ; (-)/pSilencer (3 µg/ml), 0.52 ± 0.038 .

Northern Blot Analysis

To measure the expression of MHC isoform mRNAs, we performed Northern blot analysis. Total RNA was extracted from approximately 8×10^5 cultured VSM cells using a PURESRIPT® RNA Isolation Kit cell and tissue kit (GentraSystem, Minneapolis, Minn., USA). For Northern blotting, each of 10 µg RNAs was denatured and loaded on a 1.1% agarose-formaldehyde gel. Subsequently, RNAs were transferred onto a positively charged nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany), and UV cross-linked with a HybriLinker HL-2000 (UVP Inc., Upland, Calif., USA). The templates of cDNA probes for SMemb, PAI-1 and glyceraldehydes-3-phosphate dehydrogenase (GAPDH), which was used as an internal control, were prepared by RT-PCR as previously described [18]. The cDNA probe was labeled with [α -³²P] dCTP using a Rediprime™ II random prime labeling system (Amersham Biosciences, Little Chalfont, UK), and hybridized with ExpressionHyb solution (BD Biosciences Clontech, Palo Alto, Calif., USA) at 68°C for 1 h. The membrane was washed with $2 \times$ SSC, 0.05% SDS solution for 20 min at room temperature twice; this was followed by a $0.1 \times$ SSC, 0.1% SDS solution with continuous shaking at 50°C for 20 min twice. The radioactivity on the membrane was measured by Fuji imaging plate type BAS-IIIIs (Fuji Film, Kanagawa, Japan) using Fuji film BAS-1500. The intensities of the bands were measured by NIH Image®.

Comparative RT-PCR

To measure the expression of SM1 and SM2 mRNAs, we performed comparative RT-PCR as previously reported [17, 19]. The primers for MHC isoforms (SM1 and SM2) and GAPDH were used as previously described [17]. The PCR products were separated on 2.5% agarose gel containing ethidium bromide with a half concentration of Tris-borate-EDTA electrophoresis buffers (pH 7.5). The fluorescent intensities of amplified cDNA bands were estimated by NIH Image.

Immunofluorescence Staining of Cultured VSM Cells

For immunofluorescence, we employed mouse anti-rabbit SMemb monoclonal antibody (Yamasa Corp., Chiba, Japan) according to the method given in the previous report [21]. Forty-eight hours after introduction, the cultured rabbit VSM cells were fixed with 10% formalin neutral buffer solution (Wako, Osaka, Japan) for 40 min at room temperature. The fixed VSM cells were treated with 0.2% Triton X-100 in PBS for 5 min to increase their membrane permeability. The cells were incubated with 3% nonfat milk in PBS for 1 h at room temperature, and then the cells were incubated for 1 h with mouse anti-rabbit SMemb antibody, which

was diluted to 1:3,000 by 3% nonfat milk in PBS. After washing 4 times with 0.05% Tween 20 in PBS for 10 min each, the cells were incubated for 1 h with 1:3,000 diluted tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). The cell images were analyzed using a Nikon ECLIPSE E600 microscope (Nikon, Tokyo, Japan) with the B-2E/C fluorescence filter for FITC staining and the UV-2E/C fluorescence filter for DAPI staining.

Western Blot Analysis

Crude myosin extracts were prepared after 48-hour treatment according to the method by Rovner et al. [22] with a minor modification. To measure the protein level of SMemb after treatment of the cultured VSM cells with ORF-2/pSilencer, Western blots were performed as previously reported [17]. To separate the proteins in the crude myosin extracts, SDS-PAGE with a 4–10% gradient gel was performed using a PhastSystem (GE Healthcare UK Ltd., Chalfont St Giles, UK). The proteins in the gel were electrically blotted onto polyvinylidene difluoride membrane (Immune-Blot PVDF Membrane; Bio-Rad Laboratories, Hercules, Calif., USA) at 4°C, 1.5 mA/cm² for 2 h. After blotting, the membrane was blocked with 3% nonfat milk in PBS for 2 h at room temperature, and then the membrane was incubated at 4°C overnight with a monoclonal antibody against SMemb (Yamasa Corp.), which was diluted to 1:1,000 by 3% nonfat milk in PBS. After 5 times washing with 0.1% Tween 20 in PBS (PBST) for 10 min each, the membrane was incubated with 1:3,000 diluted horseradish peroxidase-conjugated sheep anti-mouse IgG_{2b} (The Binding Site, Birmingham, UK) in 1% nonfat milk in PBST at room temperature for 1 h. After washing 4 times by PBST and 2 times by PBS, immune complex was visualized using ECL Plus Western blotting detection reagents (GE Healthcare). The signal intensities of SMemb protein bands were analyzed by NIH Image.

Cell Proliferation and Migration Assay

Cell proliferation assay of the cultured VSM cells was examined by the cell proliferation reagent WST-1 or the cell proliferation ELISA 5-bromo-2'-deoxyuridine (BrdU, colorimetric; Roche Diagnostics). Ten microliters of the cell proliferation reagent WST-1 or BrdU labeling solution was added to each well. After 1-hour incubation of the cells with the WST-1 reagent, the absorbance at 415 nm (reference wavelength of 550 nm) was measured by an MTP-300 microplate reader (Corona Electric, Ibaragi, Japan). To examine the effects of pSilencer vectors on bound thrombin-induced cell proliferation, the cells were cultured for 48 h in the absence (PBS) or presence of bound α -thrombin (10 U/ml). After that, the cultured cells were trypsinized, and then 3.0×10^3 cells (in 100 µl of 10% FBS-DMEM) were plated into each well of a 96-well microplate (Corning Co., Corning, N.Y., USA) and incubated for another 48 h with the vectors [(-)/pSilencer, GAPDH/pSilencer and ORF-2/pSilencer] at a concentration of 1 µg/ml. Cell proliferation was evaluated by n-fold increase in the number of viable cells before and after 48-hour treatment with the vectors. The blank value was the absorbance of the culture medium without cells. The absorbance was converted to cell numbers based on comparisons with known numbers of cultured cells. For the determination of DNA synthesis, BrdU incorporation was added at 72 h after seeding the cells (24 h prior to completion of treatment with ORF-2/pSilencer) and quantitated at 24 h after addition of

Fig. 1. Expression of SMemb mRNA in the cells after treatment with ORF-2/p*Silencer*. **a** Northern blot analysis of SMemb and GAPDH mRNA expressions after 48-hour treatment with different concentrations of ORF-2/p*Silencer* (0–1 μ g/ml). **b** Bar graph showing the summary data of **a**. Data represent relative mean values $\pm$ SE of the ratio of SMemb/GAPDH mRNA from 7 different experiments. * $p < 0.05$ when compared with 0 μ g/ml (lipofectamine alone). **c** Comparative RT-PCR study on the off-target effects of ORF-2/p*Silencer* (0–1 μ g/ml) on SM1 and SM2 mRNA expressions. Data represent relative mean values $\pm$ SE of ratios of SM1 or SM2/GAPDH mRNA from 8 different experiments.

the BrdU solution. The cells were denatured and fixed by incubation for 30 min with the FixDenat solution (Roche Diagnostics). For detection of the incorporated BrdU, the cells were incubated for 2 h with a peroxidase-conjugated anti-BrdU antibody (1:100 dilutions). After 3 washes by PBS, a substrate (tetramethylbenzidine) was added to each well, and then the absorbance at 370 nm (reference at 492 nm) was measured after 10 min of incubation.

For migration assay, 1.0×10^4 cells of the cultured VSM cells were seeded onto the 8- μ m FALCON® HTS FluoroBlok® Inserts (Becton Dickinson), which were precoated with type I collagen from rat tail. In the lower wells of each 24-well plate, 500 μ l of 10% FBS-DMEM were added. After incubation for 23 h at 37°C, the fluorescent dye calcein-AM (Dojindo, Kumamoto, Japan) was added to each insert at a final concentration of 2 μ g/ml, and the cells were further incubated for another 1 h to label them. The VSM cells on the inserts were fixed by 10% formalin-PBS. The membranes were removed and mounted on glass slides with coverslips. The VSM cells that had migrated to the lower side of the membrane were counted under the microscope (magnification $\times 400$) with the B-2E/C fluorescence filter inserted. The migration activity was expressed as the averaged number of migrated cells in 4 different high-power fields (HPF). To test the effect of bound thrombin with or without ORF-2/p*Silencer* on cell migration activity, the cultured VSM cells (in 25-cm² flasks) were stimulated by bound thrombin for 48 h, followed by treatment with ORF-2/p*Silencer* for another 48 h. After that, the cells were trypsinized, suspended in 10% FBS-DMEM, and plated onto the insert at a density of 1.0×10^4 cells/insert for cell migration assay.

Data Analysis

Statistical analysis was performed by either one-way analysis of variance (ANOVA) followed by Bonferroni/Dunn's multiple comparison tests or Student's unpaired t test, using StatView version 4.5 (Abacus Concepts, Berkeley, Calif., USA). $p < 0.05$ was defined as statistical significance.

Results

To select which of the 3 SMemb-siRNA/p*Silencer* exert the most effective inhibition of SMemb mRNA expression, 0.5 μ g/ml of the 3 kinds of SMemb-siRNA/p*Silencer* (ORF-1, ORF-2 and 3' UTR SMemb/p*Silencer*) were introduced into the cultured VSM cells. Based on the number of FITC-positive cells, transfection efficiency was about 80% when the cells were treated with 1 μ g/ml of FITC-labeled ORF-2/p*Silencer* (data not shown). The ORF-2/p*Silencer* was the most effective in inhibiting the SMemb mRNA (data not shown). ORF-2/p*Silencer* down-regulated SMemb mRNA expression in a dose-dependent manner (fig. 1a). ORF-2/p*Silencer* significantly reduced SMemb mRNA expression (relative to GAPDH mRNA expression) to 0.24 ± 0.05 at the concentration of 1.0 μ g/ml, compared with 0 μ g/ml (0.46 ± 0.06 , $p < 0.01$; fig. 1b). In order to test specificity of ORF-2/p*Silencer* on downregulation of SMemb, off-target effects of ORF-2/p*Silencer* were tested using other MHC isoforms (SM1 and SM2). Comparative RT-PCR was performed to test the effect of ORF-2/p*Silencer* treatment on the mRNA expressions of SM1 and SM2 after 48 h. Consequently, mRNA expressions of SM1 and SM2 (relative to GAPDH mRNA expression) were not changed by the treatments with various concentrations of ORF-2/p*Silencer*, compared with 0 μ g/ml (lipofectamine alone; fig. 1c).

Western blot analysis was performed to measure the protein level of SMemb after 48-hour treatment with

ORF-2/p*Silencer* (fig. 2a). SMemb protein in crude myosin extracts from cultured VSM cells was increased compared with that from rabbit aorta. SMemb protein level was decreased to 43.5% in the treatment with ORF-2/p*Silencer*, compared with that with lipofectamine alone (fig. 2a). To further investigate whether the SMemb-siRNA/p*Silencer* inhibits the expression of SMemb protein in individual cells, immunofluorescence study was performed. In figure 2b, although the number of nuclei (stained by DAPI) in the treatment with ORF-2/p*Silencer* was larger than that with lipofectamine, the number of TRITC fluorescence-positive cells was decreased to 41% by ORF-2/p*Silencer* at the concentration of 1 μ g/ml, compared with lipofectamine alone.

The level of SMemb mRNA in total RNA was measured by Northern blot analysis after 48-hour stimulation of VSM cells by PBS, fibrinogen (10 μ g/ml), thrombin (10 U/ml) and bound thrombin (10 U/ml; fig. 3a). Bound thrombin significantly increased the SMemb mRNA expression (1.4 ± 0.01 , $p < 0.01$ vs. PBS treatment; fig. 3b). SMemb mRNA expression, however, was not increased by treatment with fibrinogen or thrombin. Another VSM phenotype marker, PAI-1 mRNA expression, was also measured. The bound thrombin significantly increased PAI-1 mRNA expression (2.65 ± 0.69 , $p < 0.01$ vs. PBS treatment), whereas no significant increase in PAI-1 mRNA expressions was found when the cells were treated with fibrinogen and thrombin (fig. 3c).

We investigated whether ORF-2/p*Silencer* could prevent SMemb mRNA expression in bound thrombin-stimulated cultured VSM cells. After the VSM cells were grown to confluence, they were stimulated by 10 U/ml of bound thrombin or treated with PBS (control). After 48-hour stimulation by bound thrombin, culture medium was changed, and then ORF-2/p*Silencer* was introduced into cultured VSM cells. After 48-hour transfection, total RNA was extracted for Northern blot analysis to measure SMemb mRNA expressions, and the cells were fixed by 10% formalin for immunofluorescence to detect SMemb protein expressions in individual cells.

Northern blot analysis revealed that bound thrombin significantly increased SMemb mRNA expression in (-)/p*Silencer* (empty) vector-treated cells [1.58 ± 0.28 , $p < 0.05$ vs. PBS and (-)/p*Silencer*-treated cells; fig. 4a and b]. In contrast, bound thrombin-induced upregulation of SMemb mRNA expression was abolished in ORF-2/p*Silencer* vector-treated cells [0.97 ± 0.08 , $p < 0.05$ vs. bound thrombin and (-)/p*Silencer*-treated cells; fig. 4a and b]. Furthermore, bound thrombin increased PAI-1 mRNA expression in cultured cells treated with (-)/p*Silencer* by

Fig. 2. Expression of SMemb protein in the cells after treatment with ORF-2/p*Silencer*. **a** Effect of ORF-2/p*Silencer* on the SMemb protein expression in cultured VSM cells. Crude myosin extracts prepared from cultured rabbit aortic VSM cells 48 h after treatment with ORF-2/p*Silencer*. Lane 1 = Cultured VSM cells treated with lipofectamine alone; lane 2 = GAPDH/p*Silencer*; lane 3 = ORF-2/p*Silencer*. Intensity of the SMemb protein bands was valued by NIH Image and normalized by the value of lipofectamine. Summary data of percentage changes in SMemb protein after 48-hour treatment by ORF-2/p*Silencer* are shown. Data are represented as means $\pm$ SE from 3 separate experiments. * $p < 0.05$, one-way ANOVA followed by post hoc test. **b** Immunofluorescence staining for SMemb protein expressions. The cells were fixed with 10% formalin-PBS at 48 h after treatment, and then immunofluorescence staining for SMemb protein expression was performed (see Materials and Methods). The cells were treated with lipofectamine alone and 1 μ g/ml of ORF-2/p*Silencer*. Fluorescence by TRITC represents SMemb protein expression. Counterstaining for cell nuclei was performed using DAPI.

1.50 ± 0.34 , but there was no statistical significance [$p = 0.085$ vs. PBS and (-)/p*Silencer*-treated cells; fig. 4a and c]. ORF-2/p*Silencer* decreased PAI-1 mRNA expression in bound thrombin-stimulated VSM cells (fig. 4a and c).

Fig. 3. Effect of bound thrombin on SMemb and PAI-1 mRNA expressions. **a** Representative Northern blot data on the effect of bound thrombin on the mRNA expressions of SMemb, PAI-1 and GAPDH. PBS, 10 μ g/ml of rabbit fibrinogen (fibrinogen) and 10 U/ml of bovine α -thrombin (thrombin) were used for comparison. **b, c** Summarized bar graphs showing the effects of bound

thrombin on SMemb (**b**) and PAI-1 (**c**) mRNA expressions from 4 different experiments each. Ratios of SMemb or PAI-1 to GAPDH mRNA expressions were calculated and expressed as relative ratio to PBS. Data represent means $\pm$ SE (n = 4). * p < 0.01 when compared with PBS, one-way ANOVA, followed by Bonferroni/Dunn's multiple comparison test.

Immunofluorescence study revealed that TRITC-fluorescent signals in (-)/pSilencer-treated cells representing SMemb protein expressions were increased by bound thrombin, compared with PBS treatment (intensity in arbitrary units: bound thrombin, 4.5 ± 7.2 ; PBS, 2.1 ± 4.3). In addition, TRITC-fluorescent signals in ORF-2/pSilencer-treated cells were not increased by bound thrombin (intensity in arbitrary units: bound thrombin, 8.9 ± 11.1 ; PBS, 8.4 ± 12.3).

Cell proliferation of cultured VSM cells was evaluated either by increase in the number of viable cells or by DNA synthesis activity measured as the amount of BrdU incorporation. The change in the number of viable cells during 96-hour incubation with bound thrombin (lipofectamine was added to the medium in the latter half of incubation) showed no significant increase: PBS, 1.9 ± 0.17 -fold; bound thrombin, 1.8 ± 0.34 -fold (fig. 5a). In addition, 48-hour treatment of the cultured cells with (-)/pSilencer, GAPDH/pSilencer or ORF-2/pSilencer had no effect in bound thrombin-stimulated cells (fig. 5a). Although incorporation of BrdU was substantially increased by bound thrombin treatment, there was no statistical significance compared to PBS treatment (fig. 5b). Moreover, (-)/pSilencer, GAPDH/pSilencer and ORF-2/pSilencer had no effect on the incorporation of BrdU by bound thrombin (fig. 5b).

To investigate the role of SMemb in the basal level of cell migration seen without stimulation, the cells were treated for 48 h with (-)/pSilencer, GAPDH/pSilencer and ORF-2/pSilencer after addition of PBS. Consequent-

ly, ORF-2/pSilencer treatment significantly decreased the migration activity to 55.8% (6.83 ± 3.7 cells/HPF), compared with PBS plus lipofectamine (12.25 ± 1.5 cells/HPF). Migration activity was significantly increased by about 1.4-fold in the bound thrombin plus lipofectamine group compared to the PBS plus lipofectamine group: PBS, 12.25 ± 1.5 cells/HPF; bound thrombin, 17.4 ± 1.6 cells/HPF (p < 0.05; fig. 6). To investigate the role of SMemb in bound thrombin-stimulated migration, the cells were treated for 48 h with (-)/pSilencer, GAPDH/pSilencer and ORF-2/pSilencer after stimulation by bound thrombin. Consequently, ORF-2/pSilencer treatment significantly reduced the bound thrombin-stimulated migration activity to 69% compared to (-)/pSilencer treatment: (-)/pSilencer, 17.0 ± 1.9 cells/HPF; ORF-2/pSilencer, 11.8 ± 0.15 cells/HPF (p < 0.05; fig. 6).

Discussion

In the present in vitro study, the knockdown of SMemb gene expression by transfection with ORF-2/pSilencer significantly inhibited bound thrombin-induced migration of cultured rabbit VSM cells. In addition, our data suggest that SMemb plays an important role in cell migration of VSM cells. Although there were many methods to knockdown SMemb gene expression, we employed siRNA expression plasmid vectors because they (1) were resistant to breakdown by RNase, (2) had long-term ex-

Fig. 4. Effect of ORF-2/pSilencer on bound thrombin-induced up-regulation of SMemb mRNA expressions. **a** Northern blot data on the effect of ORF-2/pSilencer on the mRNA expressions of SMemb, PAI-1 and GAPDH under PBS or bound thrombin-stimulated condition. **b, c** The cells were treated with 1 μ g/ml of either (-)/pSilencer (open bars) or ORF-2/pSilencer (gray bars) for 48 h. Bar graphs summarize the data on the effect of ORF-2/pSilencer on SMemb (**b**) and PAI-1 (**c**) mRNA expressions from 4 different experiments each. Ratios of SMemb/GAPDH and PAI-1/GAPDH mRNA expression were calculated and expressed as relative ratio to PBS. * $p < 0.05$ when compared with PBS; + $p < 0.05$ when compared with bound thrombin/empty vector, one-way ANOVA followed by Bonferroni/Dunn's multiple comparison test.

pression in the cells and (3) were applicable to an in vivo study. Since we planned to study whether local delivery of ORF-2/pSilencer by gene-eluting stents prevents balloon denudation-induced vascular restenosis in rabbits,

Fig. 5. Cell proliferation assay in ORF-2/pSilencer-treated cultured rabbit VSM cells after stimulation by bound thrombin. **a** After 48-hour stimulation of cultured VSM cells by PBS (open bars) or bound thrombin (gray bars), the numbers of cells were counted by the WST-1 reagent before and after 48-hour treatments with 1 μ g/ml of different plasmid vectors [(-)/pSilencer, GAPDH/pSilencer and ORF-2/pSilencer] or lipofectamine alone. n-Fold increase was calculated by dividing the number of cells after 48-hour treatment with the vectors by that before treatment. Data represent means $\pm$ SE ($n = 3$). **b** DNA synthesis activity was determined by measuring the incorporation of BrdU into the cells by ELISA. Data represent means $\pm$ SE ($n = 4$).

we took the first step to test the ORF-2/pSilencer plasmid in cultured rabbit VSM cells.

Coagulation factors, including thrombin and tissue factor, are involved in the development of vascular restenosis.

Fig. 6. Cell migration assay in ORF-2/pSilencer-treated cultured rabbit VSM cells after stimulation by bound thrombin. The migration activity is expressed as the averaged number of migrated cells in 4 different HPF. $\times 400$. To test the effect of ORF-2/pSilencer on the basal (PBS, open bars) and bound thrombin-induced (gray bars) cell migration activity, the cultured VSM cells were stimulated by bound thrombin for 48 h, followed by treatment with 1 $\mu\text{g}/\text{ml}$ of different plasmid vectors [(-)/pSilencer, GAPDH/pSilencer and ORF-2/pSilencer] or lipofectamine alone for another 48 h. After that, the cells were trypsinized, suspended in 10% FBS-DMEM and plated onto the insert at a density of 1.0×10^4 cells/insert for cell migration assay. Data represent means $\pm$ SE ($n = 4-8$). * $p < 0.05$ when compared with PBS plus lipofectamine; + $p < 0.05$ when compared among the groups of the stimulation by bound thrombin.

nosis after interventional treatment [12, 13]. We hypothesized that bound thrombin in the thrombus plays an important role in the development of restenosis. This idea was supported by our previous comparative RT-PCR data demonstrating that bound thrombin enhanced the phenotype conversion of VSM cells towards a synthetic type by upregulating SMemb and PAI-1 mRNA expressions [17]. In the present study, bound thrombin upregulation of SMemb and PAI-1 mRNA expressions was further evidenced by Northern blot analysis (fig. 3). However, the mechanism by which bound thrombin upregulated those mRNA expressions remains unknown.

Cell proliferation was not changed by bound thrombin in our study (fig. 5a). Although cell number was not increased by bound thrombin, the incorporation of BrdU tended to be increased by bound thrombin (fig. 5b). These data suggest that bound thrombin simultaneously increases mitogenic activity and apoptosis of VSM cells. It was reported that PAI-1 induced a remarkable increase in

apoptosis of human umbilical vein endothelial cells and VSM cells via activation of caspase 3 [23]. Therefore, bound thrombin-induced increase in PAI-1 observed in our study may produce apoptosis of VSM cells.

Migration of VSM cells was regulated by many factors, including platelet-derived growth factor, insulin-like growth factor 1 and basic fibroblast growth factor [24, 25]. Although thrombin is known to induce proliferation of VSM cells, the effect of thrombin on migration of VSM cells is less known. Our data demonstrated that bound thrombin promoted migration activity of the cultured VSM cells, which were abolished by knockdown of SMemb gene expression by ORF-2/pSilencer (fig. 6). The effect of knockdown of SMemb gene expression on migration activity induced by other stimulants (for example, platelet-derived growth factor-BB) was not measured to test if the inhibitory effect of SMemb knockdown on cell migration was specific to that induced by bound thrombin. Because the basal migration activity was inhibited by SMemb knockdown, the inhibitory effect of SMemb knockdown on the cell migration is not likely specific to that induced by bound thrombin. Since bound thrombin is composed of α -thrombin and N-terminal regions of the α - and γ -chains of fibrinogen, bound thrombin can activate the thrombin receptor (protease-activated receptor-1) or bind to integrin $\alpha_v\beta_3$ on the membrane of VSM cells [26]. Protease-activated receptor 1 activation was reported to lead to $[\text{Ca}^{2+}]_i$ mobilization, thereby promoting cell migration activity [25]. Alternatively, binding of bound thrombin to $\alpha_v\beta_3$ of VSM cells upregulated matrix metalloprotease-2 expression, thereby promoting cell migration activity [27]. In addition, since PAI-1 has been shown to be a potent regulator of vascular cell migration in vitro [28], bound thrombin may promote cell migration activity through upregulation of PAI-1.

In summary, of the 3 SMemb siRNA expression vectors, ORF-2/pSilencer exerted the strongest inhibition on SMemb mRNA expression. Bound thrombin significantly increased SMemb mRNA expression and migration of VSM cells, and these actions were abolished by knockdown of SMemb gene expression by ORF-2/pSilencer. Thus, SMemb gene expression could be necessary for bound thrombin-stimulated migration of VSM cells. Since bound thrombin might be involved in the development of restenosis after angioplasty, ORF-2/pSilencer could be used to inhibit the SMemb gene and suppress the migration of VSM cells, thereby preventing restenosis.

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