Ets-1 mediates platelet-derived growth factor-BB-induced thrombomodulin expression in human vascular smooth muscle cells

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Received 26 July 2008; revised 17 November 2008; accepted 10 December 2008; online publish-ahead-of-print 17 December 2008

Time for primary review: 25 days

Aims Thrombomodulin (TM), a potent anticoagulant, is not detected in quiescent vascular smooth muscle cells (VSMCs). In diseased vessels, VSMC expresses TM, but the mechanisms are unclear. This study examined molecular mechanisms for TM expression in VSMCs.

Methods and results Platelet-derived growth factor-BB (PDGF-BB) induced TM expression in cultured human aortic VSMCs. PDGF-induced TM is functional in activating protein C. TM induction was eliminated by inhibitors of Src kinase, phosphatidylinositol 3-kinase (PI3-kinase), and mammalian target of rapamycin (mTOR) and by expressing dominant-negative Akt while expressing active Akt-stimulated TM expression. PDGF-BB activated the TM promoter, and the deletion of a sequence segment −394/−255 drastically reduced TM promoter activity. Transcription factor E26 transformation-specific sequence-1 (Ets-1) was upregulated by PDGF-BB in a PI3-kinase- and mTOR-dependent manner. RNA interference of Ets-1 inhibited PDGF induction of TM, and overexpressing Ets-1 increased TM expression. Chromatin immunoprecipitation and electrophoretic mobility shift assay detected increased Ets-1 binding to the TM promoter after PDGF treatment. Following carotid artery ligation of C57/BL6 mice, PDGF-BB and TM were co-expressed in the media and neointima.

Conclusion In VSMCs, PDGF-BB stimulates TM expression that is mainly mediated by Ets-1 via the Src kinase/PI3-kinase/Akt/mTOR signalling pathway. Furthermore, PDGF-BB may regulate TM expression in VSMCs during vascular remodelling.

1. Introduction

Thrombomodulin (TM), a transmembrane glycoprotein highly expressed by endothelial cells (ECs), plays a critical role in maintaining vascular thromboresistance.1 Thrombin forms a complex with TM and thereby changes its substrate to catalyse the activation of protein C. Activated protein C inhibits blood coagulation by neutralizing the feedback loop of thrombin generation via factors Va and Vilia. Therefore, TM plays a critical role in anticoagulant pathway. Recent studies have revealed multiple biological roles for TM in addition to its anticoagulant function. TM is essential for embryogenesis, as TM deficiency causes pre-natal death of mice resulting from defective placental development.2 In addition, TM functions as an anti-inflammatory molecule through both direct and indirect mechanisms. Protein C activated by TM dulls inflammatory activities by inhibiting macrophage expression of tissue factor, tumour necrosis factor (TNF)-α, and leukocyte adhesion molecules.3 The lectin-like domain of TM exhibits potent anti-inflammatory activity that suppresses EC activation through mitogen-activated protein (MAP) kinase and NF-κB pathways.4 Recombinant TM fragments containing epidermal growth factor-like domain and serine/threonine-rich region stimulate endothelial migration and proliferation in vitro and induce angiogenesis in vivo.5 Furthermore, TM modulates pathological changes of the vessel wall that occur in...
restenosis and vein graft atherosclerosis. Overexpressing TM or systemic administration of recombinant TM reduces inflammatory cell infiltration and neointima formation in several animal models. These findings suggest that TM plays critical roles in vascular biology.

TM was initially discovered in ECs and considered an endothelial marker. In disease states associated with inflammation such as atherosclerosis and vein graft thrombosis, endothelial TM is markedly downregulated. Interestingly, in advanced atherosclerotic plaques, TM is detected in vascular smooth muscle cells (VSMCs) of both media and intima. Thus, neointimal VSMCs were proposed to be a relevant source of TM and form a surface relatively resistant to thrombosis. However, little is known about the regulation of TM expression in VSMCs, whose proliferation and migration are central to the pathogenesis of atherosclerosis and injury-induced intimal hyperplasia. In cultured VSMCs, thrombin, cAMP, and COX-2-derived prostaglandins were reported to upregulate TM expression. Mechanisms regulating TM expression in VSMCs stimulated by growth factors or inflammatory cytokines remain unclear.

Among growth factors and cytokines released at the site of vascular injury, platelet-derived growth factor-BB (PDGF-BB) is the most potent mitogen and chemotactant of VSMCs in vitro and functions as an important mediator in the pathogenesis of vascular diseases. Stimulation of PDGF receptors on VSMCs activates several signalling pathways, including those mediated by Src family kinases, extracellular signal-regulated kinases 1/2 (ERK1/2), p38 MAP kinase (p38 MAPK), and phosphatidylinositol 3-kinase (PI3-kinase). Whether PDGF-BB regulates TM expression in VSMCs and in vascular remodelling is currently unknown. This study investigated the effect of PDGF-BB on TM expression in VSMCs and mechanisms mediating TM expression.

2. Methods

2.1 Materials

Human recombinant PDGF-BB was obtained from R&D Systems (Lake Placid, NY, USA). U0126, SB203580, LY294002, human protein C, and antithrombin III were purchased from Calbiochem (San Diego, CA, USA). Chromoyme PCa was purchased from American Diagnostica Inc. (Greenwich, CT, USA). Fugene HD was purchased from Roche (Basel, Switzerland). PP2 was purchased from Tocris (Bristol, UK). Cycloheximide, rapamycin, heparin, and monoclonal antibody against β-actin were obtained from Sigma (St Louis, MO, USA). Mouse monoclonal antibody against human TM was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibody against mouse TM was produced using recombinant TM lectin-like domain as the antigen. Monoclonal antibody against phospho-ERK1/2 and polyclonal antibodies against Akt and phospho-Akt were purchased from Cell Signaling Technologies (Beverly, MA, USA). Horseradish peroxidase-conjugated horse anti-mouse immunoglobulin (IgG) and goat anti-rabbit IgG were obtained from Vector Laboratories (Burlingame, CA, USA) and Antibodies Incorporated (Davis, CA, USA). Human aortic VSMCs (HASMCs), medium 231, and smooth muscle growth supplement (SMGS) were purchased from Cascade Biologies (Portland, OR, USA).

2.2 Cell culture

HASMCs were maintained in medium 231 containing SMGS at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Cells (between passages 3 and 7) were grown to 80–90% confluence, made quiescent by serum starvation [0.2% fetal bovine serum (FBS)] for 24–48 h, and treated with PDGF-BB. Unless indicated otherwise, cells were treated with 10 ng/mL PDGF.

2.3 Western blot analysis

HASMCs were serum-starved for 48 h and stimulated with PDGF. Cell lysis and western blotting were performed as described previously. Protein bands were visualized by enhanced chemiluminescence using ECL-Plus (PerkinElmer, Inc.) and quantified densitometrically.

2.4 RNA extraction and semi-quantitative reverse transcriptase-polymerase chain reaction

Total RNA from cultured HASMCs was isolated using TRI REAGENT, treated with DNase I, and re-extracted. The first-strand cDNA was synthesized from 2 μg of total RNA. A 502 bp GAPDH fragment and a 963 bp GAPDH fragment were co-amplified with PCR for semi-quantitative analysis. PCR products were resolved on 2% agarose gels. The detailed methods and sequences for PCR primers are provided in Supplementary material.

2.5 Thrombomodulin expression in isolated mouse aortae

Aortae were isolated from anaesthetized 8-week-old male C57BL/6 mice, placed in Hank's buffered salt solution (HBSS), denuded endothelium, and cleaned the adventitia. Aortae were treated with PDGF, washed with phosphate-buffered saline (PBS), homogenized, and analysed by western blot analysis. All experimental procedures conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication No. 85–23, revised 1996) and were approved by the institutional animal care and use committee.

2.6 Thrombomodulin-dependent protein C activation assay

HASMCs cultured in 96-well plates were washed with PBS and incubated in HBSS containing 5 mg/mL BSA, 2 U/mL thrombin, and 5 μg/mL human protein C. After 80 min incubation at 37°C, the reaction was terminated by adding equal volumes of HBSS containing 50 μg/mL antithrombin III and 200 U/mL heparin and mixing for 5 min at 37°C. The activity of the activated protein C was determined by adding chromozyme PCa and measuring absorbance at 405 nm.

2.7 Adenovirus infection

The recombinant adenoviral constructs encoding haemagglutinin (HA)-tagged constitutively active Akt (myrAkt) and dominant-negative Akt (AAA-Akt) were kindly provided by Kenneth Walsh (Boston University School of Medicine, Boston, MA, USA) and prepared as described previously. An adenoviral vector expressing GFP was used as a control. HASMCs were infected with adenoviral constructs (1:10 to 1:50 dilution) for 24 h, incubated in fresh medium for 24 h, serum-starved for 24 h, and treated with PDGF.

2.8 Transient transfection and luciferase activity assay

A luciferase reporter plasmid (pXP-1) bearing a promoter region (nucleotides −1519/+151 bp) of the human TM gene was constructed (pXP-TM). Transient transfection of cells with plasmids was performed with Fugene HD, according to the manufacturer’s instructions. Briefly, HASMCs and NIH-3T3 cells at 80% confluence in six-well plates were co-transfected with 600 ng of pXP-TM or plasmids containing 5′ sequence deletion constructs and 150 ng of the expression vector pRL-SV40 in medium containing 5% FBS. The co-transfection of pRL-SV40 that contains the reporter renilla luciferase serves as the control to correct for the variation of transfection efficiency. Three hours after transfection, the cells were
stimulated with PDGF (20 ng/mL) and harvested 24 h later for luciferase activity assay using the Dual-Luciferase Reporter Assay System (Promega).

2.9 siRNA knock-down of ETS-1

For small interfering (si)RNA studies, E26 transformation-specific sequence-1 (ETS-1) sense (ACUUGCUACAUCCCGUAAC-dTdT) and antisense (GUACGGGAAUGGUGACAAGU-dTdT) siRNAs and control-scrambled siRNAs (sense, ACGGAAUAUUGCAGGCUU-dTdT; antisense, AAGGUCG AAAAAUCUCU-dTdT) were obtained from Invitrogen, according to a previous report.22 HASMCs (2 × 10⁵ cells/well) were transfected with Ets-1 or scrambled siRNA using Fugene HD. Transfected cells were maintained in complete medium for 24 h, serum-starved for 24 h, and stimulated with PDGF.

2.10 Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChiP) assays were performed according to a published procedure,23 with minor modifications. The detailed methods and sequences for PCR primers are provided in Supplementary material.

2.11 Electrophoretic mobility shift assay

Nuclear extracts were isolated from HASMCs with or without 6 h PDGF (20 ng/mL) treatment, and electrophoretic mobility shift assay (EMSA) was performed according to a published method.24 The detailed methods are provided in Supplementary material.

2.12 Ets-1 and thrombomodulin double immunofluorescence

HASMCs were transfected with a plasmid containing human Ets-1 (pBK-CMV-Ets-1), kindly provided by Dr Hironori Nakagami, Osaka University, Graduate School of Medicine, Osaka, Japan). Following fixation and permeabilization, cells were incubated with anti-Ets-1 and anti-TM antibodies, followed by Alexa 488-conjugated goat anti-rabbit IgG (Ets-1) and Alexa 546-conjugated goat anti-mouse IgG (TM) and observed with a fluorescence microscope. Additional details are provided in Supplementary material.

2.13 Statistical analysis

Data are presented as mean ± SEM of n independent experiments. Statistical analysis was performed with one-way analysis of variance, followed by Dunnett’s multiple comparison. P-values less than 0.05 were considered significant.

3. Results

3.1 Platelet-derived growth factor stimulates thrombomodulin protein expression in human aortic vascular smooth muscle cells in vitro and ex vivo

PDGF induced TM protein expression in cultured HASMCs in a time-dependent manner. PDGF markedly increased TM protein expression between 3 and 72 h of treatment and peaked at 6 h (7.6 ± 0.4-fold control) (Figure 1A). In addition, PDGF upregulated TM protein levels in a dose-dependent manner with a steep increase at a concentration of 10 ng/mL (Figure 1B). In endothelial-denuded, isolated mouse aortae, PDGF stimulated TM expression, reaching a maximal stimulation of 2.3-fold at 6 h, sustaining at 12 h, and gradually declining at 24 h (Figure 1C). To determine whether PDGF-induced TM expression translates into increased function, TM activity was measured by the production of active protein C. HASMCs treated with 5 and 10 ng/mL of PDGF exhibited 1.55 ± 0.11- and 1.88 ± 0.11-fold active protein C relative to untreated control (Figure 1D).

3.2 Platelet-derived growth factor-stimulated thrombomodulin mRNA expression requires new protein synthesis

We next examined whether PDGF stimulates TM mRNA expression. Following PDGF exposure, TM mRNA levels increased at 1 h, peaked at 3 h, and sustained at 6 h, reaching five-fold the control level (see Supplementary material online, Figure S1A). After that, TM mRNA levels gradually declined towards baseline. To determine whether the inducible TM gene expression is dependent on de novo protein synthesis, HASMCs were pre-treated with cycloheximide, a protein translational inhibitor, prior to PDGF treatment (see Supplementary material online, Figure S1B). Cycloheximide pre-treatment blocked PDGF-induced TM mRNA, indicating that the increase is dependent on de novo protein intermediates.

3.3 Platelet-derived growth factor-induced thrombomodulin expression is mediated by PI3-kinase/Akt/mammalian target of rapamycin signalling pathway

To delineate the signalling pathways mediating PDGF-induced TM expression, we examined the roles of PI3-kinase. HASMCs were treated with PDGF for 6 h after pre-incubation with either PI3-kinase inhibitors, LY294002 (10–50 μM) or wortmannin (0.05–0.5 μM). The efficacy of these inhibitors was assessed by Akt phosphorylation, a well-established downstream target for PI3-kinase. LY294002 (50 μM) abolished PDGF-induced Akt phosphorylation and TM upregulation (Figure 2A). Similar results were obtained with wortmannin treatment.

To further ascertain the role of Akt signalling in PDGF-induced TM expression, HASMCs were transfected with adenoviral vectors expressing either the constitutively active Akt (myr-Akt) or the dominant-negative Akt mutant (AAA-Akt). Suppressing Akt signalling by infection with HA-tagged adeno-AAA-Akt markedly reduced PDGF-induced TM expression (Figure 2B). In contrast, infection with adenomyrAkt increased basal TM expression with a little further increase by PDGF.

Among Akt-stimulated downstream targets, we investigated the involvement of mTOR in TM induction.25 Pre-treatment with rapamycin (0.1, 1, or 10 ng/mL) resulted in dose-dependent inhibition on PDGF-induced TM expression, reaching 63 ± 5.8% inhibition with 10 ng/mL rapamycin (Figure 2C).

3.4 Src kinases are required for platelet-derived growth factor-induced PI3-kinase activation and thrombomodulin expression

Src kinases were shown to regulate PI3-kinase activity in PDGF signalling,26 we therefore examined whether PDGF-stimulated PI3-kinase activation requires Src activity. Pre-treatment of an Src kinase inhibitor, PP2 (5, 10, or 20 μM), abolished PDGF-stimulated Akt phosphorylation and TM expression (Figure 3A and B). The efficacy of PP2
was confirmed by its ability to abolish PDGF-induced Src phosphorylation (data not shown).

3.5 Extracellular signal-regulated kinase activation partially mediates platelet-derived growth factor-induced thrombomodulin expression

The roles of ERK1/2 and p38 MAPK in PDGF-induced TM upregulation were examined with a selective MEK1/2 inhibitor U0126 and a p38 MAP kinase inhibitor SB203580. The efficacy of U0126 was assessed by ERK1/2 phosphorylation. PDGF-induced TM protein expression was inhibited 26 and 58%, respectively, by 10 and 20 μM U0126, whereas ERK1/2 phosphorylation was abolished (see Supplementary material online, Figure S2A). In contrast, inhibition of p38 MAP kinase with SB203580 (up to 10 μM) exhibited no effect on PDGF-induced TM expression (see Supplementary material online, Figure S2B).

3.6 Platelet-derived growth factor regulates thrombomodulin promoter

To investigate whether PDGF regulates transcriptional activity of TM, HASMCs were transiently transfected with a mammalian expression plasmid encoding TM promoter region spanning −1519/+151 bp. PDGF treatment increased luciferase reporter activity 2.4-fold (n = 3, P < 0.01) compared with untreated controls (Figure 4A).

Because much greater transfection efficiency is obtained with NIH-3T3 cells compared with HASMCs and TM expression in NIH-3T3 cells is induced by PDGF (data not shown), we used NIH-3T3 cells to analyse promoter elements involved in PDGF-stimulated TM induction. PDGF treatment induced luciferase activity by 13.7-fold in cells transfected with TM-1519 (Figure 4B). Progressive deletion of the promoter sequence from −1519 to −394 did not affect PDGF induction. Further deletion between −394 and −255 markedly attenuated PDGF induction from 11.8 to 6.9-fold. Similar PDGF induction was detected with constructs between TM-255 and TM-111, whereas construct TM-34 exhibited promoter activity similar to that of the promoter-less plasmid, pXP-1. These results localize PDGF responsive elements of the TM promoter to the regions between −394 and −255 bp and between −111 and −34 bp.

3.7 Ets-1 plays an essential role in platelet-derived growth factor-stimulated thrombomodulin expression

The sequence analysis of the TM promoter between −394 and −255 bp revealed the presence of three consensus Ets binding sites (−385TTCC−382, −358TTCC−355, and −352GGAA−349) and one Sp1 binding site (−269GGCCGG−264). To evaluate the
role of Ets-1 in PDGF-induced TM expression, PDGF-stimu-
lated Ets-1 expression was examined with and without ly294002 (10 and 20 μM) (Figure 5A) or rapamycin (0.1, 1, and 10 ng/mL) (Figure 5B). PDGF treatment increased Ets-1 expression ~2.5-fold (n = 4, P < 0.01), which was inhibited by both ly294002 and rapamycin. We next examined the effect of Ets-1 siRNA on PDGF-induced TM expression. Compared with scrambled siRNA, Ets-1 siRNA dose-dependently and significantly reduced PDGF-induced TM expression (Figure 5C). In contrast, overexpressing Ets-1 in HASMCs increased basal expression of TM (Figure 5D).
3.8 Ets-1 interacts with the thrombomodulin promoter in human aortic vascular smooth muscle cells

To investigate whether Ets-1 binds chromatin at the regions identified by promoter activity assay, we performed ChIP assay. PCR primer sets were made for three different regions of the TM promoter denoted ChIP1 through ChIP3. As shown in Figure 6A, ChIP1 spans from -183 to -45 and covers four Ets-1 motifs; ChIP2 spans from -421 to -256 and covers three Ets-1 motifs; and ChIP3 spans from -1250 to -1110 and covers three Ets-1 motifs. With PDGF treatment, Ets-1 binding to the ChIP2 region markedly increased, whereas binding to the ChIP1 region increased to smaller degrees. No binding was detected in the ChIP3 region.

We next performed EMSA using oligonucleotide probes.
encoding Ets-binding sites located between −369 and −345 (−369/−345) or between −75 and −52 (−75/−52). As shown in Figure 6B, PDGF increased Ets-1 binding to the −369/−345 segment (lane 3, 2.02 ± 0.18-fold) when compared with the unstimulated control (lane 2). The addition of a molar excess of unlabelled probe (lane 4) or an antibody directed against Ets-1 (lane 5) abolished complex formation, whereas isotype-matched IgG had no effect (lane 6, 1.99 ± 0.25-fold). In contrast, Ets-1-binding to the −75/−52 segment was not stimulated by PDGF (see Supplementary material online, Figure S3). These results indicate that the TM promoter is a direct target of Ets-1 upon PDGF stimulation with the major binding region located between −369 and −345 bp.

3.9 Localization of thrombomodulin and platelet-derived growth factor-BB in ligated carotid artery

The expression of TM and PDGF-BB in mouse carotid arteries after ligation was evaluated by immunohistochemistry (see Supplementary material online, Figure S4). TM and PDGF-BB were barely detectable in non-ligated carotid arteries, but were markedly up-regulated in the media at 1 week post-ligation. Two weeks post-ligation, the ligated artery developed significant neointima, which became more pronounced at 4 weeks post-ligation. TM and PDGF-BB expression was clearly detected in neointimal and medial cells of ligated arteries at 2 and 4 weeks post-ligation. Frequently, the cells expressed TM at the PDGF-BB-positive sites.

4. Discussion

TM expression in VSMCs is detected during vascular remodeling in atherosclerosis and following vascular injury; however, the regulatory mechanisms remain unclear. In this study, we have the following major findings: (i) PDGF-BB upregulates TM expression at both mRNA and protein levels in cultured HASMCs and isolated mouse aorta. (ii) PDGF-induced TM expression is mediated by Src kinases and PI3-kinase/Akt/mTOR-dependent signalling pathway. (iii) PDGF increases Ets-1 binding to the TM promoter and Ets-1 is required for TM upregulation by PDGF. (iv) Both PDGF-BB and TM are markedly expressed in the media and neointima following carotid artery ligation.

Characterization of factors and signalling pathways that modulate TM expression in the vasculature are important for unraveling both pathogenesis and therapeutic targets of vascular diseases. Previous studies showed that thrombin and COX-2-derived prostaglandins, such as PGE₂ and PGI₂, stimulate TM expression in VSMCs in vitro.15,17 Several lines of evidence from this study clearly indicate that PI3-kinase signalling pathway plays an essential role in PDGF-BB-induced TM expression in VSMCs. PI3-kinase activation occurs through tyrosine phosphorylation by either receptor or non-receptor tyrosine kinases.26 Our results indicated that Src kinases act upstream of PI3-kinase as PDGF-stimulated Akt phosphorylation and TM expression were both blocked by Src inhibition.
Moreover, expressing dominant-negative Akt mutant abolished PDGF-induced TM expression, whereas expressing constitutively active Akt mutant alone induced TM expression, which strongly support a critical role for PI3-kinase/Akt in TM expression in VSMCs. Akt acts through mTOR to activate p70 S6k, which phosphorylates S6RP and increases translation.25 Our results showed that inhibition of mTOR repressed both PDGF-BB-induced Ets-1 upregulation and TM expression. This result provides the first evidence that mTOR regulates Ets-1 expression. Insulin-like growth factor-I (IGF-I) is a potent activator of the PI3-kinase/Akt/mTOR pathway.25 Similar to PDGF, IGF-I treatment increased TM expression in HASMCs and the effect is blocked by PI3-kinase inhibitors (I.-C. Lo et al., unpublished results). In addition, the MEK1/2 inhibitor U0126 partially but significantly inhibited PDGF-induced TM expression. Taken together, these results indicate that PDGF-BB induces TM expression mainly through Src/PI3-kinase/Akt/mTOR and in part via ERK1/2 signalling pathways.

PDGF stimulation on TM expression is detected at both mRNA and protein levels and requires de novo protein synthesis, suggesting the regulation at the transcriptional level. Indeed, using EMSA, ChIP, and siRNA-mediated knockdown, we have identified Ets-1 as an essential transcriptional factor mediating PDGF-induced TM expression in VSMCs. Two sequence segments of the TM promoter responsible for PDGF-BB stimulation, located at -394/-255 and -111/-34, contain three and four Ets-binding elements, respectively. The promoter segment -394/-255, which accounts for the major portion of TM promoter activity in VSMCs, exhibited increased Ets-1 binding upon PDGF stimulation. In contrast, Ets-1 binding to the segment -111/-34 is relatively weak without apparent stimulation by PDGF. These results contrast strongly to those reported in ECs. The promoter segment -111/-34 contains PyPu box that is both critical for high basal activity and responsible for TNF-α-induced repression in ECs. In contrast, the segment -394/-255 is not involved in regulating TM expression in ECs.27 The differential roles of these Ets-binding elements in TM transcriptional activation in ECs and VSMCs point to the complex mechanism involved in regulating TM transcription. In ECs, several Ets transcription factors, Ets-1, Ets-2, and Erg, were shown to bind to the TM promoter. TNF-α suppresses TM transcription by reducing the binding of p300, a transcription coactivator for Ets, to the TM promoter in an NF-κB-dependent manner without directly affecting Ets binding.28 Among Ets family members, Ets-1 is expressed at low levels in resting VSMCs and ECs and is induced in response to various stimuli including angiotensin II, PDGF-BB, and thrombin. Interestingly, Erg is constitutively expressed in ECs but is not detected in VSMCs.29 It is plausible that TM expression in ECs and VSMCs is regulated by different Ets factors.

A recognition motif for Sp-1 is present in the TM promoter region -394/-255. Ets-1 and Sp1 were shown to interact to stimulate gene transcription in various cell types including VSMCs.29 Although Sp1 expression is not regulated by PDGF-BB (I.-C. Lo and M.J. Jiang, unpublished results), Akt was reported to increase Sp1 phosphorylation, thereby increasing its transcriptional activity.30 It is possible that PDGF increases Ets-1 transcriptional activity via Sp1 phosphorylation, increasing the interaction between Ets-1 and Sp1.

Results from previous studies suggest that TM functions as a protective factor in cardiovascular diseases. In human, plasma TM levels are inversely correlated with the incidence of coronary artery disease.31 In a rabbit model with mechanical overdistension-induced injury, local TM overexpression prevents arterial thrombosis and inhibits neointima formation.6 In mouse models with balloon-induced arterial injury and carotid ligation, injection of recombinant TM fragment containing extracellular domains decreases neointima formation.7,8 In contrast, endothelium-specific loss of TM causes spontaneous and progressive coagulopathy.9,10 However, TM expression in ECs in vivo is markedly downregulated by inflammatory mediators such as TNF-α.27,28 TM expression in vivo is decreased in endothelium overlying atherosclerotic lesions and following arterial ligation.6,10 The decreased anticoagulant and anti-inflammatory activity on the endothelial surface resulting from TM downregulation may exacerbate atherosclerosis progression. Therefore, PDGF-induced TM expression in VSMCs can provide a mechanism to reduce thrombosis and inflammation in the vascular wall and to maintain vascular homeostasis. Results from this and a previous study,17 showing that TM expressed in VSMCs is capable of generating active protein C, support this notion.

In conclusion, PDGF stimulates the expression of functional TM in VSMCs. The PDGF induction is mainly mediated by Src kinases and PI3-kinase/Akt/mTOR signalling pathways, with Ets-1 playing a critical role in TM transcription. Furthermore, the co-localization of PDGF-BB and TM following carotid artery ligation implies that PDGF-BB is involved in TM induction in VSMCs during vascular remodelling.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

Acknowledgements
We thank Kenneth Walsh (Boston University, USA) for the recombinant adenoviral constructs containing myr-Akt and AAA-Akt. We also thank Hironori Nakagami (Osaka University, Japan) for a human Ets-1 plasmid, pBK-CMV-hEts-1.

Conflict of interest: none declared.

Funding
This study was supported by the MOE Program for Promoting Academic Excellence of Universities 91-B-FA09-2-4 and the National Science Council Grants NSC 94-2320-B-006-052 (to M.J.J.), NSC95-2752-B-006-003-PAE (to H.L.W.), and NSC95-2752-B-006-005-PAE (to G.Y.S.) of Taiwan.

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