A mitogen for growth-arrested cultured bovine aortic smooth muscle cells was purified to homogeneity from the supernatant of cultured human umbilical vein endothelial cells by heparin affinity chromatography and reverse-phase high performance liquid chromatography. This mitogen was revealed to be tissue factor pathway inhibitor-2 (TFPI-2), which is a Kunitz-type serine protease inhibitor. TFPI-2 was expressed in baby hamster kidney cells using a mammalian expression vector. Recombinant TFPI-2 (rTFPI-2) stimulated DNA synthesis and cell proliferation in a dose-dependent manner (1–500 nM). rTFPI-2 activated mitogen-activated protein kinase (MAPK) activity and stimulated early proto-oncogene c-fos mRNA expression in smooth muscle cells. MAPK, c-fos expression and the mitogenic activity were inhibited by a specific inhibitor of MAPK kinase, PD098059. Thus, the mitogenic function of rTFPI-2 is considered to be mediated through MAPK pathway. TFPI has been reported to exhibit antiproliferative action after vascular smooth muscle injury in addition to the ability to inhibit activation of the extrinsic coagulation cascade. However, structurally similar TFPI-2 was found to have a mitogenic activity for the smooth muscle cell.

The proliferation of smooth muscle cells is closely related to the pathogenesis of atherosclerosis and the restenosis after percutaneous transluminal coronary angioplasty (1). However, the precise mechanism of the proliferation is unknown. In this study, we purified a mitogen for growth-arrested bovine aortic smooth muscle cells from the supernatant of cultured HUVEC and identified it as TFPI-2, whose physiological function has been unknown.

TFPI-2 is structurally related to TFPI. TFPI is synthesized in endothelial cells and exists on the endothelial surface and in plasma (2–5). TFPI inhibits the initial steps of the extrinsic coagulation pathway and regulates the hemostasis. Recently, an antiproliferative action of TFPI has been reported. In the atherosclerotic rabbit arterial injury model, treatment with recombinant TFPI reduced angiographical restenosis and decreased neointimal hyperplasia (6). Inhibition of TF-mediated coagulation by recombinant TFPI administration during the first 24 h after balloon-induced arterial injury at the carotid artery of minipigs seems to be effective for attenuating subsequent neointimal formation and luminal stenosis (7). TFPI exhibits inhibitory activity toward cultured human neonatal aortic smooth muscle cells (8). However, TFPI-2 has a mitogenic activity despite its similarity of structure to TFPI. This new function of TFPI-2 may play an important role in the pathogenesis of atherosclerosis and neointimal hyperplasia after percutaneous transluminal coronary angioplasty.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human umbilical vein endothelial cells were cultured as described previously (9). Bovine aortic smooth muscle cells were isolated from the medial layers of adult bovine aorta by a modification of the explant technique of Ross as described previously (10). In brief, they were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were grown at 37°C in a humidified atmosphere of 5% CO₂ in air. Culture medium (DMEM + 10% FCS) was changed every 3 days, and a confluent smooth muscle cell monolayer was obtained after about 7 days. Cells were used from the second to the sixth passage. Cells were harvested with 0.1% trypsin-0.02% EDTA solution and plated at a density of 2.0 × 10⁵ cells in 10-cm dish (Nalge Nunc) for 48 h, after which their growth was arrested with DMEM containing 0.1% FCS. After 48 h, fresh medium (DMEM + 0.1% FCS) and different concentrations of mitogen (TFPI-2) were added simultaneously to the growth-arrested cells. After 48 h, cells were recovered using the trypsin/EDTA solution, and cell counts were performed with a hemocytometer. For the detection of DNA synthesis, cells were plated at a density of 3,000 cells/well in 96-well plates. Biotrak Cell proliferation enzyme-linked immunosorbent assay system (Amersham Pharmacia Biotech) was used. 5-Bromo-2′-deoxyuridine (BrdUrd) was added 24 h after TFPI-2 addition. After 9 h, incorporated BrdUrd was assayed.

**Purification and Identification of TFPI-2**—A mitogen was purified from the conditioned HUVEC medium. Medium (800 ml) was concentrated to 20 ml using a ultrafiltration membrane (YM 10, Amicon) at 4°C. This 20-ml solution was applied to HiTrap Heparin affinity column (5 ml, Amersham Pharmacia Biotech) previously equilibrated with 50 mM Tris-HCl (pH 7.4). Following sample application, the column was washed with 50 ml of 50 mM Tris-HCl (pH 7.4) solution. Mitogenic activity was eluted with 20 ml of 50 mM Tris-HCl (pH 7.4) containing 0.1 M NaCl. The eluent was concentrated to 0.5 ml by Centriprep 10 (Amicon). This sample was injected to ProRPC HR 5/10 column (Amersham Pharmacia Biotech) using LC-6A high performance liquid chromatography system (Shimadzu Co.). The flow rate was 1 ml/min. The peak was monitored at 280 nm. The column was eluted with a gradient formed from 0.1% trifluoroacetic acid in 20% acetonitrile to 0.1% trifluoroacetic acid in 100% acetonitrile. The mitogenic fraction eluted
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from the column was freeze-dried. Amino acid sequence analysis was carried out by automated Edman degradation using an Applied Biosystems 470 A gas-phase sequencer (Perkin-Elmer).

**Electrophoresis**—SDS-polyacrylamide gel electrophoresis was performed using Phast System (Amersham Pharmacia Biotech). The protein was stained with Coomassie Brilliant Blue. The molecular size marker used was obtained from Amersham Pharmacia Biotech.

**Construction of TFPI-2 cDNA Expression Vector**—The mammalian expression vector pK4K was used for the construction of the expression vector designated as pK4KT2 (11). This vector contains unique restriction sites for HindIII and BamHI. The TFPI-2 cDNA was made using polymerase chain reaction (PCR) with a sense oligonucleotide 5'-'AGGACCCCGCTCGCC-3' and antisense oligonucleotide 5'-'GCCATTAGAAGACAAACAGAT-3', which corresponded to nucleotides 39–54 and 761–782 of TFPI-2, respectively. The template was pBluescript II SK(−) containing TFPI-2 cDNA (Stratagene). This PCR product was blunted (DNA Blunting Kit, Takara Biochemicals) and ligated into the above vector after blunting. The sequence of PCR product was confirmed with automated DNA sequencer 373A (Applied Biosystems, Perkin-Elmer).

**Transfection and Cell Culture**—Nontransfected baby hamster kidney (BHK) tk−ts13 cells were grown in DMEM supplemented with 5% FCS, streptomycin (30 μg/ml), and penicillin (30 units/ml). BHK cells (2 × 10⁵ cells) were transfected with 5 μg of pK4KT2 by a modified CaPO₄ precipitation technique using the CellPhect transfection kit (Amersham Pharmacia Biotech). The transfected cells, BHKT2, were grown in DMEM containing 5% FCS. After selection with 250 nM methotrexate, cell culture supernatants were collected and used for the isolation of recombinant TFPI-2 (rTFPI-2).

**Purification of TFPI-2**—rTFPI-2 was purified from conditioned BHK medium by the method described under "Purification and Identification of TFPI-2." rTFPI-2 was identified by the retention time on the chromatogram, SDS-PAGE analysis, and the peptide sequence analysis (data not shown).

**MAPK Phosphorylation by MAPK Kinase**—Serum-starved cells in 24-well plates were exposed to 500 nM TFPI-2 in DMEM + 0.1% FCS. After incubation for the periods indicated in Fig. 6, the supernatant was removed. 100 μl of 10 molar Tris-HCl (pH 8.0), 1 mM EDTA, 2.5% SDS, and 5% mercaptoethanol was added to the well. The dissolved cell fractions were separated on 8–25% gels by SDS-polyacrylamide gel electrophoresis using Phast System. The proteins were then blotted onto nitrocellulose ( Hoeffer Scientific Instruments) by semi-dry electrobblotting with Phast Transfer (Amersham Pharmacia Biotech) for 30 min. The blots were blocked for 1 h with 10% bovine serum albumin in Tris-buffered saline (20 mM Tris-HCl (pH 7.6) and 157 mM NaCl). The blots were then washed five times in the same buffer containing 0.1% Tween-20. This washing was performed between each subsequent step. The blots were incubated sequentially with the monoclonal antibody (Promega Inc.) against phosphorylated MAPK (P44/ERK1 and P42/ERK2, which show no cross-activity with nonphosphorylated MAPK) diluted in Tris-buffered saline (25 ng/ml) for 1 h, with the biotinylated Fab'₂ rabbit anti-mouse immunoglobulin G (Serotec Ltd.) for 1 h, and with the streptavidin-alkaline phosphatase conjugate for 1 h. Finally, nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt were added to detect specific proteins, and the reaction was stopped by washing in distilled water.

**MAPK Activity**—Bovine aortic smooth muscle cells were plated in 60-mm dishes (Nalge Nunc) for 48 h, after which their growth was arrested with DMEM containing 0.1% FCS. After 48 h, fresh medium (DMEM + 0.1% FCS) and different concentrations of mitogen (TFPI-2) were added simultaneously to the growth-arrested cells. PD098059 was added 30 min before rTFPI-2 stimulation. After incubation, MAPK activity was measured using a P44/42 MAP kinase assay kit (New England Biolabs, Beverly, MA) according to the manufacturer's instructions.

**TABLE I**

| Purification step | Total activity | Specific activity | Recovery |
|-------------------|----------------|-------------------|----------|
| Concentrated HUVEC | 2,366,840 | 5.53 | 100       |
| Heparin affinity  | 863,848 | 49.06 | 36        |
| ProRPC HR5/10    | 92,872  | 2321.8  | 3.9       |

**FIG. 1**. Reverse-phase high performance liquid chromatography on a ProRPC HR5/10 column. Eluent from HiTrap Heparin column (5 ml) was concentrated by Centriprep 10 to 0.5 ml. This sample was injected into ProRPC HR5/10 column. The gradient is shown by the dotted line from 0.1% trifluoroacetic acid in 20% acetonitrile to 0.1% trifluoroacetic acid in 100% acetonitrile. The solid peak shows the cell proliferating activity for the growth-arrested bovine aortic smooth muscle cells. The flow rate was 1 ml/min, and the peak was monitored at 280 nm.
muscle cells. Bovine aortic smooth muscle cells were plated (2.0 × 10^6 cells) in 10-cm dish in DMEM + 0.1% FCS. After growth-arrested cells were exposed to rTFPI-2 in the presence of 0.1% FCS. rTFPI-2 (500 nM) was newly changed every 48 h. After 2, 4, and 6 days, cell numbers were evaluated with a hemocytometer. The control experiment was also shown. Data are the means ± S.E. (n = 4).

Mitogenic effect of rTFPI-2 on bovine aortic smooth muscle cells. Bovine aortic smooth muscle cells were plated (2.0 × 10^5 cells) in 10-cm dish in DMEM + 0.1% FCS. After 48 h, growth-arrested cells were exposed to rTFPI-2 in the presence of 0.1% FCS. rTFPI-2 (500 nM) was newly changed every 48 h. After 2, 4, and 6 days, cell numbers were evaluated with a hemocytometer. Data are the means ± S.E. (n = 4).

**RESULTS**

Purification and Identification of TFPI-2—Table I shows the step for purification of mitogen for growth-arrested bovine aor-
tic smooth muscle cells. In Fig. 1, results of ProRPC HR5/10 column chromatography are shown. The solid column shows the peak in cell growth activity. On SDS-PAGE analysis, the molecular mass of this peak was 32 kDa (Fig. 2B). The peptide sequence analysis revealed the N-terminal sequence to be DAAQEPTGNNAEI, which was identical to TFPI-2 or placental protein 5 (3, 5).

**rTFPI-2**—We produced the rTFPI-2 by using the mammalian expression vector in the BHK cells. The supernatant in the

**TABLE II**

|                      | Optical density |
|----------------------|-----------------|
| rTFPI-2 (500 nM)     | 0.90 ± 0.12     |
| rTFPI-2 (500 nM) +   | 0.88 ± 0.30     |
| anti-human PDGF-AB   |                 |
| antibody (10 µg/ml)  |                 |
| PDGF (100 ng/ml)     | 1.4 ± 0.24      |
| PDGF (100 ng/ml) +   | 0.2 ± 0.01*     |
| anti-human PDGF-AB   |                 |
| antibody (10 µg/ml)  |                 |

*p < 0.01, compared with PDGF by Dunnett's test.

**FIG. 5.** Mitogenic effect of rTFPI-2 on bovine aortic smooth muscle cells. Bovine aortic smooth muscle cells were plated (3,000 cells/well) in 96-well plates in DMEM + 0.1% FCS. After 48 h, growth-arrested cells were exposed to rTFPI-2 in the presence of 0.1% FCS. After 24 h, BrdUrd was added, and its incorporation into DNA was determined after 12 h. Data are the means ± S.E. (n = 6).

**FIG. 6.** Mitogenic effect of rTFPI-2 on bovine aortic smooth muscle cells. Bovine aortic smooth muscle cells were plated (3,000 cells/well) in 96-well plates in DMEM + 0.1% FCS. After 48 h, growth-arrested cells were exposed to rTFPI-2 in the presence of 0.1% FCS. After 24 h, BrdUrd was added, and its incorporation into DNA was determined after 12 h. Data are the means ± S.E. (n = 6).

**FIG. 7.** Phosphorylation of MAPK by MAPK kinase. Growth-arrested bovine aortic smooth muscle cells (5 × 10⁵ cells) were stimulated to 2 h with 500 nM rTFPI-2 in the presence of 0.1% FCS. Cells were analyzed for phosphorylated MAPK by Western blot analysis as described under "Experimental Procedures." PD098059 (100 µM) was pretreated 30 min before rTFPI-2 administration.

**FIG. 8.** Time course of MAPK activity stimulated by 500 nM rTFPI-2. Densitometric analysis was performed by NIH image. Data are the means ± S.E. (n = 4).

**FIG. 9.** Mitogenic effect of rTFPI-2 on bovine aortic smooth muscle cells. In Fig. 1, results of ProRPC HR5/10 column chromatography are shown. The solid column shows the peak in cell growth activity. On SDS-PAGE analysis, the molecular mass of this peak was 32 kDa (Fig. 2B). The peptide sequence analysis revealed the N-terminal sequence to be DAAQEPTGNNAEI, which was identical to TFPI-2 or placental protein 5 (3, 5).

**rTFPI-2**—We produced the rTFPI-2 by using the mammalian expression vector in the BHK cells. The supernatant in the
transfected BHK was collected and purified by the same method employed for the cultured HUVEC. Results of SDS-PAGE of the purified rTFPI-2 are presented in Fig. 2C.

**Mitogenic Effect of rTFPI-2 on the Growth-arrested Bovine Aortic Smooth Muscle Cells**—Fig. 3 shows the effect of rTFPI-2 on the cell proliferation of the growth-arrested bovine aortic smooth muscle. In a dose-dependent manner (1–500 nM), rTFPI-2 increased the cell growth. Fig. 4 shows the time course of the cell proliferation. Cell counts increased from day 0 to day 6. To confirm the mitogenic character of rTFPI-2, we assayed BrdUrd incorporation into DNA. In Fig. 5, BrdUrd was found to be incorporated into DNA by rTFPI-2 stimulation dose-dependently. In the Fig. 6 experiment, instead of 0.1% FCS, 10 ng/ml PDGF was used. In the presence of PDGF, the increased incorporation of BrdUrd was also observed.

**Effect of Anti-Human PDGF-AB Antibody on rTFPI-2 or PDGF-induced BrdUrd Incorporation into Smooth Muscle Cell**—BrdUrd incorporation by rTFPI-2 was not inhibited by anti-human PDGF-AB antibody. PDGF-induced BrdUrd incorporation was inhibited by anti-human PDGF-AB antibody (Table II).

**MAPK Activation and MAPK Activity**—To study the phosphorylation of MAPK in rTFPI-2-induced smooth muscle cell growth, the Fig. 7 experiment was done. Using an antibody specific for dually phosphorylated MAPK (P44/ERK1 and P42/ERK2), a rapid and transient phosphorylation was found to occur after the stimulation by rTFPI-2. A specific inhibitor of MAPK kinase, PD098059 (100 μM), inhibited these phosphorylations. Fig. 8 shows the time course of MAPK activity stimulated by rTFPI-2 PD098059 inhibited MAPK activity and BrdUrd incorporation in a dose-dependent manner (Fig. 9).

**Transcriptional Activation of c-fos by rTFPI-2 and Inhibition by PD098059**—rTFPI-2 increased the c-fos promoter activity by luciferase assay. PD098059 was found to inhibit the promoter activity (Fig. 10).

**Effect of rTFPI-2 on c-fos Expression and the Inhibition by PD098059**—Northern blotting analysis was done to study the induction of proto-oncogene c-fos (Fig. 11). After stimulation with rTFPI-2, there was a rapid increase in c-fos expression. At 30 min, a rapid increase was found in c-fos. After 2 h, c-fos expression disappeared. This c-fos expression was inhibited dose-dependently by PD098059 (Fig. 12).

**DISCUSSION**

To study the effect of the endothelial cells on the smooth muscle cell growth, the following co-culture system was used; a culture insert with a 1.0 μM membrane (3102, Becton Dickinson) along with cultured HUVEC was placed over bovine aortic smooth muscle cells grown in 6-well micro-test plates (3502, Becton Dickinson). When endothelial cells existed in the insert, the smooth muscle cell growth was found to be stimulated (data not shown). Based on this finding, we purified the mitogenic substance from the conditioned medium of the cultured HU-
Smooth muscle cells (10^8 cells) were incubated with 500 nM TFPI-2 and PD098059 in the presence of 0.1% FCS for 30 min, and Northern blot analysis was performed. PD098059 was pretreated 30 min before the experiment. Expression of c-fos and the housekeeping glucose-3-phosphate dehydrogenase (G3PD) was determined as described under "Experimental Procedures." Depicted is an autoradiogram that represents three independent experiments.

Inhibition of c-fos expression by PD098059. Growth-arrested smooth muscle cells (10^8 cells) were incubated with 500 nM TFPI-2 and PD098059 in the presence of 0.1% FCS for 30 min, and Northern blot analysis was performed. PD098059 was pretreated 30 min before the experiment. Expression of c-fos and the housekeeping glucose-3-phosphate dehydrogenase (G3PD) was determined as described under "Experimental Procedures." Depicted is an autoradiogram that represents three independent experiments.

FIG. 12. Inhibition of c-fos expression by PD098059. Growth-arrested smooth muscle cells (10^8 cells) were incubated with 500 nM TFPI-2 and PD098059 in the presence of 0.1% FCS for 30 min, and Northern blot analysis was performed. PD098059 was pretreated 30 min before the experiment. Expression of c-fos and the housekeeping glucose-3-phosphate dehydrogenase (G3PD) was determined as described under "Experimental Procedures." Depicted is an autoradiogram that represents three independent experiments.

TFPI-2 has not been reported previously. To elucidate the mechanism of the mitogenic activity of TFPI-2, we studied the signal transduction pathway for smooth muscle cell growth. TFPI-2 activated the 44- and 42-kDa MAPK rapidly and transiently (Fig. 7). MAPK/ERK is a key intermediate in a signal transduction pathway that links many types of cell surface receptors with nuclear events that initiate mitosis (12–14). TFPI-2 induced the activation of c-fos promoter (Fig. 10) and subsequent rapid and transient expression of c-fos mRNA (Fig. 11). PD098059, a specific inhibitor of MAPK kinase inhibited dose-dependently the activation of MAPK (Figs. 7 and 9), c-fos promoter activation (Fig. 10), and expression of c-fos mRNA (Fig. 12). These data suggest that TFPI-2 stimulates cell proliferation through MAPK activation and subsequent c-fos expression.

Vascular smooth muscle cell growth is a key event in atherogenesis and the restenosis after percutaneous transluminal coronary angioplasty. In animal experiments, inhibition of TFPI-mediated coagulation with rTFPI has been reported to be effective in preventing neointimal formation and stenosis (6, 7, 15). Thrombin is a potent mitogen for smooth muscle cells. TFPI is an inhibitor of factor Xa alone or factor VIIa-TF complex in the presence of factor Xa. Factor Xa and factor VIIa-TF cause thrombin generation. Thus, the inhibitory action of cell proliferation and restenosis by rTFPI is considered due to the reduced thrombin generation secondary to inhibition of VIIa/TF and factor Xa. After balloon angioplasty, TFs exposed on the luminal surface of the vessel and factor X activation seem to play an important role in thrombus formation and the generation of thrombin. As a mechanism of prevention of restenosis by rTFPI, the direct inhibitory effect of rTFPI on the proliferation was proposed using cultured human neonatal aortic smooth muscle cells (8). The following mechanisms are proposed for mitogenic activity of rTFPI-2: (i) rTFPI-2 directly binds to its receptor on smooth muscle cell and stimulates the cell proliferation and (ii) similar to factor Xa (16), TFPI-2 functions via the stimulation of PDGF. However, this second possibility seems to be unlikely, because BrdUrd incorporation was not inhibited by anti-human PDGF-AB neutralizing antibody (Table II).

In conclusion, TFPI has been reported to exhibit antiproliferative action against vascular smooth muscle after arterial injury in addition to the inhibition of the activation of the extrinsic coagulation cascade. However, the structurally similar TFPI-2 has been found to be mitogenic for smooth muscle cell. There may be a new mechanism by which these two peptides regulate smooth muscle cell proliferation.

REFERENCES
1. Ross, R. (1993) Nature 362, 801–809
2. Jesty, J., Wun, T. C., and Lorenz, A. (1994) Biochemistry 33, 12686–12694
3. Sprecher, C. A., Kusiel, W., Mathewes, S., and Foster, D. C. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 3353–3357
4. Peterson, L. C., Sprecher, C. A., Foster, D. C., Blumberg, H., Hamamoto, T., and Kusiel, W. (1996) Biochemistry 35, 266–273
5. Miyagi, Y., Koshikawa, N., Yasumitsu, H., Miyagi, E., Hirahara, F., Aoki, I., Misugi, K., Umeda, M., and Miyazaki, K. (1994) J. Biochem. (Tokyo) 116, 939–942
6. Jang, Y., Guzman, L. A., Lincoff, M., Gottsaman-Wolf, M., Forudi, F., Hart, C. E., Courtman, D. W., Ezban, M., Ellis, S. G., and Topol, E. J. (1995) Circulation 92, 3041–3050
7. Oltra, L., Speidel, C., Rech, D., Wiekline, S. A., Eisenberg, P. R., and Abendschein, D. R. (1997) Circulation 96, 646–652
8. Kamikubo, Y., Nakahara, Y., Takekota, S., Hamuro, T., Miyamoto, S., and Funatsu, A. (1997) FEMS Lett. 407, 116–120
9. Seichiru, W., Miyahara, K., Toda, K., Ogoshi, S., Ochi, S., Mitsui, Y., Yui, Y., Kawai, C., and Shizuta, Y. (1995) Biochem. Biophys. Res. Commun. 216, 729–735
10. Shirou, M., Yui, Y., Hattori, R., and Kawai, C. (1990) J. Pharmacol. Exp. Ther. 299, 738–744
11. Nidome, T., Teramoto, T., Murata, Y., Tanaka, J., Seto, T., Sawada, K., Mori, Y., and Katayama, K. (1994) Biochem. Biophys. Res. Commun. 203, 1821–1827
12. Straeten, F. S., Muller, R., Currin, T., Beveren, C. V., and Verma, I. M. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 3183–3187
13. Muller, J. M., Krauss, B., Kaatsch, M., Bauere, P. A., and Rupke, R. A. (1997) J. Biol. Chem. 272, 23435–23439
14. Yamakawa, T., Eguchi, S., Yamakawa, Y., Motley, E. D., Numaguchi, K., Utsunomiya, H., and Inagami, T. (1998) Hypertension 31, 248–253
15. Brown, D. M., Kaina, N. M., Choi, E. T., Lantieri, L. A., Pasia, E. N., Wun, T. C., and Khouri, R. K. (1996) Arch. Surg. 131, 1086–1090
16. Ko, P. N., Yang, Y. C., Huang, S. C., and Ou, J. T. (1996) J. Clin. Invest. 98, 1483–1501