

# Tissue factor regulates tumor angiogenesis of retinoblastoma via the extracellular signal-regulated kinase pathway

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**Abstract.** Retinoblastoma, a well-vascularized tumor that is dependent on a very robust angiogenic response, is the most common intraocular malignancy in children. Tissue factor (TF) is known to regulate tumor progression and in the present study we demonstrated that TF regulates tumor angiogenesis of retinoblastoma. In an orthotopic transplantation model of retinoblastoma, TF was selectively expressed in the proliferative area of retinoblastoma including tumor vessels as well as tumor cells, where TF expression was co-localized with endothelial cells of tumor vessels. TF expression progressively increased with fibroblast growth factor-2 (FGF-2)-induced proliferation of human umbilical vein endothelial cells (HUVECs), which was effectively inhibited by blockade of the TF pathway by TF pathway inhibitor (TFPI). In addition, FGF-2-induced angiogenic processes of migration and tube formation of vascular endothelial cells were also effectively suppressed by TFPI, which would be mediated by inhibition of extracellular signal-regulated kinase activation. Therefore, further to our previous report that TF is involved in tumor cell proliferation of retinoblastoma, our current data suggest that blockade of the TF pathway by TFPI could effectively inhibit tumor growth by suppressing tumor cell proliferation and tumor angiogenesis at the same time.

## Introduction

Retinoblastoma is the most common intraocular malignancy in children, with an incidence rate of 11.8 per million children aged 0-4 years in the United States (1). Histopathologically, retinoblastoma tumor shows perivascular sleeves consisting of viable neoplastic cells and necrotic tissue peripherally (2), which shows a close association of tumor cells with blood vessels. Marback *et al* suggested relatively high vascular area of the tumor could be a poor prognostic factor of retinoblastoma (3). Moreover, intraocular extension to the choroid and the optic nerve, a well-known poor prognostic factor of retinoblastoma, was found to be associated with extensive tumor necrosis, which was accompanied by thrombosis of the central retinal vessels (4,5). The majority of cancer patients are in the hyper-coagulated status and activation of coagulation might be involved in tumor progression (6). Therefore, therapeutic agents modulating tumor angiogenesis and thrombosis at the same time could be a good treatment modality for retinoblastoma.

Tissue factor (TF), a 47-kDa membrane-bound glycoprotein, is a well-known cellular activator of the coagulation cascade (7), which primarily binds to factor VII (FVII) and forms the TF:FVIIa complex (8). TF itself also can simulate angiogenesis-independent FVIIa, and the TF:FVIIa complex is involved in signaling pathway through cell-bound protease-activated receptors (PARs), inducing proangiogenic and immune modulating cytokines, chemokines and growth factors (7,9). In addition, TF is a well-known tumor pro-coagulant, and is also considered to be associated with tumor angiogenesis (10,11). Therefore, tumor angiogenesis could be effectively inhibited by blockade of the tissue factor pathway.

Tissue factor pathway inhibitor (TFPI), which is mainly synthesized in the vascular endothelium and present mainly in the endothelium and plasma, consists of Kunitz-type domains, where the first domain binds to FVIIa, the second to activated factor X (FXa), and inactivates the TF:FVIIa complex and FXa (12). Therefore, the dual inhibitory effect of TFPI on the TF:FVIIa complex and FXa could be a promising candidate modulating thrombosis and tumor angiogenesis at the same time.

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We previously reported that TF is expressed in tumor cells of retinoblastoma cells, whose expression is closely associated with proliferation of tumor cells in retinoblastoma (13). However, it remains to be elucidated whether TF is involved in tumor angiogenesis of retinoblastoma.

Herein, we demonstrated for the first time that TF regulates tumor angiogenesis of retinoblastoma. We found that TF is expressed by endothelial cells of retinoblastoma, whose expression is upregulated with the proliferation of endothelial cells. In addition, blockade of the TF pathway by TFPI effectively inhibits FGF-2-induced proliferation of endothelial cells. Moreover, FGF-2-induced angiogenic processes of migration and tumor formation of endothelial cells are suppressed by TFPI, which would be mediated by blockade of the extracellular signal-regulated kinase (ERK) pathway.

## Materials and methods

**Orthotopic transplantation mouse model of retinoblastoma.** BALB/c female nude mice were purchased from Oriental (Korea). Care, use and treatment of all animals in this study were in agreement with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. As in our previous report (14), cultivated SNOU-Rb1 cells ( $1 \times 10^6$  cells) were harvested, suspended in cold phosphate-buffered saline and injected into the intravitreal cavity of BALB/c nude mice. Tumor development was observed by indirect ophthalmoscopic examination twice a week for 4 weeks. Four weeks after inoculation, the mice were sacrificed and enucleated.

**Immunofluorescence staining.** The enucleated eyes were formalin-fixed, paraffin-embedded and then sectioned ( $4 \mu\text{m}$ ). The slides were de-paraffinized and incubated with proteinase K at  $37^\circ\text{C}$ . After blocking endogenous peroxidase activity with hydrogen peroxide and non-specific binding with blocking kit (Zymed Laboratories Inc., South San Francisco, CA, USA), slides were incubated overnight with anti-TF (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-CD31 (1:200; BD Biosciences, San Jose, CA, USA) or anti-Ki67 (BD Biosciences) at  $4^\circ\text{C}$ . Alexa Fluor 594 donkey IgG (1:500; Molecular Probes, Eugene, OR, USA), Alexa Fluor 594 donkey IgG (1:200; Molecular Probes), Alexa Fluor 546 donkey IgG (1:100; Molecular Probes) were used as secondary antibodies. The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich Co., St. Louis, MO, USA). The slides were mounted Faramount Aqueous mounting medium (Dako, Glostrup, Denmark) and observed under a fluorescence microscope (BX50, Olympus, Tokyo, Japan).

**Immunohistochemistry.** Slides were prepared as for immunofluorescence staining and incubated with anti-TF (1:200; Santa Cruz Biotechnology) at  $4^\circ\text{C}$  for 12 h. Then, a biotinylated goat antibody (Dako) was used for the avidin/biotin complex (Vectastain kit; Vector Laboratories, Burlingame, CA, USA) and the 3-amino-9-ethyl-carbazole chromogen (AEC, Dako). The slides were mounted Faramount Aqueous mounting medium (Dako) and observed under a light microscope (Carl Zeiss, Chester, VA, USA). Primary antibody was omitted for negative control.

**Cell culture.** Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Basel, Switzerland) and cultured on 0.15% gelatin-PBS coated plates in M199 medium (Gibco-BRL, Rockville, MD, USA) supplemented with 20% fetal bovine serum (Gibco-BRL), 3 ng/ml fibroblast growth factor-2 (FGF-2; Millipore, Bedford, MA, USA), 10 U/ml heparin (Sigma-Aldrich) and 1% antibiotic-antimycotic solution (Invitrogen, Carlsbad, CA, USA) at  $37^\circ\text{C}$  in a moist atmosphere of 95% air and 5%  $\text{CO}_2$ . The medium was changed every third day. Cultured cells were observed daily under a phase-contrast microscope (Carl Zeiss). HUVECs used in this study were taken from passage 4 to 8. When required, FGF-2 (10 ng/ml; Sigma-Aldrich) or TFPI (0.1-1 nM; American Diagnostica GmbH, Pfungstadt, Deutschland) treatment was carried out.

**Western blot analysis.** Cells were harvested, washed with ice-cold phosphate buffer solution, and lysed with buffer containing 50 mM of Tris-HCl (pH 7.4), 150 mM of NaCl, 1% Nonidet P40, 2 mM of sodium orthovanadate and a protease inhibitor cocktail (Roche). An equal amount (15  $\mu\text{g}$ ) of the samples was separated on sodium dodecyl sulfate-polyacrylamide gel and then transferred onto nitrocellulose filters (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were immunoblotted with primary antibodies against TF (1:1000; Santa Cruz Biotechnology), ERK 1/2 (1:1000; Cell Signaling Technology, Beverly, MA, USA) or phospho-ERK 1/2 (1:1000; Cell Signaling Technology). To ensure the equal loading of protein in each lane, the blots were stripped and re-probed with an antibody against  $\beta$ -actin.

**Cell proliferation assay.** Cell proliferation was evaluated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay modified from our previous description (15). HUVECs ( $5 \times 10^3$  cells) were seeded onto 96-well plates and cultured overnight. The cells were treated with FGF-2 (10 ng/ml; Sigma-Aldrich) under various concentrations of TFPI (0.1-1 nM; American Diagnostica GmbH) for 24 h. Following incubation, the medium was carefully removed from the plate, and dimethyl sulfoxide was added to solubilize formazan produced from MTT by the viable cells. Absorbance was measured at 540 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

**Wound migration assay.** The migration of endothelial cells was evaluated with wound migration assay modified from our previous study (16). HUVECs ( $1 \times 10^6$  cells) were seeded and cultured onto gelatin-coated 60-mm culture dishes at 90% confluence and monolayers of cells were wounded with a micropipette tip. After rinsing with serum-free medium, the wounded monolayers were incubated with treatment of 0.5 or 1.0 nM TFPI (American Diagnostica GmbH) and 10 ng/ml FGF-2 (Sigma-Aldrich) for 12 h. Migration was measured by counting the number of cells that moved across the reference line under a light microscope (Carl Zeiss).

**Tube formation assay.** The tube formation of endothelial cells was assayed as previously described (17). HUVECs ( $1 \times 10^5$  cells) were inoculated on the surface of the Matrigel with treatment of 0.5 or 1.0 nM TFPI (American Diagnostica

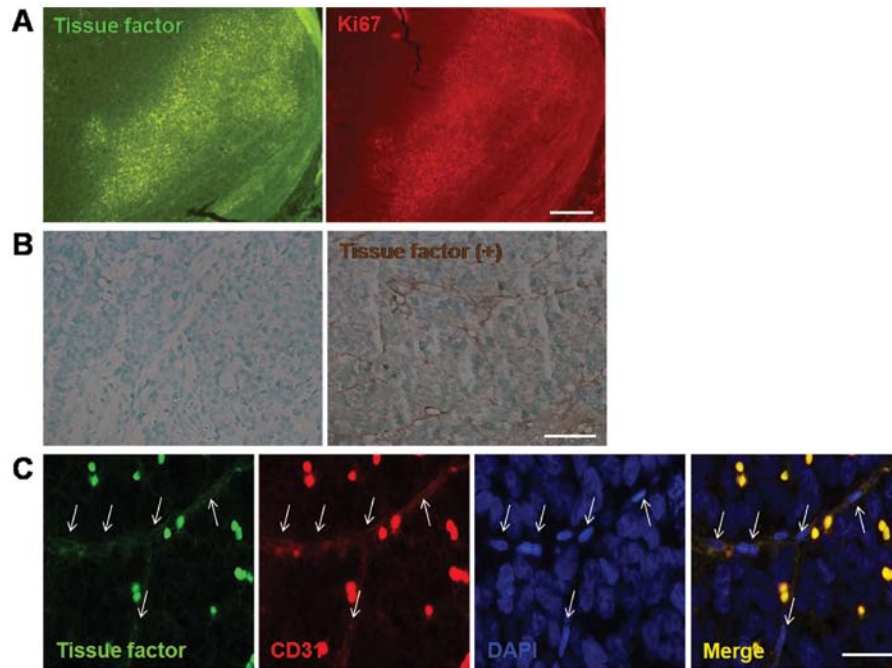


Figure 1. TF is expressed on tumor vessels of the orthotopic transplantation model of retinoblastoma. Four weeks after the inoculation of SNUOT-Rb1 cells ( $1 \times 10^6$  cells per  $20 \mu\text{l}$ ) into vitreous cavities of BALB/c-nude mice, the mice were sacrificed and enucleated. (A) Immunofluorescence staining for TF and Ki67 as a proliferation marker was performed. (B) Immunohistochemistry for TF was performed. (C) TF expression around tumor cells and vessel walls (B). Immunofluorescence staining for TF and CD31 as an endothelial cell marker was performed. For staining of nuclei, slides were stained with DAPI. Each figure was selected as representative data from three independent experiments. Scale bars: (A),  $100 \mu\text{m}$ ; (B),  $25 \mu\text{m}$ ; (C),  $20 \mu\text{m}$ .

GmbH) and with  $10 \text{ ng/ml}$  FGF-2 (Sigma-Aldrich) for 12 h. Tube formation was observed under a light microscope (Carl Zeiss) and photographed at a  $\times 400$  magnification. Tube formation was quantified by counting the number of connected cells divided by the total number of cells in randomly selected fields at a  $\times 400$  magnification.

**Statistical analysis.** Statistical differences between groups were evaluated with the Mann-Whitney U test. Data were recorded as the mean  $\pm$  SD. P-values of  $\leq 0.05$  were considered to indicate statistically significant differences.

## Results

**TF is expressed on tumor vessels of the orthotopic transplantation model of retinoblastoma.** Four weeks after the inoculation of retinoblastoma cells, the vitreous cavity was almost completely occupied by the tumor. Based on our recent report that TF immunopositive cells are mainly detected in the proliferative area of retinoblastoma (13), immunoreactivity for TF in retinoblastoma was analyzed with Ki67, a proliferation marker in the orthotopic transplantation mouse model of retinoblastoma. As shown in Fig. 1A, TF in retinoblastoma was prominently expressed in the area of highly proliferative activity. In addition, TF expression was confirmed in retinoblastoma tissue through immunohistochemistry, markedly where we found that TF is expressed on the tumor vessels as well as around tumor cells of the mouse model of retinoblastoma (Fig. 1B).

Therefore, given that proliferative tumor cells of high metabolic activity in retinoblastoma are supplied by abundant tumor angiogenesis (2,3), we investigated whether TF is

expressed on tumor vessels of retinoblastoma. As expected, there was strong immunoreactivity of TF on tumor vessels of retinoblastoma, which was furthermore co-localized with CD31, as an endothelial cell maker (Fig. 1C). Thus, TF expression on tumor vessels of retinoblastoma suggests a correlation of TF with tumor angiogenesis of retinoblastoma.

**TF regulates proliferation of vascular endothelial cells.** To examine whether TF is related to tumor angiogenesis of retinoblastoma, we determined temporal expression of TF in the proliferation of vascular endothelial cells. As demonstrated in Fig. 2A, TF expression in HUVECs progressively and significantly increased with proliferation induced by FGF-2, a well-known mitogen of retinoblastoma tumor ( $^*P < 0.05$ ) (18).

Next, we evaluated whether proliferation of vascular endothelial cells could be directly inhibited by blockade of the TF pathway. As shown in Fig. 2B, proliferation of HUVECs was significantly increased by FGF-2 treatment ( $^*P < 0.05$ ), which was prevented by co-treatment with TFPI in a dose-dependent manner ( $^*P < 0.05$ ).

**TF regulates angiogenic processes of migration and tube formation of vascular endothelial cells.** Based on our data that proliferation of vascular endothelial cells is regulated by the TF pathway, we investigated whether the TF pathway is involved in the regulation of angiogenic processes of migration and tube formation of vascular endothelial cells. The migration of HUVECs increased 1.7-fold with FGF-2 treatment ( $^*P < 0.05$ ), whereas the migratory activity was completely inhibited by co-treatment with TFPI ( $^*P < 0.05$ , Fig. 3A). Moreover, FGF-2 induced extensive formation of capillary-like networks, which was 1.6-fold compared to control ( $^*P < 0.05$ ), was nearly abol-

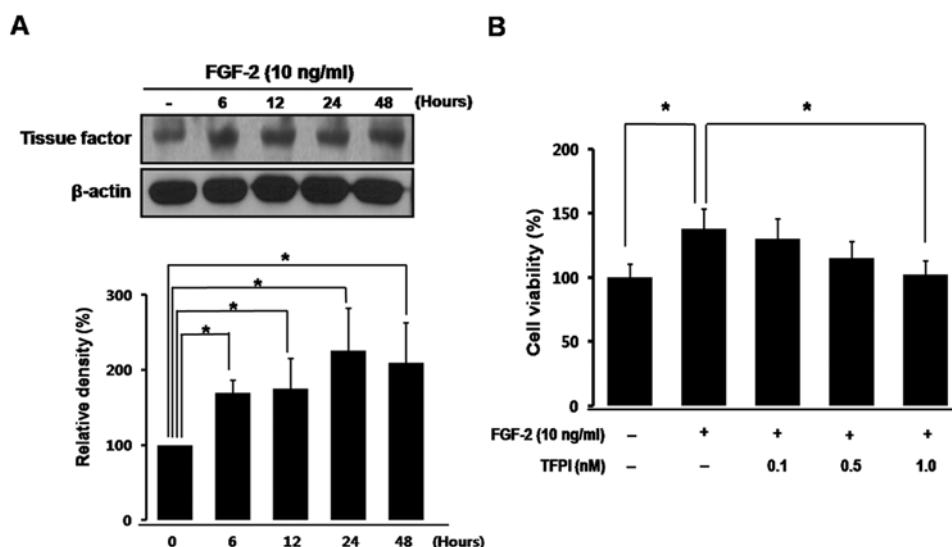


Figure 2. TF regulates proliferation of vascular endothelial cells. FGF-2 (10 ng/ml) with or without TFPI (0.1-1.0 nM) was treated on HUVECs. (A) Western blot analysis for TF was performed.  $\beta$ -actin served as the loading control. Figures were selected as representative data from three independent experiments. Quantitative analysis was performed by measuring the intensity relative to the control. Each value represents the means  $\pm$  SE from three independent experiments ( $^*P < 0.05$ ). (B) The proliferation of HUVECs was evaluated by MTT assay. Quantitative analysis was performed by measuring the intensity relative to the control. Each value represents the means  $\pm$  SE from three independent experiments ( $^*P < 0.05$ ).

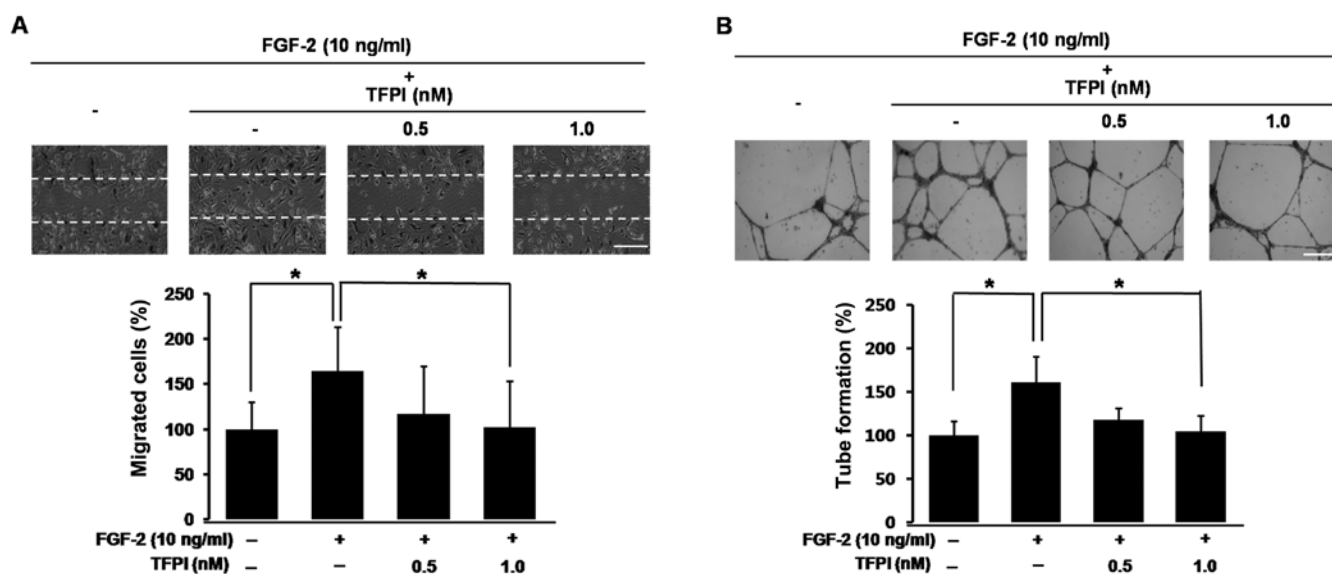


Figure 3. TF regulates angiogenic processes of migration and tube formation of vascular endothelial cells. (A) HUVECs were plated onto gelatin-coated culture dishes and wounded with a razor blade. The wounded monolayers were incubated with treatment of FGF-2 (10 ng/ml) or TFPI (0.5 and 1.0 nM). Figures were selected as representative data from three independent experiments. The migration was quantified by counting the number of cells that moved across the reference line. Quantitative analysis was performed by measuring the intensity relative to the control. Each value represents the mean ( $\pm$  SD) of three independent experiments ( $^*P < 0.05$ ). (B) HUVECs were inoculated on the surface of the Matrigel and treated with FGF-2 (10 ng/ml) or TFPI (0.5 and 1.0 nM). Figures were selected as representative data from three independent experiments. The tube formation was quantified by counting the number of connected cells in randomly selected fields, and dividing that number by the total number of cells in the field. Quantitative analysis was performed by measuring the intensity relative to the control. Each value represents the mean ( $\pm$  SD) of three independent experiments ( $^*P < 0.05$ ).

ished by co-treatment with TFPI ( $^*P < 0.05$ , Fig. 3B). These results suggest that in addition to FGF-2 mediated proliferation of vascular endothelial cells, FGF-2 mediated angiogenic processes of migration and tube formation could be regulated by the TF pathway.

*TF regulates FGF-2-induced angiogenic processes through ERK activation.* Given that the sustained activation of the ERK

pathway is required for FGF-2-induced angiogenesis (19), we confirmed whether TF could regulate FGF-2-induced angiogenic processes via ERK activation. ERK-1/2 phosphorylation was significantly increased with FGF-2 treatment in HUVECs ( $^*P < 0.05$ ), which was completely blocked by co-treatment with TFPI ( $^*P < 0.05$ , Fig. 4). Therefore, it was clearly demonstrated that blockade of the TF pathway could effectively inhibit tumor angiogenesis mediated by FGF-2 of retinoblastoma.

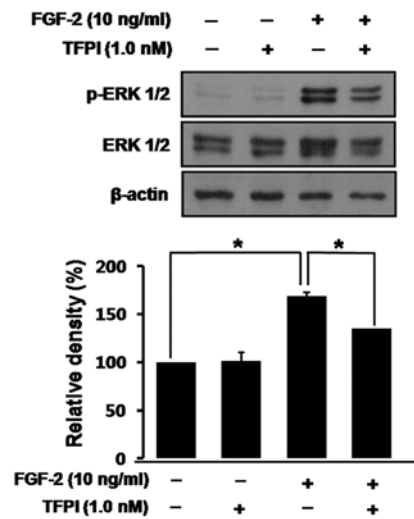


Figure 4. TF regulates FGF-2-induced angiogenic processes through ERK activation. FGF-2 (10 ng/ml) with or without or TFPI (1.0 nM) was treated on HUVECs. Western blot analysis for p-ERK 1/2 and ERK 1/2 was performed.  $\beta$ -actin served as the loading control. Figures were selected as representative data from three independent experiments. Quantitative analysis was performed by measuring the intensity relative to the control. Each value represents the means  $\pm$  SE from three independent experiments (\* $P < 0.05$ ).

## Discussion

Although TF has been reported to be expressed in several malignant tumors such as colon cancer, breast cancer, leukemia and small cell lung cancer (20,21), the role of TF in retinoblastoma had yet to be elucidated. Our findings show that TF is expressed in retinoblastoma cells, and could be involved in the proliferation of tumor cells (13). In addition to tumor cells, it was also indicated that TF expression in tumor could be related to tumor angiogenesis (9-11). Herein, we demonstrated for the first time that TF regulates tumor angiogenesis of retinoblastoma.

Growth factors in tumor are known to be involved in growth, progression and drug resistance (22). Among variable growth factors, FGF-2 was reported to be produced in retinoblastoma cells to contribute to tumor progression as a major underlying mitogen of retinoblastoma (18,22). FGF-2 was found in a vascular pattern of a transgenic mouse model of retinoblastoma, which suggests that FGF-2 in the tumor microenvironment of retinoblastoma plays a direct role in supporting tumor angiogenesis (23). Moreover, FGF-2 expression in the transgenic mouse model of retinoblastoma is prominently localized to the tumor vasculature (23). First, we discovered that TF is selectively expressed in the proliferative area of retinoblastoma including the tumor vessels as well as tumor cells. Then we showed that in addition to FGF-2-induced proliferation of vascular endothelial cells, FGF-2-induced angiogenic processes of migration and tube formation of vascular endothelial cells were directly regulated by the TF pathway, which would be mediated by the ERK pathway.

Although it is not clear how TF is involved in tumor angiogenesis, many supporting results have been reported. For example, increased expression of TF in pancreatic cancer was associated with VEGF expression and increased microvessel

density (9), whereas Low-TF mice showed reduced tumor blood vessel size in B16F1 melanoma (24). In addition, TF is believed to play a role in angiogenesis indirectly by clotting-dependent mechanisms or by modulating angiogenic properties of tumor cells, or directly by clotting-independent mechanisms (25). In particular, specific inhibitors to the TF:FVIIa complex impaired angiogenesis, in contrast to the FXa inhibitor that could not (26). Thus, a direct mechanism through TF:FVIIa complex mediated signaling may play a crucial role in angiogenesis, which suggests that the inhibitory effects of TFPI on angiogenic processes are probably mediated by inhibiting TF:FVIIa-mediated signaling, not by FXa. Based on the fact that TFPI could inhibit FGF-2-stimulated angiogenic processes of vascular endothelial cells (27,28), we demonstrated that blockade of the TF pathway by TFPI could inhibit FGF-2-induced angiogenic processes of migration and tube formation as well as proliferation of vascular endothelial cells via the ERK pathway. The TF:FVIIa complex leads to activation of the G-protein coupled receptor called protease-activated receptor 2 (PAR2) (29), whose activation could activate several mitogen-activated protein kinase pathways (25). Therefore, the anti-angiogenic activity of TFPI would be mediated by TF:FVIIa-PAR2-MAPK signaling.

In addition to our previous report that TF expression is increased in proliferating tumor cells of retinoblastoma, which could be inhibited by blockade of the TF pathway, TFPI (13), in our current study we demonstrated that TF is also expressed on tumor vessels of retinoblastoma, which could be involved in the angiogenic processes of tumor angiogenesis in retinoblastoma. Given that, TF is expressed in tumor vessels of retinoblastoma as well as in tumor cells, which is directly involved both in the proliferation of tumor cells and in tumor angiogenesis. In conclusion, our results suggest that blockade of the TF pathway by TFPI could effectively inhibit tumor growth by suppressing tumor cell proliferation and tumor angiogenesis at the same time. Therefore, TFPI could be considered to be applied to retinoblastoma as an effective therapeutic targeting tumor cell proliferation and tumor angiogenesis.

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