Human recombinant tissue factor pathway inhibitor induces apoptosis in cultured human endothelial cells

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Abstract Tissue factor pathway inhibitor (TFPI) is mainly synthesized in vascular endothelial cells and exhibits a strong and specific inhibitory activity against tissue factor-mediated blood coagulation. In the present study, we demonstrate that human recombinant TFPI (h-rTFPI) inhibits the growth of cultured human umbilical vein endothelial cells (HUVECs) by inducing apoptosis. In a growth-rate assay of HUVECs, the growth of the cultured HUVECs is completely abolished by the addition of 1 μM h-rTFPI to the culture medium containing fetal bovine serum (FBS), basic fibroblast growth factor, and epidermal growth factor. In addition, h-rTFPI and h-rTFPI-C which lacks the carboxyl-terminal basic region prevent the survival of growth-arrested HUVECs which are starved in a medium containing 2% FBS alone, suggesting that h-rTFPI directly induces the death of these HUVECs. This hypothesis is supported by the finding that h-rTFPI does not inhibit the synthesis of DNA in HUVECs during proliferation, as shown by a 5-bromo-2′-deoxyuridine (BrdU) incorporation assay. Furthermore, Giemsa staining and a gel electrophoretic analysis of DNA fragmentation show that the HUVEC death mediated by h-rTFPI has the typical characteristics of apoptosis. However, the apoptosis in HUVECs is considerably inhibited in the presence of 1 μg/ml of the protein synthesis inhibitor, cycloheximide. Therefore, the process of apoptosis triggered by h-rTFPI is, at least in part, actively conducted by the cells.

Key words: Tissue factor pathway inhibitor; Human umbilical vein endothelial cell; Apoptosis

1. Introduction

Embryonic vascular development involves a complex series of events during which endothelial cells differentiate, proliferate, and undergo morphological organization in the context of their surrounding tissues [1]. Two successive processes, vasculogenesis and angiogenesis, are thought to contribute to embryonic vascular development [2,3]. Vasculogenesis is the de novo organization of blood vessels by the in situ differentiation of endothelial cells from the mesoderm. Angiogenesis is the budding and branching of vessels from pre-existing vessels. Angiogenesis in adult humans accounts for the neovascularization that accompanies the normal processes of ovulation, placental development and wound healing, as well as various clinically significant pathological processes such as tumor growth and diabetic retinopathy [4,5], while vasculogenesis appears to be a mechanism unique to embryonic development. The basic mechanisms underlying vasculogenesis and angiogenesis have been studied, and several factors with mitogenic activity have been identified [6–8]. However, the degeneration and regression of blood vessels are also thought to be equally important for the early development of the vascular system [9], since the minority of blood vessels formed during embryonic development probably persists until adulthood and most capillaries in the primitive vascular plexus regress. In addition, wound capillaries regress after wound healing. The molecular mechanisms of the regression and degeneration of vascular endothelial cells are not yet understood. We speculate that the regression of blood vessels involves induced endothelial cell death or apoptosis caused by unknown factors. In the present study, we analyze a death factor of cultured vascular endothelial cells which might play a positive role in enhancing the vascular degeneration and the regression.

Tissue factor pathway inhibitor (TFPI) is a Kunitz-type protease inhibitor which exhibits a strong and specific inhibitory activity against the tissue factor (TF)-mediated initiation of the blood coagulation cascade [10]. It is thought that TFPI plays a significant role in vivo in the regulation of blood coagulation triggered by TF expressed on vascular endothelial cells, because TFPI is synthesized mainly in vascular endothelial cells and bound to the vessel wall [11–14]. It has been suggested that TFPI is bound to glycosaminoglycans (GAGs) on the endothelium, and that heparin competes with this binding [15,16]. Iversen et al. [17] recently reported that human recombinant TFPI (h-rTFPI) binds to cultured human umbilical vein endothelial cells (HUVECs) with a dissociation constant (K_d) of 0.164 μM and that the binding does not involve GAGs. They further revealed that other unidentified molecules on HUVECs are responsible for the interaction of TFPI with HUVECs. The interaction of GAGs with TFPI is important for enhancing the inhibitory activity of TFPI against TF-induced coagulation [18]. We recently discovered that the interaction of h-rTFPI with cultured human smooth muscle cells (hSMCs), mediated via the carboxyl-terminal region, is responsible for the anti-proliferative action of h-rTFPI [19]. Therefore, it seems likely that h-rTFPI bound to endothelial cells might have some other function besides as a protease inhibitor. However, it is unknown whether TFPI has a direct action on vascular endothelial cells.

The aim of the present study was to investigate the effect of h-rTFPI on the growth of vascular endothelial cells in a culture. We found that h-rTFPI completely inhibited the growth of HUVECs and that its action was dependent on the induction of cell death, showing the typical characteristics of apoptosis but not necrosis. These results suggest that TFPI plays a significant role in regulating the development of the vascular system and in enhancing the degeneration and regression mediated via the induction of apoptosis in vascular endothelial cells.

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2. Materials and methods

2.1. Materials

The h-rTFPI was isolated from the culture medium of transformed chinese hamster ovary (CHO) cells with human TFPI cDNA by immunoaffinity chromatography using its monoclonal antibody (HTFPIK-9)-conjugated Cellulofine (Seikagaku Co., Tokyo, Japan), as described [20]. For the separation of h-rTFPI from h-rTFPI-C, which lacks the carboxyl-terminal basic region in TFPI, we performed an affinity chromatography on a heparin-Sepharose 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, whereas h-rTFPI-C ended at Lys^{249} [21]. The h-rTFPI concentration was determined by measuring the inhibition of human factor Xa activity after the incubation of factor Xa with serially diluted h-rTFPI [22]. A synthetic peptide corresponding to Lys^{254}-Met^{276} in TFPI was purchased from Sawady Biotechnology Co. (Tokyo, Japan). The concentration of the peptide was determined by an amino acid analysis. The monoclonal antibody (HTFPIK-9) against human TFPI was purified by the previously described methods [23]. The following materials were obtained from the listed companies: crystallized bovine serum albumin (BSA), cycloheximide (Sigma Chemical Co., St. Louis, MO, USA), 0.025% trypsin containing 0.01% EDTA (trypsin/EDTA) (Kurabo Co., Osaka, Japan), and 0.4% trypan blue (Flow Laboratories, Irvine, Scotland). All other chemicals, reagent grade or better, were from Wako Pure Chemical Industries (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan).

2.2. HUVEC cultures

The HUVECs were purchased from Kurabo. The cells were isolated from human umbilical cord veins and identified by an immunological analysis of von Willebrand factor. The HUVECs were cultured in the growth medium HuMedia-EG2 (Kurabo), and grown at 37°C in a humidified atmosphere at 5% of CO_2 in air. The HuMedia-EG2 medium consisted of the base medium (HuMedia-EB2) supplemented with 2% fetal bovine serum (FBS), 10 ng/ml recombinant epidermal growth factor (rEGF), 5 ng/ml recombinant basic fibroblast growth factor (r-bFGF), 10 μg/ml heparin, and 1 μg/ml hydrocortisone. Confluent adherent HUVECs were detached from 75 cm^2 flasks (Iwaki, Chiba, Japan) by exposure to a trypsin/EDTA solution at room temperature, and the detached cells (routinely over 98% viable as assessed by trypan blue assay) were then suspended in the HuMedia-EG2 medium. HUVECs were routinely used from the fourth to the fifth passage.

2.3. Assays of HUVEC growth

Three hundred microliters of HUVEC suspension was plated in a 48-well plate (Iwaki) at 2.5×10^3 cells per well and cultured in HuMedia-EG2 medium. At day 2, the culture medium was changed to a fresh HuMedia-EG2 medium containing various concentrations of h-rTFPI. The medium was changed thereafter every 2 days. For the growth-rate determination, cells were trypsinized with 100 μl of a trypsin/EDTA solution and resuspended in 4.7 ml of ISOTON-II (Japan Scientific Instruments Co., Osaka) following the neutralization of trypsin by 200 μl of FBS (Kurabo). Then, the number of cells in suspension was determined by a Coulter counter (Japan Scientific Instruments).

2.4. Preparation of growth-arrested HUVECs

For the preparation of growth-arrested HUVECs, HUVECs were starved in a growth factor-depleted medium. In brief, HUVECs were seeded at 12×10^3 cells per well in a 48-well plate and cultured in growth factor-depleted medium. After 2 days, the medium was replaced by fresh growth factor-depleted medium, and the HUVECs were further cultured for 2 days. The growth factor-depleted medium contained HuMedia-EB2 and 2% FBS, but not rEGF or r-bFGF. To determine whether h-rTFPI would induce the death of HUVECs, adherent cells were trypsinized and counted in a Coulter counter at 2 days after the cultivation in the presence of varying concentrations of h-rTFPI.

2.5. DNA synthesis assays

To test the effect of h-rTFPI on the DNA synthesis in HUVECs, we performed a 5-bromo-2′-deoxyuridine (BrdU) incorporation assay with a commercial quantification kit (Boehringer Mannheim, Tokyo) according to the manufacturer's protocol. Briefly, 100 μl of HUVEC suspension was plated in a 96-well plate (Becton Dickinson, Lincoln Park, NJ, USA) at 5×10^3 cells per well and allowed to attach to the culture plate. At 4 h, the culture medium was changed to a fresh HuMedia-EG2 medium containing h-rTFPI (1 μM), and the HUVECs were further cultured for 24 h. BrdU was added to the culture medium at 0, 6, 12, and 18 h after an addition of h-rTFPI, and incubated for 6 h for labeling the HUVECs. After labeling, the BrdU-treated cells were fixed and the DNA was denatured. The BrdU incorporated into newly synthesized cellular DNA was reacted with a peroxidase-conjugated anti-BrdU antibody, and the immune complexes were quantified by the subsequent substrate reaction. The amount of reaction product was determined by measuring the absorbance at 450 nm using a plate reader (THERMOmax, Molecular Devices Co., Menlo Park, CA, USA). The absorbance value was directly correlated to the amount of DNA synthesis and to the number of proliferative cells.

2.6. Estimation of apoptotic HUVECs

For investigating whether h-rTFPI triggers apoptosis in HUVECs, we performed an estimation of apoptotic HUVECs by using the Giemsa staining method. To obtain dead cells, adherent HUVECs were incubated with h-rTFPI in a HuMedia-EG2 medium for 2 days, and then floating cells in the medium were collected. These cells were centrifuged and used to estimate the number of apoptotic cells. To assess the cells' viability, 1 volume of trypan blue was added to 1 volume of the cells, and the viable cells were then counted in a hemocytometer. The dead cells were stained with Giemsa (Merck, Darmstadt, Germany), and were examined over 1×10^5 cells per slide by oil immersion light microscopy at a final magnification of 250. Apoptotic cells were identified according to the following criteria [24]: condensed and fragmented nuclei, and decrease in cell size.

2.7. Detection of DNA fragmentation

For further investigating HUVEC apoptosis induced by h-rTFPI, fragmented and intact DNA in dead cells was evaluated by means of an apoptosis ladder detection kit from Wako Pure Chemical Industries according to the manufacturer's protocol. Briefly, after the HUVECs floating in medium (8×10^5) were collected and centrifuged, the DNA in the cells was extracted. The DNA samples were combined with loading buffer and electrophoresed in a 1.5% agarose gel. The

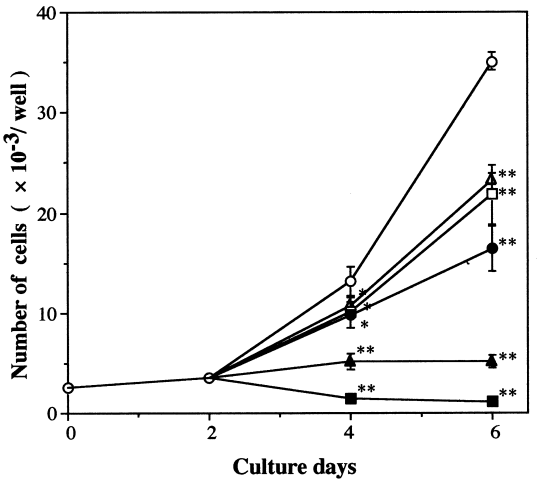

Fig. 1. Inhibition of HUVEC growth by h-rTFPI. Cells were seeded in 48-well plates at 2.5×10^3 cells per well, and the culture medium was changed at 48 h to a fresh growth medium containing various concentrations of h-rTFPI. The medium was changed every 2 days thereafter. For the growth rate determination, cells were detached from 4 wells and counted in a Coulter counter. Each point indicates the mean cell number ± S.D. ($n=4$). The data for each culture day represent a significant difference from the data for no h-rTFPI by unpaired Student's t-test; $^*P<0.05$, $^{**}P<0.01$. (○) No h-rTFPI; (△) 0.13 μM h-rTFPI; (□) 0.25 μM h-rTFPI; (●) 0.5 μM h-rTFPI; (▲) 1 μM h-rTFPI; (■) 2 μM h-rTFPI.

DNA was visualized by UV examination and photographed with a Polaroid camera. For comparison, adherent cells cultured in the absence of h-rTFPI were trypsinized, and the DNA in these cells was also analyzed.

2.8. Statistics

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

3. Results

3.1. Inhibition of the growth of HUVECs by h-rTFPI

We investigated the effect of varying concentrations of h-rTFPI on the growth of cultured HUVECs. As shown in Fig. 1, the growth of HUVECs cultured in growth medium was significantly inhibited by the addition of h-rTFPI in a dose-dependent fashion at day 4 and 6, and 1 μM h-rTFPI completely abolished the growth of HUVECs in culture. We also observed morphological changes in h-rTFPI-treated HUVECs, wherein cells rounded up and floated away into the medium. When we compared the number of cells treated by 2 μM h-rTFPI with that before the addition of h-rTFPI (corresponding to the number of cells at day 2), it was clear that the number of h-rTFPI-treated cells was much lower than that of the non-treated cells, suggesting that h-rTFPI induced cell death. Similar tests of h-rTFPI on the growth of bovine aortic endothelial cells also showed a significant inhibitory effect, while in human fibroblasts, h-rTFPI had no significant effect (data not shown).

3.2. The effect of h-rTFPI on the survival of growth-arrested HUVECs

We also examined the effect of h-rTFPI on the survival of growth-arrested HUVECs in order to clarify whether the inhibitory effect of h-rTFPI on HUVEC growth contributed to the inhibition of the proliferation of HUVECs by exogenously added growth factors such as bFGF and EGF. In this experiment, we used growth-arrested HUVECs which were starved in HuMedia-EB2 supplemented with 2% FBS. As a result, the number of adherent cells (corresponding to survived cells) was significantly decreased in a dose-dependent manner by the addition of h-rTFPI to starved HUVECs, indicating that the survival of the growth-arrested HUVECs was inhibited by h-rTFPI (Fig. 2). We thus noted that it was possible that h-rTFPI induced the death of the HUVECs and that the h-rTFPI was mainly responsible for the inhibitory effect on HUVEC growth. This possibility was further supported by the BrdU incorporation assay finding that h-rTFPI did not inhibit the synthesis of DNA in HUVECs during proliferation, indicating that h-rTFPI has no direct anti-mitogenic activity toward the HUVECs stimulated by growth factors (Fig. 3). To evaluate the functional domain in h-rTFPI, we tested the effect of h-rTFPI-C and the Lys^{254}-Met^{276} peptide on the survival of HUVECs. As shown in Fig. 4, h-rTFPI-C significantly inhibited the survival of HUVECs, whereas the Lys^{254}-Met^{276} peptide did not.

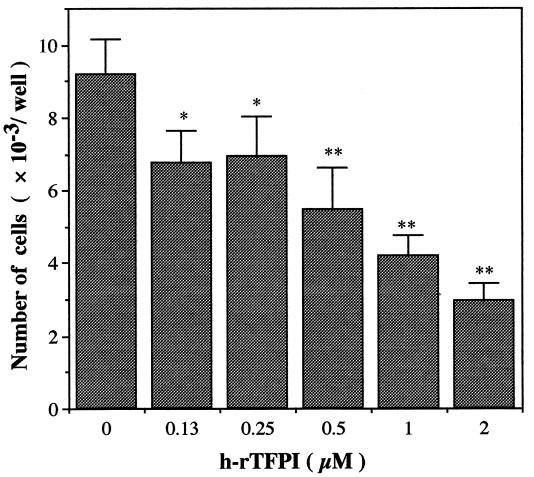

Fig. 2. The effect of h-rTFPI on the survival of growth-arrested HUVECs. HUVECs were seeded at 12×10^3 cells per well in a 48-well plate and cultured in growth factor-depleted medium which contained HuMedia-EB2 and 2% FBS. After 2 days, the medium was replaced by fresh growth factor-depleted medium supplemented with varying concentrations of h-rTFPI, and the HUVECs were further cultured in the presence of h-rTFPI for 2 days. After cultivation, adherent cells were trypsinized and counted in a Coulter counter. Each bar indicates the mean of the survived cell number ± S.D. ($n = 4$). *$P < 0.05$ or **$P < 0.01$ vs. no h-rTFPI.

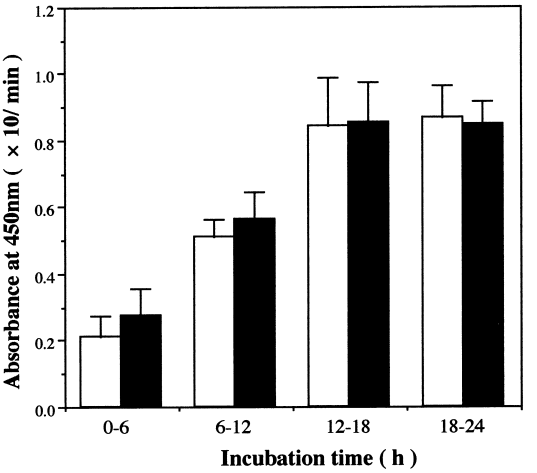

Fig. 3. No effect of h-rTFPI on DNA synthesis in HUVECs. To test the effect of h-rTFPI on DNA synthesis in HUVECs, we performed a BrdU assay as described under Section 2. The absorbance value at 450 nm directly correlated to the amount of DNA synthesis. Each point indicates the mean absorbance ± S.D. ($n = 6$). Open bars, no h-rTFPI; solid bars, 1 μM h-rTFPI.

3.3. h-rTFPI-dependent induction of apoptotic cell death

The Giemsa staining and gel electrophoretic analysis of DNA fragmentation were used to determine whether h-rTFPI triggers apoptosis in HUVECs. These morphological and biochemical analyses demonstrated the typical characteristics of apoptosis in the HUVECs incubated with 2 μM h-rTFPI. As shown in Fig. 5A, the HUVECs floating in the medium after treatment by h-rTFPI showed highly condensed and fragmented nuclei, and a decrease in cell size. The electrophoretic analysis demonstrated the characteristic pattern of fragmentation of DNA in multiples of 180–200 bp (Fig. 5B). These results suggest that the HUVEC death induced by h-rTFPI was apoptosis. Apoptosis is an active process that requires macromolecular synthesis, and the apoptosis in vascular endothelial cells is inhibited by a protein synthesis inhibitor [25]. When we cultured HUVECs in the simultaneous presence of h-rTFPI and cycloheximide (1 μg/ml), cycloheximide augmented the survival of cells at day 2 (Table 1) and the process of apoptosis triggered by h-rTFPI was prevented to a consid-

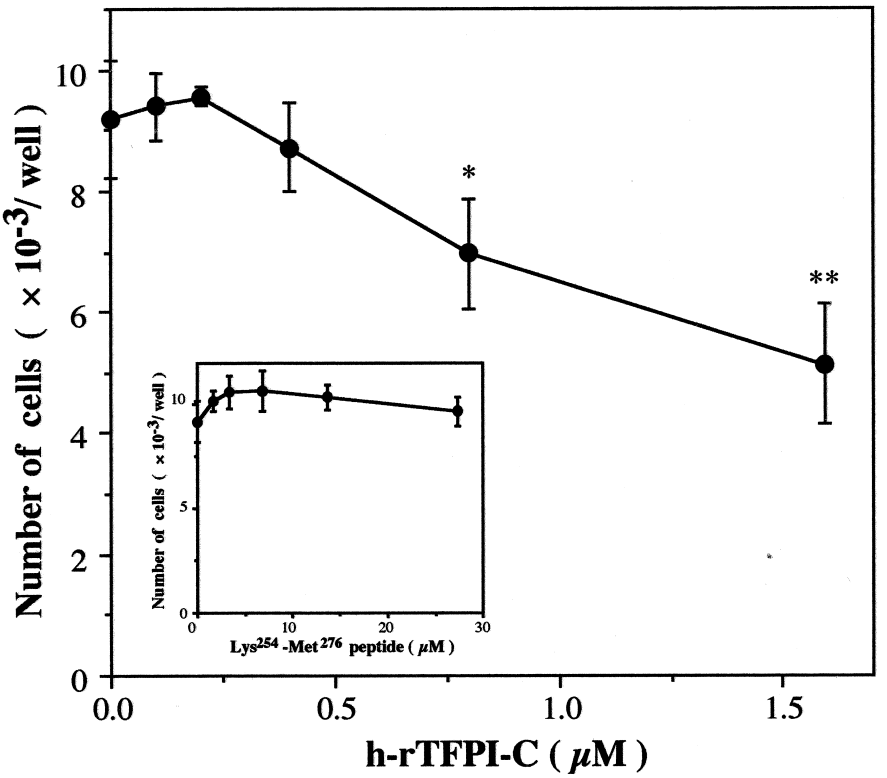

Fig. 4. The effect of h-rTFPI-C and the Lys^{254}-Met^{276} peptide on the survival of HUVECs. The effect of h-rTFPI-C and the Lys^{254}-Met^{276} peptide (inset) on the survival of HUVECs were investigated by the methods described in the legend to Fig. 2. Each point indicates the mean of the survived cell number ± S.D. ($n = 4$). *$P < 0.05$ or **$P < 0.01$ vs. no h-rTFPI-C.

erable degree, suggesting that the apoptosis was, at least in part, actively conducted by the cells. Thus, the treatment of HUVECs with h-rTFPI resulted in an active process of cell death with the morphological and biochemical characteristics of apoptosis.

4. Discussion

Apoptosis or programmed cell death is a form of cell death wherein the cells participate in their own demise; this process is morphologically and biochemically different from necrosis, and occurs under physiologic and pathophysiologic conditions [26]. Briefly, the areas in which apoptosis plays an important role can be divided as follows: (1) during embryological and fetal development (e.g. the cavitation of the embryo and limb bud development) [27]; (2) in normal homeostasis (e.g. the turnover of intestinal epithelial cells) [27]; and (3) in disease states [26]. Various factors or stimuli that induce apoptosis in vascular endothelial cells have been identified, e.g. tumor necrosis factor-α (TNF-α) [28,29], and high glucose concentration [30]. The deprivation of a growth factor such as bFGF also induces apoptosis in endothelial cells [31,32]. The apoptosis of endothelial cells induced by these factors is generally thought to contribute to vascular pathology, including atherosclerosis and inflammation [28,33]. Bombeli et al. [34] have shown that apoptotic endothelial cells become procoagulant, suggesting that the apoptotic cells may play a role in the development of thrombotic events.

Endothelial cell apoptosis is also physiologically significant in normal tissue homeostasis and embryological development [9,29]. However, the molecular mechanism of apoptosis remains poorly understood. In the present study, we investigated a death factor in vascular endothelial cells which may play a positive role in enhancing vascular degeneration and regression. We observed that this factor, TFPI, was different from the above apoptosis-inducing factors. Our experiments showed that h-rTFPI inhibits the growth of cultured HUVECs and that its action contributed to the apoptotic cell death which is mainly involved in de novo protein synthesis. Iversen et al. [17] recently demonstrated that h-rTFPI binds to cultured HUVECs with K_d value of 0.164 μM. When the concentrations of h-rTFPI used in this study were compared with the K_d value reported by Iversen et al., it was found that the concentrations of h-rTFPI required for apoptotic action are rather close to the K_d value (0.164 μM vs. 0.13 μM–2 μM). These results lead to our speculation that vascular en-

Table 1
Effect of cycloheximide on the cell death induced by h-rTFPI

h-rTFPI (μM)	Cycloheximide	Percent of viable cells (%)
0	−	100.0 ± 9.0
	+	100.0 ± 3.7
1	−	42.4 ± 5.2
	+	77.4 ± 8.9**
2	−	33.4 ± 10.2
	+	65.8 ± 3.8**

Growth-arrested HUVECs were prepared in growth factor-depleted medium and cultured in the presence of h-rTFPI and/or cycloheximide for 2 days, as detailed in Fig. 2. Cycloheximide was used at 1 μg/ml. After cultivation, adherent cells were trypsinized and counted in a Coulter counter. Each value indicates the percent of survived cells ± S.D. ($n = 4$).
**$P < 0.01$ vs. no cycloheximide. +, presence; −, absence.

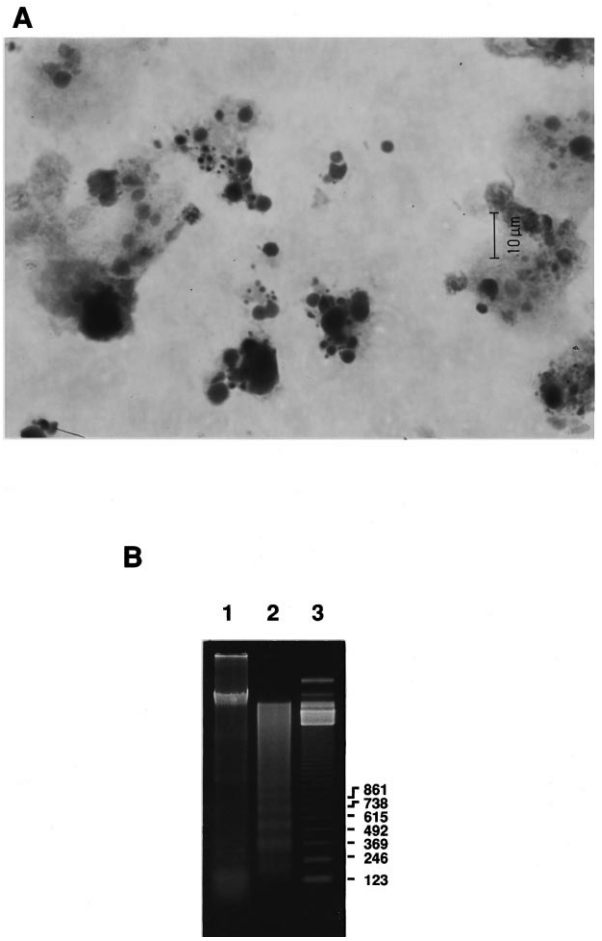

Fig. 5. Estimation of apoptotic HUVECs by using Giemsa staining and a gel electrophoretic analysis of DNA fragmentation. To determine whether h-rTFPI induces apoptosis in HUVECs, we performed the Giemsa staining and gel electrophoretic analysis of DNA fragmentation described in Section 2. A: Apoptotic HUVECs stained with Giemsa. B: Gel electrophoretic pattern of DNA fragmentation. Lane 1, DNA from living cells (adherent cells); lane 2, DNA from dead cells collected from the medium at 2 days after h-rTFPI treatment; lane 3, molecular weight markers. The molecular weights are expressed in terms of base pairs.

dothelial cells in which apoptosis is triggered by TFPI, are phagocytosed into neighboring phagocytic cells and then degraded, finally resulting in the elimination of the dying cells without the induction of the inflammatory and hypercoagulation processes, because TFPI prevents the hypercoagulation and inflammation [35]. We also discovered that h-rTFPI has an anti-proliferative effect on hSMCs [19]. Taken together, these findings suggest that TFPI plays a physiologically significant role in the establishment of the vascular system during embryological development and angiogenesis by inducing the apoptosis of vascular endothelial cells and by regulating hSMC growth.

On the basis of the present studies, h-rTFPI may transmit the direct death signal to cultured HUVECs, although neither the membrane receptor nor signalling system has been identified. TFPI is a Kunitz-type protease inhibitor which exhibits a strong and specific inhibitory activity against the coagulation factors VIIa-TF complex and Xa. However, it seems unlikely that the inhibitory activity of h-rTFPI is required for inducing the apoptosis in HUVECs, because the inhibitory activity of h-rTFPI was abolished after reduction and alkylation of h-rTFPI, but not the apoptotic action (data not shown). In addition, TFPI is a heparin-binding protein which binds via the carboxyl-terminal and third Kunitz domain [16,20]. Among the heparin-binding proteins, the significance of the anti-proliferative effect of platelet factor-4 on endothelial cells has been well established [36,37]. The effect is thought to be mainly attributable to the competition with the heparin-binding growth factors such as FGFs for cell surface GAGs. However, it seems unlikely that h-rTFPI can induce the apoptosis in HUVECs mediated by the interaction of h-rTFPI with GAGs, because the apoptotic action of h-rTFPI is observed in the presence of a high concentration of heparin (Fig. 1), and the Lys^{254}-Met^{276} peptide which contains GAG-binding region does not prevent the survival of HUVECs (Fig. 4). Therefore, it is possible that receptors other than GAGs on endothelial cells are involved in the apoptotic action of h-rTFPI, suggesting that the mechanism for endothelial cell apoptosis induced by TFPI is different from that for the anti-proliferative effect on hSMCs.

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