

Human recombinant tissue-factor pathway inhibitor prevents the proliferation of cultured human neonatal aortic smooth muscle cells

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Abstract Tissue-factor pathway inhibitor (TFPI) inhibits the procoagulant activity of the tissue-factor/factor VIIa complex. It was recently reported that TFPI prevented restenosis following tissue injury in a rabbit atherosclerotic model. In order to clarify the mechanism behind this successful prevention of restenosis, we investigated the direct effect of human recombinant TFPI (h-rTFPI) on the proliferation of cultured human neonatal aortic smooth muscle cells (hSMC). We found that h-rTFPI exhibits inhibitory activity toward hSMC proliferation, while h-rTFPI-C which lacks the carboxyl (C)-terminal region does not. Furthermore, we found that h-rTFPI binds to hSMCs with $K_d = 526$ nM but that this binding is inhibited by the addition of the synthetic C-terminal peptide, Lys²⁵⁴–Met²⁷⁶, to h-rTFPI. Thus, the interaction of h-rTFPI with hSMCs mediated via the C-terminal region is responsible for the anti-proliferative action of h-rTFPI. On the basis of these results, we presume that the anti-proliferative effect of h-rTFPI in addition to its anti-coagulant function plays a significant role in preventing restenosis following tissue injury.

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Key words: Tissue-factor pathway inhibitor; Restenosis; Human neonatal aortic smooth muscle cell; Tissue factor

1. Introduction

The proliferation of aortic smooth muscle cells (SMC) is a key event in the development of arterial lesions such as neointimal hyperplasia after vascular injury and atherogenesis [1–3]. Various types of growth factors which are secreted from platelets, macrophages, and vascular cells have been identified, and the involvement of these growth factors in the development of arterial lesions has been suspected [4]. However, the identification of growth inhibitors has received little attention, which has instead been focused on the characterization of growth promoters of SMCs. Among the inhibitors so far reported [5], heparin/heparan sulfate is well characterized and has been used for clinical intervention to prevent restenosis after balloon angioplasty [6,7]. Jang et al. [8] recently demonstrated that treatment with tissue-factor pathway inhibitor (TFPI) in a rabbit atherosclerotic model reduces angiographic restenosis.

TFPI is a Kunitz-type protease inhibitor which exhibits a strong and specific inhibitory activity against tissue factor (TF)-mediated initiation of the blood coagulation cascade [9,10]. TF expressed on cellular surface binds to factor

VII(a) and activates factors X and factor IX, leading to the triggering of the extrinsic coagulation cascade [11]. It has been reported that TF is highly expressed in human atherosclerotic plaque and in rat aortic medial SMCs following balloon injury [12,13]. Therefore, the inhibitory activity of TFPI may contribute to the inhibition of the extrinsic coagulation pathway triggered by TF in the lesions. However, we cannot rule out two possible mechanisms by which TFPI prevent restenosis: (1) the anti-proliferative effect of TFPI on SMCs, and (2) the inhibitory activity of TFPI toward the migration of SMCs from the media to the intima. In order to clarify the specific mechanism of the prevention of restenosis by TFPI, we attempted to investigate the direct effect of human recombinant TFPI (h-rTFPI) on the proliferation of cultured human aortic SMCs (hSMC). SMCs in the restenosed lesions have been shown to be 'synthetic' SMCs with high proliferative potential, and have characteristics similar to neonatal SMCs isolated from the neonates [14]. In the present study, we used neonatal hSMCs instead of SMCs in the restenosed lesions because we could not obtain SMCs from the restenosed lesions. We found that h-rTFPI inhibits hSMC proliferation and that the anti-proliferative effect of h-rTFPI is mediated by the binding of its carboxyl (C)-terminal region with hSMCs.

2. Materials and methods

2.1. Materials

h-rTFPI was isolated from the culture medium of transformed Chinese hamster ovary cells by immunoaffinity chromatography using its monoclonal antibody (HTFPIK-9)-conjugated Cellulofine (Seikagaku Co., Tokyo, Japan), as previously described [15]. For the separation of h-rTFPI from h-rTFPI-C, which lacks the C-terminal region, we performed an affinity chromatography on a heparin–Sephacel CL-6B (Pharmacia-LKB Biotechnology, Uppsala, Sweden) column (2.7×8.8 cm). A chemical analysis of the isolated materials showed that h-rTFPI was a full-length form [16], whereas h-rTFPI-C ended at Lys²⁴⁹ [17]. The h-rTFPI concentration was determined by measuring the inhibition of factor Xa activity after incubation of factor Xa with serially diluted h-rTFPI as previously described [18]. The HTFPIK-9 monoclonal antibody was prepared by the methods described in a previous paper [19]. For the ELISA, the specific polyclonal antibody against h-rTFPI was prepared by injecting h-rTFPI into the guinea pigs.

The following peptides (CTP) were purchased from Sawady Biotechnology Co. (Tokyo, Japan): CTP23, KTKRKRKKQVRVKIAYE-EIFVKNM (Lys²⁵⁴–Met²⁷⁶); CTP11, KTKRKRKKQVR (Lys²⁵⁴–Val²⁶⁴); CTP12, KIIAYEEIFVKNM (Lys²⁶⁵–Met²⁷⁶). The concentrations of these peptides were determined by amino acid analysis.

The following materials were obtained from the listed companies: unfractionated heparin (180 U/mg, Shimizu Pharma., Shizuoka, Japan), crystallized bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO), peroxidase-conjugated rabbit anti-guinea pig antibody (Dako Japan, Kyoto), Block Ace (Dainihon Pharma., Osaka, Japan), benzamide (Tokyo Kasei, Japan). All other chemicals, re-

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agent grade or better, were from Wako Pure Chemical Industries (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan).

2.2. hSMCs culture

Neonatal hSMCs were purchased from Kurabo Co. (Osaka, Japan). The cells were isolated from an aortic media in neonate, and identified by an immunological analysis of α -actin (positive) and von Willebrand factor (negative). hSMCs were cultured in growth medium, Humedia-SG2 (Kurabo Co.), and were grown at 37°C in a humidified atmosphere at 5% of CO₂ in air. Humedia-SG2 medium contained 5% fetal bovine serum (FBS), 0.5 ng/ml recombinant epidermal growth factor (rEGF), 2 ng/ml recombinant basic fibroblast growth factor (r-bFGF), and 5 μ g/ml insulin. We changed the medium every 2 days. For growth rate determination, cells were detached by treatment with 0.025% trypsin containing 0.01% EDTA (Kurabo Co.) and counted in a Coulter counter (Japan Scientific Instruments Co., Osaka). Cells were routinely used from the fifth to sixth passage.

2.3. Binding assays of h-rTFPI to hSMCs

The cells were seeded at 2500 cells/cm² in a 96-well plate (Becton Dickinson, New Jersey) and cultured in growth medium. The culture medium was changed every 2 days, and a confluent monolayer was obtained after 6 days. Confluent monolayers were washed with 200 μ l of a buffer containing 10 mM HEPES, 0.15 M NaCl, 4 mM KCl, and 11 mM glucose, pH 7.45 (buffer A) supplemented with 1 M NaCl, and then washed with 200 μ l of buffer A. Triplicate wells were incubated at 4°C with various concentrations of either h-rTFPI or h-rTFPI-C in a volume of 100 μ l of 10 mM HEPES, 0.15 M NaCl, 4 mM KCl, and 11 mM glucose, pH 7.45, supplemented with 5 mM CaCl₂ (buffer B) containing 1 mg/ml BSA. The incubation was performed at 4°C to prevent endocytosis of bound h-rTFPI. After 2 h, the incubating mixtures were removed, and the cells were washed twice in ice-cold buffer B not containing BSA as quickly as possible. After washing, the cells were scraped with 200 μ l of 0.5% Triton X-100 in phosphate-buffered saline containing 50 mM benzamidine and 10 mM EDTA, and then incubated for 10 min with 100 μ l of 25 mM NH₄OH to remove cytoskeletal elements, according to the methods outlined in Knudsen et al. [20]. The amounts of h-rTFPI in the Triton X-100 and NH₄OH aliquots were quantified by sandwich ELISA. In the current study, the total amount of h-rTFPI in Triton X-100 and NH₄OH was defined as the amount of TFPI bound to hSMCs.

2.4. Measurement of h-rTFPI antigen by a sandwich ELISA

h-rTFPI antigen was measured by a sandwich ELISA using monoclonal (HTFPIK-9) and polyclonal antibodies as described in a previous paper [19]. To evaluate the effect on the ELISA of components in the cell lysate other than TFPI, we performed the recovery studies with spiked samples which were prepared by adding various concentrations of h-rTFPI to the cell lysate. As a result, it was observed that no components had any effect on the ELISA (data not shown).

2.5. Statistics

Statistical analysis was performed by means of the unpaired Student's *t*-test. In this test, data are considered significant if $P < 0.05$.

3. Results

3.1. Effect of h-rTFPI on the proliferation of neonatal hSMCs

We investigated the effect of varying concentrations of h-rTFPI on the proliferation of the cultured neonatal hSMCs. The neonatal hSMCs did not show a hill and valley growth pattern, but grew in sheets. As shown in Fig. 1, h-rTFPI inhibited the proliferation of the hSMCs in a dose-dependent manner and that its inhibition was very dramatic at day 8. No change of morphological feature was observed in the hSMCs in the presence of h-rTFPI. To identify the functional domain in h-rTFPI, we compared the inhibitory activity of h-rTFPI with that of h-rTFPI-C which lacks the amino acid sequence of Gly²⁵⁰-Met²⁷⁶ in the C-terminal region. As shown in Fig. 2, it was clear that hSMC proliferation was not inhibited by h-rTFPI-C. In fact, h-rTFPI-C weakly enhanced the prolifera-

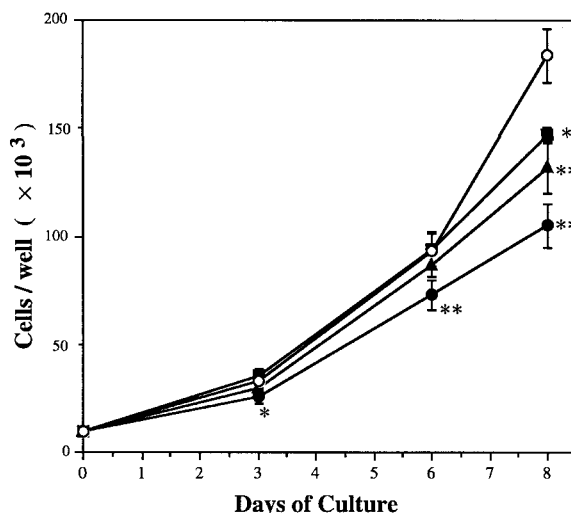


Fig. 1. Effect of h-rTFPI on cultured neonatal hSMC proliferation. hSMCs were plated sparsely (10⁴ cells/well) and at 24 h the culture medium was changed to a fresh growth medium containing various concentrations of h-rTFPI. After that, we changed the fresh medium every 2 days. For growth rate determination, cells were detached from 4 wells and counted in a Coulter counter. Each point indicates the mean cell number \pm SD ($n=4$). The data for each culture day represents a significant difference from the data for no h-rTFPI when compared by means of the unpaired Student's *t*-test if * $P < 0.05$ or ** $P < 0.01$. (○) no h-rTFPI; (■) 47 nM h-rTFPI; (▲) 236 nM h-rTFPI; (●) 1179 nM h-rTFPI.

tion of hSMCs. No cytotoxicity due to h-rTFPI was detected (data not shown).

3.2. Effects of heparin on the binding of h-rTFPI and h-rTFPI-C to hSMCs

To examine the interaction of h-rTFPI with hSMCs, we performed binding studies. As shown in Fig. 3, h-rTFPI (300 nM in buffer B) bound to hSMCs with a mean binding of 1135 ± 229 fmol/well. In the case of h-rTFPI-C, the binding was reduced by about 50% (mean binding: 533 ± 56 fmol/well). It was previously reported that one of the heparin binding sites in TFPI exists in the C-terminal region [17,21] and that the binding of h-rTFPI to the cultured ovarian carcinoma cells in the absence of factors VIIa and Xa is dramatically inhibited by an addition of heparin [22]. Therefore, we examined the effect of heparin on the binding of h-rTFPI and h-rTFPI-C, respectively, to hSMCs. The addition of heparin to h-rTFPI diminished the binding of h-rTFPI to hSMCs in a dose-dependent manner (Fig. 3), and the binding of h-rTFPI in the presence of high concentrations of heparin approached that of h-rTFPI-C, while the binding of h-rTFPI-C was not reduced at all. On the basis of these results, it was supposed that the abrogation of the binding of h-rTFPI caused by the addition of heparin (heparin-inhibitable binding) was mainly responsible for the anti-proliferative effect of h-rTFPI against hSMCs. To determine the binding constant for the heparin-inhibitable binding of h-rTFPI to hSMCs, hSMCs were incubated with increasing concentrations of h-rTFPI, and then the amount of heparin-inhibitable binding was determined by subtracting h-rTFPI's binding in the presence of heparin (60 μ g/ml) from that in the absence of heparin. The representative binding isotherms in five experiments are shown in Fig. 4, with a Scatchard plot in the inset. The binding was saturable at about 500 nM, and the Scatchard analysis showed a single

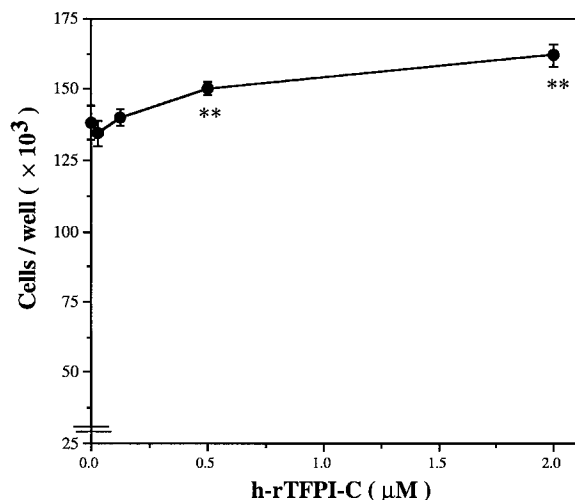


Fig. 2. Effect of h-rTFPI-C on cultured neonatal hSMC proliferation. hSMCs were cultured in a growth medium containing various concentrations of h-rTFPI-C as described in the legends for Fig. 1. Results are expressed as the mean cell number \pm SD ($n=4$) at day 8. ** $P<0.01$ versus no h-rTFPI-C.

class of binding sites. In addition, from a mean of five experiments, we determined a mean value of K_d with 526 ± 189 nM and the number of the binding sites with $5.87 (\pm 3.69) \times 10^7$ sites/cell.

3.3. Identification of the h-rTFPI sites involved in h-rTFPI's binding to hSMCs

In order to identify the h-rTFPI sites involved in h-rTFPI's binding to hSMCs, we performed a competitive binding assay

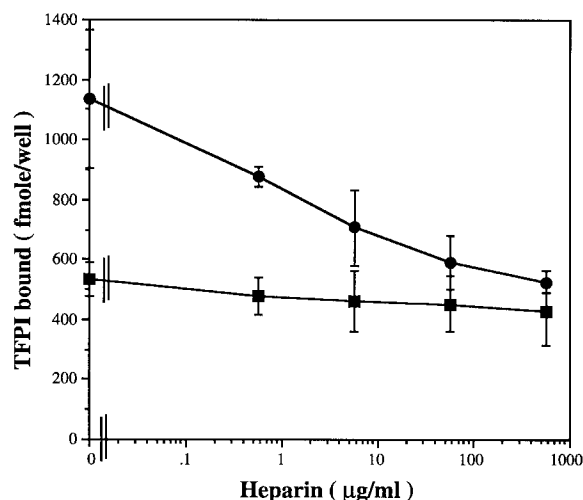


Fig. 3. Effect of heparin on the binding of h-rTFPI and h-rTFPI-C to hSMCs. hSMCs were incubated with either h-rTFPI or h-rTFPI-C in the presence of increasing concentrations of heparin at 4°C. After incubation for 2 h, hSMCs were washed, and the bound TFPI was determined by the ELISA as described under Section 2. Results are expressed as the mean amount of TFPI bound \pm SD (fmol/well) ($n=3$): (●) 257 nM h-rTFPI; (■) 320 nM h-rTFPI-C.

using TFPI-related peptides. As shown in Fig. 5, the addition of the CTP23 peptide (Lys²⁵⁴–Met²⁷⁶) to h-rTFPI abolished h-rTFPI's binding in a dose-dependent manner. To further investigate the role of the basic amino acid cluster region involved in the CTP23 peptide, CTP11 (Lys²⁵⁴–Val²⁶⁴) and CTP12 (Lys²⁶⁵–Met²⁷⁶) peptides were used in a competitive assay. Fig. 5 shows that the CTP11 peptide drastically inhib-

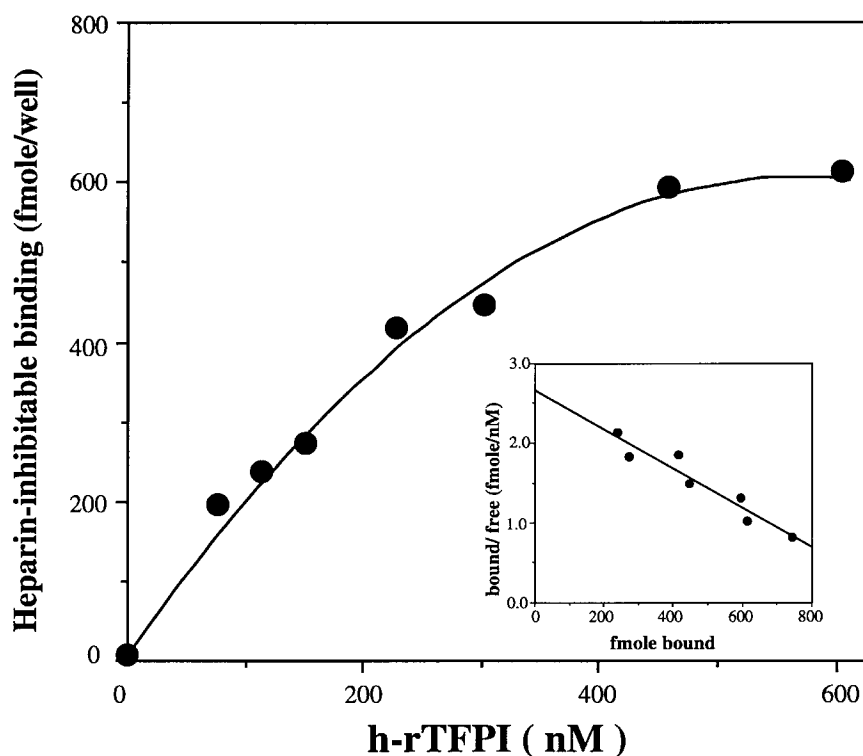


Fig. 4. Determination of the kinetic constant for the heparin-inhibitable binding of h-rTFPI to hSMCs. The heparin-inhibitable binding was determined by subtracting the h-rTFPI bound in the presence of heparin (60 μ g/ml) from that bound in the absence of heparin. Inset: Scatchard plot for heparin-inhibitable binding of h-rTFPI to hSMCs.

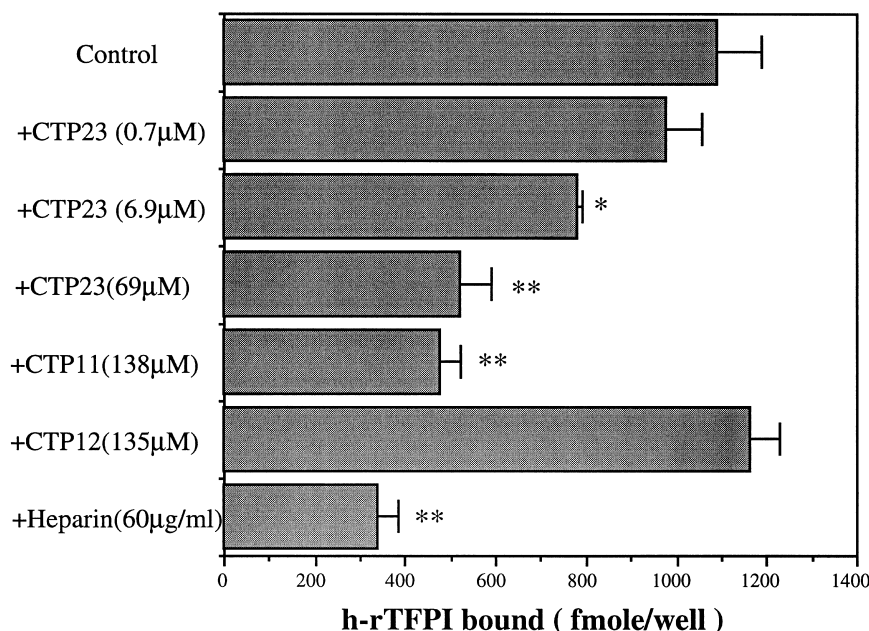


Fig. 5. Effect of the TFPI-derived C-terminal peptides on binding of h-rTFPI to hSMCs. After 300 nM h-rTFPI was incubated with hSMCs in the presence of some peptides or heparin (60 μg/ml) for 2 h at 4°C, the bound TFPI was determined by the ELISA. In the experiments, we used the CTP23 peptide (Lys²⁵⁴–Met²⁷⁶) (0.7–69 μM), 138 μM CTP11 peptide (Lys²⁵⁴–Val²⁶⁴), and 135 μM CTP12 peptide (Lys²⁶⁵–Met²⁷⁶) as the C-terminal peptide. Columns and error bars represent the mean and SD of triplicate samples, respectively. **P* < 0.05 and ***P* < 0.01 versus the control groups which contain h-rTFPI only.

ited h-rTFPI's binding, while the CTP12 peptide did not. On the basis of these results, it seems likely that the basic amino acid cluster region in TFPI plays a significant role in h-rTFPI's binding to hSMCs.

4. Discussion

Many biological processes of SMCs are very important in the development of atherosclerosis and restenosis following angioplasty [1,2], and the hypercoagulation triggered by TF in arterial lesions is one such significant process [12]. Jang et al. [8] recently found that TFPI treatment in a rabbit atherosclerotic model reduced restenosis and suggested that TFPI contributes to the inhibition of the TF-mediated extrinsic coagulation pathway. Sato et al. also reported that TF induces the migration of cultured aortic SMCs in concert with factor VIIa and that its induction is completely prevented by an addition of h-rTFPI [23]. However, the direct effect of h-rTFPI on the proliferation of SMCs has not yet been reported. Therefore, in the present study, we investigated the effect of h-rTFPI on the proliferation of cultured hSMCs. We found that h-rTFPI inhibits neonatal hSMC proliferation in growth medium, while h-rTFPI-C does not. Experiments on the binding of h-rTFPI to hSMCs suggested that the anti-proliferative effect of h-rTFPI is mediated by the binding of its C-terminal region with hSMCs. As expected, a very weak anti-proliferative effect of h-rTFPI on the hSMCs taken from media of adult arteries and on human fibroblasts was observed (data not shown). Therefore, it appears that the anti-proliferative effect of h-rTFPI is specific to the cultured hSMCs with high proliferative potential and that the C-terminal basic region in h-rTFPI contributes to this effect. The cultured neonatal hSMCs have some characteristics similar

to the 'synthetic' hSMCs in the restenosed lesions [14]. On the basis of these results, we think that h-rTFPI is a strong candidate to prevent restenosis following tissue injury through its anti-proliferative function against SMCs.

In the present study, we could not locate a precise mechanism for the anti-proliferative function mediated via the C-terminal region in h-rTFPI nor could we identify the specific binding substances or receptors on the surface of hSMCs. Novotny et al. previously reported that TFPI covalently binds to apolipoprotein A-II [24]. And also, apolipoprotein A-II reduces EGF-induced DNA synthesis in SMCs [25]. Therefore, it is possible that the anti-proliferative effects of h-rTFPI may be mediated through the interaction between h-rTFPI and apolipoprotein A-II, although the precise mechanism still remains unknown. It is well known that the C-terminal basic region of TFPI is a main heparin-binding region [17,21]. Among the heparin binding proteins, the significance of the anti-proliferative effect of platelet factor-4 (PF-4) [26] and histidine-rich glycoprotein (HRG) [27] have been well evaluated using endothelial cells and fibroblasts, respectively. The effect is thought to be mainly attributable to the competition with the heparin-binding growth factors such as FGFs for cell surface glycosaminoglycans (GAG), because both PF-4 [28] and HRG [27] can bind to the GAGs on the surface of cultured cells. In addition, it was recently reported that h-rTFPI could bind to heparan sulfate on ryudocan prepared from endothelium-like cells with a high affinity [29]. Therefore, it seems likely that h-rTFPI can prevent the proliferation of hSMCs by binding to GAGs.

Low-density lipoprotein receptor-related protein (LRP) has been reported to be a receptor of TFPI on cell surfaces [30]. However, it is unlikely that LRP is involved in the interaction of h-rTFPI with hSMCs, because the affinity of h-rTFPI for

LRP is much higher than that in our present study, as compared with the K_d value (30 nM versus 526 nM) [30]. In addition to this receptor, it has been recently demonstrated that TFPI synthesized by endothelial cells localizes in caveolae and that its localization is mediated by glycosyl phosphatidylinositol (GPI) [31]. Therefore, GPI is considered to be a possible receptor for h-rTFPI binding.

In conclusion, h-rTFPI exhibits inhibitory activity toward the growth of hSMCs in addition to its anticoagulant activity. Therefore, h-rTFPI may be a promising medicine for preventing restenosis postangioplasty.

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