

# Protease Dependent Activation of Endothelial Cells by Peritoneal Dialysis Effluents

Michael Krebs<sup>1</sup>, Christoph Kaun<sup>1</sup>, Matthias Lorenz<sup>2</sup>, Marianne Haag-Weber<sup>2</sup>, Margarethe Geiger<sup>1</sup>, Bernd R. Binder<sup>1</sup>

From the <sup>1</sup>Department of Vascular Biology and Thrombosis Research and <sup>2</sup>Department of Medicine III, Division of Nephrology, University of Vienna, Vienna, Austria

## Key words

Peritoneal dialysis, endothelial cells, tissue factor, adhesion molecules

## Summary

Incubation of cultured human umbilical vein endothelial cells (HUVECs) with dilutions of peritoneal dialysis effluents (PDEs) from 11 individual patients undergoing continuous ambulatory peritoneal dialysis (CAPD) induced cellular procoagulant activity in a dose and time dependent manner. This procoagulant activity could be attributed to tissue factor (TF) expression since it was blocked by rabbit anti-TF IgG. These data were confirmed by FACS analysis yielding surface TF expression; In addition PDEs induced the expression of E-selectin in HUVECs. This TF and selectin inducing activity was heat labile and could be inhibited by protease inhibitors. Partial purification could be achieved using a benzamidine-Sepharose column. The TF inducing activity could not be attributed to LPS, IL-1, TNF- $\alpha$ , mast cell tryptase, active thrombin, or complement factor D. We therefore conclude that the peritoneal cavity contains a protease activity that induces a procoagulatory and proinflammatory phenotype in HUVECs.

## Introduction

Continuous ambulatory peritoneal dialysis (CAPD) is now an established alternative treatment for patients with chronic renal failure (1). One of the major concerns is the reduction in viability of the peritoneal membrane that has been repeatedly demonstrated even in the absence of peritonitis (2, 3). The high rate of intraperitoneal fibrin turnover in clinically stable CAPD patients (4, 5) may indicate a nonbacterial inflammatory state caused by the physical and chemical irritation of the peritoneum during CAPD (6). Furthermore, pathological changes in the peritoneal vasculature in CAPD patients have been linked to ultrafiltration failure (7). We were interested in identifying factors in peritoneal dialysis effluents (PDE) that might cause local endothelial activation in CAPD.

Endothelial cells (EC) form a dynamic barrier at the inside of blood vessels. Upon activation by cytokines or bacterial lipopolysaccharides (LPS), EC become procoagulant and proadhesive. These changes in the phenotype are mediated by the upregulated expression of tissue factor (TF) and adhesion molecules.

TF is a transmembrane glycoprotein that serves as the principal initiator of coagulation in vivo by binding blood clotting factor VII/VIIa and subsequent activation of factors X and IX (for review see 8). TF expression in EC is controlled largely on the transcriptional level (9) and can be induced in these cells by inflammatory mediators [tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) (10), interleukin-1 (IL-1) (11), LPS (12)], complement components (13, 14), vascular endothelial growth factor/vascular permeability factor (15), and angiotensin II (16). Furthermore it has been shown that thrombin leads to upregulation of TF in EC via the thrombin receptor (17). This receptor belongs to the family of protease-activated receptors that are activated by proteolytic cleavage of their extracellular amino terminus (18). The thrombin receptor and the protease-activated receptor 2 (PAR-2) are expressed on EC (19).

When activated by specific agonists such as inflammatory cytokines and LPS, EC bind more white blood cells due to upregulation of cell surface adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) (20), vascular cell adhesion molecule-1 (VCAM-1) (21) and the endothelial selectins, E-selectin and P-selectin (22). These proteins interact with leukocyte receptors and act at different steps of white blood cell extravasation (reviewed in 23). It has been shown, that also thrombin and thrombin receptor-activating peptide stimulate E-selectin expression and monocytic cell adhesion in EC (24).

We report here for the first time that an activity in peritoneal fluid causes a marked increase in TF-activity and TF surface antigen expression in human umbilical vein endothelial cells (HUVECs). We also show, that endothelial selectins are upregulated in response to PDE, while VCAM and ICAM expression is not affected. These effects can be attributed in part to a protease activity contained in PDEs.

## Materials and Methods

### Materials

Rabbit anti-human complement factor D-IgG, used as irrelevant IgG, was prepared following standard protocols by immunizing a New Zealand white rabbit with purified complement factor D. IgG was purified from the serum of this rabbit as described (25). Phosphate buffered saline (PBS) contained 2 mmol/l KH<sub>2</sub>PO<sub>4</sub>, 8 mmol/l Na<sub>2</sub>HPO<sub>4</sub>, and 140 mmol/l NaCl (all: Merck, Darmstadt, Germany) at pH of 7.4. Human recombinant TNF $\alpha$  was obtained from Boehringer Mannheim (Mannheim, Germany). Other materials used in the methods described below are specified in the respective sections.

### Cell Culture

Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as described previously (26). In all experiments only cells between passage 2 and 4 were used. Prior to experiments, EC were densely seeded in gelatin coated 6-, 24-, or 96-well plates (Corning, New York) and kept in medium 199 (M199; Sigma, St. Louis, MO) supplemented with 20% heat inactivated supplemented calf serum (SCS; Hyclone, Logan, UT) and antibiotics for 24 h.

Correspondence to: Dr. Margarethe Geiger, Department of Vascular Biology and Thrombosis Research, University of Vienna, Schwarzspanierstraße 17, A-1090 Vienna, Austria – Tel.: +43 1 4277 62506; Fax: +43 1 42 77 96 25; E-mail: Margarethe.Geiger@univie.ac.at

### Peritoneal Dialysis Effluents and Peritoneal Fluids

Peritoneal dialysis effluents (PDEs) were obtained from patients [n = 11, 7 men and 4 women, mean age 60 years (range 44 to 78)] receiving CAPD treatment for end-stage kidney failure. Nine of them had renal hypertension and 3 subjects suffered from coronary or peripheral vascular disease. These patients had no signs of infection as judged from the absence of clinical symptoms, leukocyte count in blood (<10 G/l) and dialysis effluents (<0.1 G/l), and serum CRP levels (<1 mg/100 ml). Six to 14 h after intra-peritoneal infusion of 2 l of fresh peritoneal dialysis solution (Baxter Healthcare Ltd, Thetford, Norfolk, UK or Fresenius, Bad Homburg, Germany) containing only low molecular weight substances, low glucose concentration, and no other additives, the PDEs were drained from the patients and stored in aliquots at -70° C until used. To control for effects caused by cells or cell debris in the dialysis effluents 10 ml aliquots of fresh PDE were centrifuged (1500 × g for 30 min) immediately after the harvest. After centrifugation the supernatant was carefully removed and the pellets were separately stored frozen in approximately 50 µl of remaining peritoneal fluid.

Informed consent was obtained from all patients after the purpose, nature, and potential risks of the procedure were explained to them.

Dialysis of PDE was performed against PBS over night at 4° C using dialysis tubing (12-14 kD molecular weight cut off; Visking; Serva-Boehringer Ingelheim Bioproducts Partnership, Heidelberg, Germany).

### Assay for TF Procoagulant Activity

Confluent HUVECs grown in 24-well plates were incubated with 500 µl PDE or PBS diluted 1:4 or 1:5 with M199 (supplemented with 4% SCS and antibiotics) at 37° C in a 5% CO<sub>2</sub> atmosphere. At the time points indicated cells were washed twice with PBS; remaining liquid was carefully removed from the wells and the cells were lysed by repeated freezing and thawing. Thereafter the HUVEC lysate was suspended in 210 µl of clotting buffer (12 mmol/l sodium acetate, 8 mmol/l barbital sodium, 130 mmol/l sodium chloride, pH 7.4) at room temperature. The cell lysates were assayed in a one-stage clotting assay for TF activity. 100 µl of cell lysates were incubated with 100 µl of prewarmed pooled citrated normal plasma or coagulation factor X-deficient plasma (Sigma, St. Louis, MO) for 1 min at 37° C in prewarmed plastic tubes. Clotting was initiated by the addition of 100 µl of prewarmed CaCl<sub>2</sub> (20 mmol/l) and clotting time was measured using a coagulometer (Amelung, Lemgo, Germany). TF activity was quantified by means of a standard curve (log-log plot) constructed with rabbit brain thromboplastin (Sigma, St. Louis, MO). One unit of TF activity was set equivalent to the procoagulant activity of a 10<sup>5</sup>-fold dilution of thromboplastin. The clotting activity observed was due to TF activity because no procoagulant activity was detected when factor X-deficient plasma was used instead of normal plasma.

To analyze the contribution of proteases in PDE the protease inhibitors PPACK (Calbiochem-Novabiochem, Bad Soden, Germany) and diisopropyl-fluorophosphate (DFP; Fluka, Buchs, Switzerland) were used. One µl of DFP was added to 1 ml of native, undialyzed PDE or PBS (final concentration 5.7 mmol/l). After an incubation time of 1 h at room temperature followed by 1 h at 4° C the samples were dialyzed over night against PBS at 4° C. When the induction of procoagulant activity by PPACK-treated PDE was analyzed, 1 ml of PDE was treated with 2 µl of PPACK (final concentration 10 µM) and incubated for 30 min at 37° C. Thereafter the samples were dialyzed extensively over night against PBS to remove free protease inhibitor. In case of cell-ELISA experiments (see below) 2 µl of PPACK were added to 200 µl of PDE or PBS. Thereafter 800 µl of cell culture medium were added to obtain a final inhibitor concentration of 10 µmol/l.

### FACS Analysis of Surface TF Antigen

Confluent HUVECs grown in gelatin coated 6-well plates were incubated with PDE or PBS, diluted 1:2 with M199 (supplemented with 8% SCS and antibiotics) for 6 h as described above. Thereafter the cells were carefully detached using trypsin-EDTA (Bio Whittaker, Verviers, Belgium), transferred to polyethylene vials (Falcon; Becton Dickinson, San José, CA) and centrifuged

for 5 min at 400 × g. The supernatants were discharged and the pellets were resuspended and incubated with 40 µl of either 10 µg/ml rabbit anti-TF IgG (American Diagnostica, Greenwich, CT) or 10 µg/ml rabbit anti-complement factor D IgG diluted in PBS containing 1% bovine serum albumin (BSA; Behring, Marburg, Germany). After an incubation time of 1 h on ice the cells were washed with 3 ml of ice-cold PBS containing 0.1% BSA and centrifuged as described. The supernatants were carefully removed and the pellets were resuspended in 40 µl of a 50-fold dilution of fluorescein anti-rabbit IgG (H+L) FI-1000 (Vector, Burlingame, CA) in PBS containing 1% BSA and incubated for 30 min on ice. The cells were washed with 3 ml PBS as described above and the fluorescence of the individual cells was measured using a FACSsort (Becton Dickinson, San José, CA).

### Cell-ELISA for Quantification of Adhesion Molecule Expression

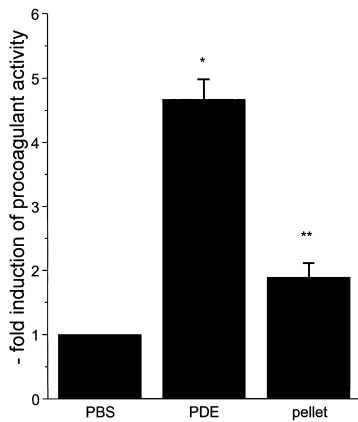
Confluent HUVECs grown in 96-well plates were incubated with 200 µl of PDE or PBS diluted 1:5 in M199 containing 4% SCS and antibiotics. After an incubation time of 4 h (determination of selectins) or 6 h (determination of ICAM and VCAM), respectively, the monolayers were washed twice with PBS (250 µl/well) and fixed using 0.1% glutaraldehyde (Merck-Schuchardt, Hohenbrunn, Germany) in PBS (100 µl/well). After 15 min incubation at 4° C the cells were washed twice with PBS containing 0.05% Tween 20 (Merck-Schuchardt, Hohenbrunn, Germany) and remaining binding sites were blocked with 5% BSA in PBS containing 0.05% Tween 20 (100 µl/well for 1 h at 37° C). Afterwards the cell layers were washed with PBS containing 0.05% Tween 20 as described above and incubated with dilutions of monoclonal anti-human E-selectin/P-selectin-IgG (1:1000), anti-human E-selectin-IgG (1:1000), anti-human ICAM-IgG (1:5000), or anti-human VCAM-IgG (1:500) (all: R&D systems, Minneapolis, MN), respectively, in PBS containing 1% BSA for 1 h at 37° C. Thereafter the cells were rinsed three times and incubated with a 5000-fold dilution of a peroxidase labeled anti mouse antibody (Pierce, Rockford, IL) in PBS containing 1% BSA. After an incubation time of 1 h at 37° C the cells were washed five times. The amount of bound peroxidase was quantified using the peroxidase substrate 1,2 phenyldiamin-dihydrochloride (OPD; Sigma, St. Louis, MO) (100 µl/well of 0.1% OPD in 0.1 M Na<sub>2</sub>HPO<sub>4</sub>, 0.05M citric acid, 0.03% H<sub>2</sub>O<sub>2</sub>). The reaction was stopped after 15 min at room temperature by the addition of 50 µl/well 3N H<sub>2</sub>SO<sub>4</sub>. Absorption was measured at 492 nm using an ELISA-reader (Anthos 2001, Salzburg, Austria).

### Determination of Cytokines and Proteases in PDEs

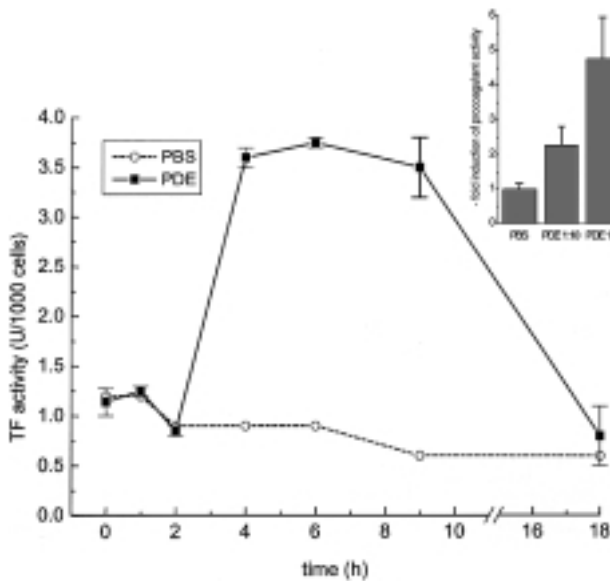
Human IL-1α, IL-1β, and TNFα were determined by immunoassays, using commercial kits (Amersham, Little Chalfont, Buckinghamshire, UK) according to the manufacturers protocols. Mast cell tryptase was determined using a commercial fluoro-immuno-assay (FIA) (Pharmacia, Uppsala, Sweden). Thrombin activity was assayed by means of a clotting test similar to the thrombin time method. Briefly, 100 µl of prewarmed pooled citrated normal human plasma were incubated with 100 µl of prewarmed H<sub>2</sub>O for 30 s at 37° C. Clotting was initiated by the addition of 100 µl of prewarmed PDEs or various concentrations of thrombin (Sigma, St. Louis, MO). Clotting time was measured using a coagulometer. The detection limit of the assay was 75 mU/ml thrombin.

### Affinity Chromatography with Benzamidine-Sepharose 6B

Forty ml of PDE were dialyzed against 0.02 mol/l NaCl, 0.01 mol/l Tris-HCl, pH 8 and applied to a benzamidine-Sepharose 6B (Pharmacia, Uppsala, Sweden). The column (1 × 10 cm) was washed with loading buffer and then eluted with a linear salt gradient from 0.02 mol/l to 1 mol/l NaCl in 0.01 mol/l Tris. Fractions (2.5 ml) were collected and screened for protein (absorbance at 280 nm), amidolytic activity, and TF induction in HUVECs. Amidolytic activity was assayed using the nonspecific substrate H-D-isoleucyl-L-prolyl-L-arginine-p-nitroaniline-dihydrochloride (S2288; Chromogenix, Mölndal, Sweden). Wells of a microtiter plate (Greiner, Kremsmünster, Austria) were filled with 20 µl of the respective fractions. Thereafter 80 µl S2288 (1 mmol/l final concentration) in 100 mmol/l Tris, 106 mmol/l NaCl, pH 8.4 were added



**Fig. 1** Procoagulant activity of HUVECs incubated with PDEs or PDE derived cellular material from patients undergoing CAPD. Native PDEs from 10 individual CAPD patients were diluted 1:4 with cell culture medium and incubated with confluent HUVECs. Alternatively HUVECs were incubated with lysed cells and cell debris contained in the pellets, dissolved in 1/16 of the original volume. PBS was used as control. After an incubation time of 6 h the HUVECs were lysed and procoagulant activity was measured as described in Methods. The results are the mean values  $\pm$  SEM of duplicate determinations (\* $p < 10^{-10}$ , \*\* $p < 0.001$  compared with the PBS control)



**Fig. 2** Dose and time dependence of procoagulant activity in HUVECs in response to PDE. Confluent HUVECs were exposed to PDE from a representative donor (dialyzed against PBS) or PBS (both diluted 1:5 in cell culture medium) for up to 18 h. At the time points indicated the cells were washed twice with PBS and frozen at  $-70^{\circ}$  C. Simultaneously, identically treated cells grown in different wells were detached using trypsin and counted. Cellular TF procoagulant activities for all time points were measured in parallel. Results are expressed as mean values  $\pm$  SEM for duplicate determinations.

The inset shows the procoagulant activity of HUVECs in response to increasing concentrations PDE. PDE from the same donor was dialyzed against PBS, diluted 1:10 and 1:5 with cell culture medium, and incubated for 6 h with confluent monolayers of HUVECs. PBS was used as control. TF procoagulant activity was determined as described in Methods. Data presented are the mean  $\pm$  SEM of triplicate determinations. Comparable results were obtained with PDE samples from different donors

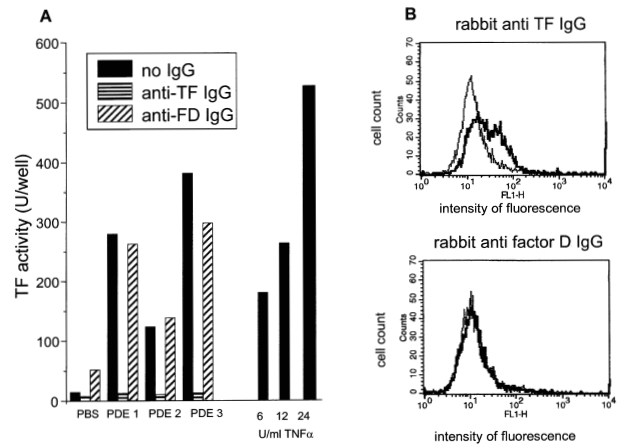
to the wells and the plate was incubated at room temperature for 12 h. The absorbance at 405 nm was measured in an ELISA reader (Anthos 2001, Salzburg, Austria), and used to quantify amidolytic activity in the individual fractions. The induction of TF-procoagulant activity in HUVECs by the respective fractions was assessed by means of the clotting assay described above. The fractions from the column were first dialyzed against PBS and the cells were then stimulated for 5 h with the fractions diluted 1:5 with M199 containing 4% SCS. The fractions were further analyzed by SDS-PAGE (8-25% gradient gels) using a PhastSystem (Pharmacia, Uppsala, Sweden).

*Statistical analysis.* Statistical significance was assessed using one way ANOVA.

**Results**

*Induction of TF and Selectins in HUVECs by PDE*

When cultured HUVECs were incubated with 1:4 dilutions of native peritoneal dialysis effluents (PDEs) from 10 individual CAPD patients their procoagulant activity increased to values three to six-fold of the control (Fig. 1). To rule out the possibility that substances contained in the small cellular fraction of these PDEs contributed to this effect, the induction of procoagulant activity by pellets obtained by centrifugation of these 10 PDE samples was analyzed. PDEs were centrifuged as described in Methods and the supernatants were carefully discharged



**Fig. 3** Identification of the induced procoagulant activity as tissue factor. (A) Effect of rabbit anti-TF IgG on the procoagulant activity of HUVEC-lysates. PDEs (dialyzed against PBS) from three individual patients or PBS were diluted 1:5 with cell culture medium and incubated with confluent monolayers of HUVECs for 6 h. TNF $\alpha$  diluted in cell culture media was used as positive control. Thereafter the cells were washed, lysed by repeated freezing and thawing, and suspended in clotting buffer. The cell lysates were incubated with 100  $\mu$ g/ml rabbit anti-human TF IgG, 100  $\mu$ g/ml rabbit anti-human complement factor D IgG, or solvent for 30 min at  $37^{\circ}$  C and procoagulant activity was measured as described in Methods. Data represent the mean values of duplicate determinations

(B) TF-antigen expression on the surface of HUVECs in response to PDE. PDEs (dialyzed against PBS) or PBS were diluted 1:2 in cell culture medium (containing 8% SCS) and incubated with confluent HUVECs as described in Methods. Thereafter the cells were washed, detached using trypsin, and incubated with rabbit anti-human TF IgG or rabbit anti-human complement factor D IgG, respectively. Bound rabbit IgG was detected using fluorescein labeled anti rabbit antibodies and FACS analysis. The thin line shows the pattern of fluorescence of the PBS treated cells and the thick line represents the intensity of fluorescence of the PDE treated cells

except for 50  $\mu$ l. The cells in the pellets were lysed by repeated freezing and thawing, the resulting lysates were diluted 1:50 in cell culture medium, and the upregulation of procoagulant activity was determined in parallel to the experiment presented above. The described procedure resulted in an approximately 16-fold concentration of cellular material in the stimulation medium compared to the 4-fold dilutions of PDEs. Only a 1.9-fold induction (range: 1 to 2.6-fold the control value) of TF activity could be detected in these pellets (Fig. 1).

To further characterize this activity all PDEs used in the experiments described below were dialyzed extensively against PBS prior to incubation with the cells using dialysis tubing with 12-14 kD molecular weight cut off, to eliminate possible influences of changes in pH, osmolarity, or electrolyte composition. When HUVECs were incubated with such prepared PDEs, induction of procoagulant activity occurred in a dose and time-dependent manner. With PDEs diluted 1:10 and 1:5, respectively a 2.3- and 4.8-fold induction of procoagulant activity was observed after 6 h of stimulation (Fig. 2, inset). TF activity reached maximum levels after 4-6 h and returned to basal values 18 h after the exposure (Fig. 2).

As presented in Fig. 3A, procoagulant activity of HUVEC lysates was completely blocked by rabbit anti-TF IgG. Rabbit anti-complement factor D IgG had no inhibitory effect in the same concentration. These data confirm that the described assay was specific for TF activity. The intensity of the stimulation of TF activity by PDE could be compared to the TF inducing activity of approximately 12 U/ml TNF $\alpha$  (Fig. 3A).

FACS analysis was used to correlate the described increase in TF activity with the expression of TF on the cell surface. TF antigen was detected using rabbit anti-TF IgG and fluorescein labeled anti-rabbit antibodies. As demonstrated in Fig. 3B, the incubation of HUVECs with PDE resulted in an increase in fluorescence of the cells. The me-

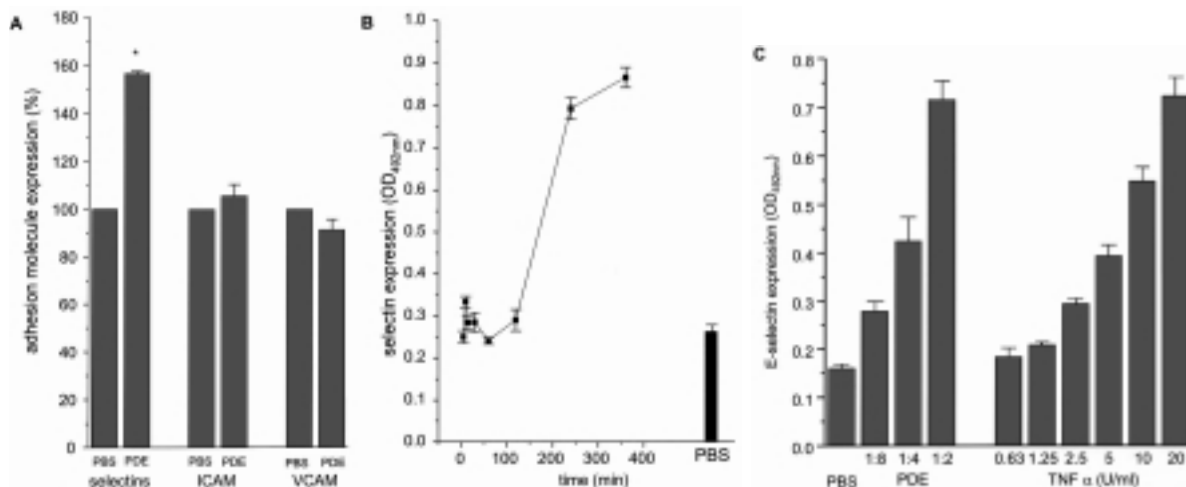
dian fluorescence intensity raised from 12.30 arbitrary units to 22.88 arbitrary units. When rabbit anti-complement factor D IgG was used instead of anti-TF IgG no shift in fluorescence-intensity could be detected.

Northern blot analysis was performed with 20  $\mu$ g of extracted total RNA to determine whether PDE enhances TF mRNA levels in HUVECs. While no TF message could be detected in untreated control cells, a faint specific signal was seen after exposure of HUVECs to PDE (data not shown).

A cell ELISA was used to quantify the expression of adhesion molecules in HUVECs. As shown in Fig. 4A, exposure of HUVECs to PDEs resulted in upregulation of endothelial selectins. The expression of ICAM and VCAM antigen was unchanged compared to that of PBS treated control cells. Selectin expression reached maximum levels after 4-6 h (Fig. 4B). The finding that incubation times of at least 4 h are needed to induce selectins in HUVECs suggests that E-selectin is the upregulated molecule, as P-selectin expression is usually very rapid and short lived (22). Monospecific anti E-selectin-IgG was used to confirm that E-selectin is upregulated in response to PDE in a dose-dependent manner (Fig. 4C). The intensity of the PDE induced E-selectin expression was compared to the E-selectin inducing activity of TNF $\alpha$ .

#### Characterization of the TF and Selectin Upregulatory Activity in PDE

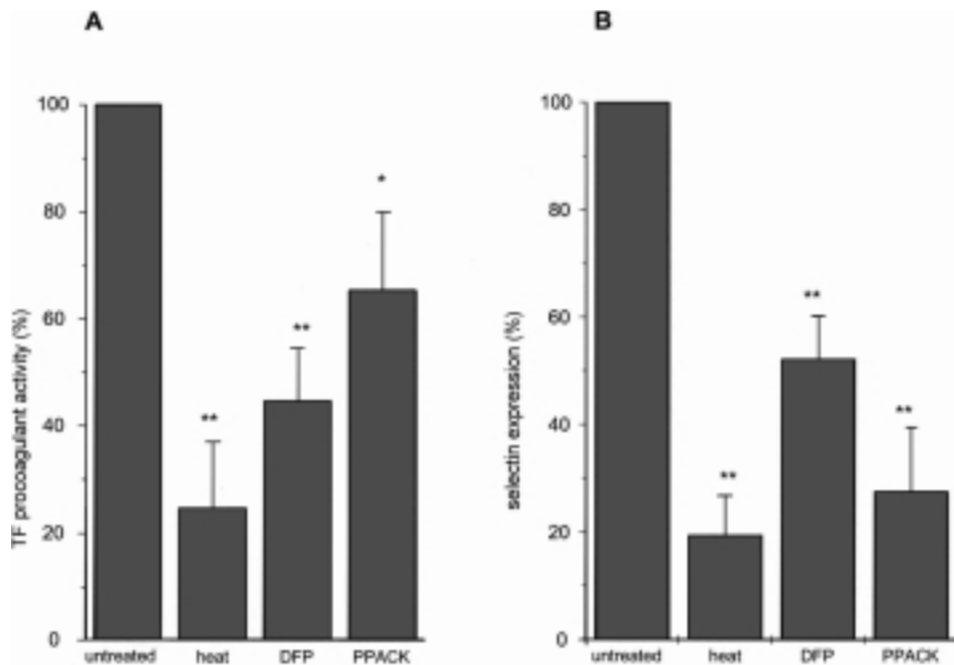
This endothelial cell activating activity was further characterized using randomly selected PDE samples from 3 individual patients. As shown in Fig. 5 (A and B), heat treatment abrogated the TF and selectin upregulatory activity in these samples. These data therefore exclude the possibility that induction of TF and selectins was due to contaminating LPS, since LPS is known not to be inactivated by heat treatment (10, 14).



**Fig. 4** Expression of adhesion molecules on HUVECs in response PDE. (A) PDE (dialyzed against PBS) or PBS were diluted 1:5 with cell culture medium and incubated with confluent monolayers of HUVECs. After an incubation time of 4 h (determination of selectins) or 6 h (determination of ICAM, VCAM), the cells were fixed and adhesion molecules were detected using a cell-ELISA as described in Methods. The adhesion molecule expression of unstimulated (PBS) control cells was set at 100%. The results are the mean  $\pm$  SEM values of three experiments performed in duplicate with PDE samples from the same three patients as in Fig. 5 (\* $p < 10^{-7}$  compared with the PBS control).

(B) PDE from the same three patients as in Panel A and Fig. 5. was pooled and dialyzed against PBS. Pooled PDE was diluted 1:5 with cell culture medium and incubated with confluent monolayers of HUVECs for up to 6 h. Cells incubated with PBS diluted 1:5 for 6 h were used as control. Selectin expression was quantified using a cell-ELISA as described in Methods. Data presented are the mean  $\pm$  SEM of quadruplicate determinations.

(C) Pooled PDE was prepared as described above, diluted 1:8, 1:4, and 1:2 with cell culture medium, and incubated for 4 h with confluent monolayers of HUVECs. PBS was used as control. The final concentrations of supplemented calf serum in the media were adjusted to 4%. HUVECs incubated with the indicated concentrations of TNF $\alpha$  diluted in cell culture media served as positive control. E-selectin expression was quantified using a monospecific antibody as described in Methods. Data presented are the mean  $\pm$  SEM of quadruplicate determinations



**Fig. 5** The effect of heat and protease inhibitor treatment on TF and selectin inducing activity of PDEs. (A) PDEs from 3 patients were subjected to heat treatment for 10 min at 100° C or were treated with the protease inhibitors DFP or PPACK as described in the Methods section. Confluent monolayers of HUVECs were incubated with such treated or untreated samples. After an incubation time of 6 h cellular TF procoagulant activity was assessed as described. Data presented are the mean  $\pm$  SEM values of three individual PDE samples each assessed in duplicate (\* $p$  < 0.05, \*\* $p$  < 0.01 compared with the untreated controls) (B) PDE samples (dialyzed against PBS) from 3 individual patients were subjected to heat treatment for 10 min at 100° C or were treated with the protease inhibitors PPACK or DFP as described in Methods. HUVECs were incubated with such treated or untreated PDEs (diluted 1:5 in cell culture medium). After an incubation time of 4 h the expression of endothelial selectins was quantified using a cell ELISA. Data presented are the mean  $\pm$  SEM values of three individual PDE samples each assessed in duplicate (\*\* $p$  < 0.01 compared with the untreated controls)

To analyze a possible contribution of TNF $\alpha$ , IL-1 $\alpha$ , or IL-1 $\beta$  to the observed effect, PDEs were screened for the presence of these cytokines. No TNF $\alpha$  antigen, no IL-1 $\alpha$  antigen, and no IL-1 $\beta$  antigen could be detected in the 3 PDE samples used in the experiments described above by means of commercial antigen ELISA-kits with a sensitivity of 5 pg/ml, 2 pg/ml, and 1 pg/ml respectively.

Also thrombin is known to upregulate TF and selectins in EC (24, 27). Therefore in the 3 PDE samples thrombin activity was analyzed. The lowest thrombin concentration that lead to clot formation in the assay used was 75 mU, and no clot formation was observed with the 3 PDE samples excluding thrombin as a major factor in the activation of EC by PDEs.

Besides thrombin also other proteases might be capable of upregulating TF and selectin expression in EC. Therefore the effect of protease inhibitors on HUVEC activation by PDEs was studied. To avoid interference of active protease inhibitors with the clotting assay for TF procoagulant activity the irreversible protease inhibitors DFP and PPACK were chosen and the inhibitor treated PDE samples were dialyzed extensively against PBS prior to incubation with the cells to remove free inhibitors. Treatment of PDEs with DFP and PPACK partially inhibited the induction of procoagulant activity (Fig. 5A). Consistent with the results obtained for TF expression, also induction of selectins in HUVECs by PDEs was blocked by the addition of the protease inhibitors (DFP, PPACK) (Fig. 5B).

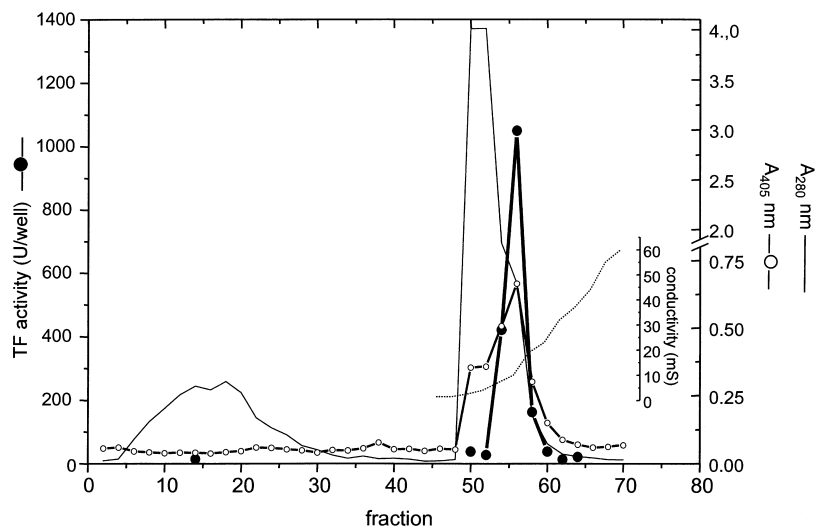
Since proteases seem to be at least partially responsible for EC activation by PDEs, benzamidine-Sepharose was employed to attempt partial purification of the TF inducing protease activity contained in PDEs. This Sepharose carries the synthetic inhibitor paraaminobenzoamide

and can be used for the isolation of trypsin-like serine proteases (28, 29). As demonstrated in Fig. 6, TF upregulatory activity was bound to benzamidine-Sepharose, as no activity could be detected in the fall through fractions. When the benzamidine-Sepharose column was eluted with a linear salt gradient, high TF inducing activity eluted as a single peak, well separated from the main protein peak. Amidolytic activ-

**Table 1** Comparison of A<sub>280nm</sub> and selectin upregulatory activity of PDE prior and after chromatography on a column of Bio Rex 70

	A <sub>280nm</sub>	Selectin antigen (%)		
		Untreated	heat treated	PPACK treated
starting material	3.505	100	40	55
fall through	2.349	107	30	47

10ml of a pool of 3 PDEs were dialyzed against loading buffer (40mmol/l NaCl, 2mmol/l KH<sub>2</sub>PO<sub>4</sub>, 8mmol/l Na<sub>2</sub>HPO<sub>4</sub>) and applied to a Bio Rex 70 column (1 x 4.5 cm) equilibrated in loading buffer. The fall through was collected in aliquots and screened for absorption at 280nm. As soon as the fall through reached constant low A<sub>280nm</sub> values the fractions were pooled. Starting material and the pooled fall through were subjected to heat treatment (100°C for 10 minutes) or were treated with PPACK as described in Methods. Induction of selectin antigen in cultured HUVECs was determined using a cell-ELISA. The selectin concentration induced by untreated starting material (dialyzed PDE) was set at 100%. The A<sub>280nm</sub> values given were extrapolated from values obtained from material diluted 1:10 with deionized water. Data given are mean values of triplicate determinations.



**Fig. 6** Affinity chromatography of PDE with benzamidine-Sepharose. Fourty ml of native PDE were dialyzed against loading buffer (0.02 mol/l NaCl, 0.01 mol/l Tris-HCl, pH 8) and applied to a benzamidine-Sepharose column. The column was washed with loading buffer, and then eluted with a linear salt gradient from 0.02 mol/l NaCl to 1 mol/l NaCl in 0.01 mol/l Tris-HCl, pH 8. Fractions were collected and assessed for protein [absorbance at 280 nm (—)], amidolytic activity [absorbance at 405 nm (—○—)], TF upregulatory activity [TF procoagulant activity (—●—)], and conductivity (mS) (.....) as described. The experiment was repeated twice with different PDE samples and comparable results were obtained

ity in this peak was concentrated approximately 10-fold compared to PDE (data not shown). The fractions with peak protease activity, determined by the cleavage of the synthetic p-nitroaniline substrate S2288, and with peak TF upregulatory activity coincided (fraction 56). In the fraction from the benzamidine-Sepharose column that contained peak protease activity and peak TF inducing activity no active thrombin could be detected using a clotting assay.

Another protease possibly present in PDE is complement factor D. Both proteases thrombin and factor D bind to Bio-Rex 70, and Bio-Rex 70 has been used not only to purify factor D (30), but also to remove thrombin from biological samples (31). TF upregulatory activity in PDE did not bind to a Bio-Rex 70 column. Furthermore purified human complement factor D did not induce procoagulant activity in cultured

HUVECs in a concentration of 1  $\mu\text{g/ml}$  (data not shown). Table 1 shows that chromatography of PDE on a column of Bio Rex 70 did not influence selectin upregulatory activity although the  $A_{280\text{ nm}}$  was lower in the fall through compared to the starting material. The selectin upregulatory activity in the starting material and in the fall through could be inhibited by heat and PPACK to a similar degree. Trypsin is a serine protease that is secreted by mast cells and is able to activate protease-activated receptors (32). Therefore trypsin levels in the 3 PDEs were also determined using an antigen assay. The concentrations of trypsin were below the assay detection limit of 1 ng/ml.

To analyze the possibility that during the incubation of the PDEs with the EC active mediators were formed by the action of proteases contained in PDE from substrates in the cell culture medium or in the PDE, PDE was preincubated with the medium prior to the addition of PPACK. As demonstrated in Table 2, no selectin upregulatory activity was detected in these samples after the addition of PPACK.

**Table 2** Selectin upregulatory activity after preincubation of PDE with cell culture medium prior to the addition of PPACK

	Selectin antigen ( $A_{492\text{nm}}$ )
PBS	0.163 $\pm$ 0.010
PBS + PPACK	0.143 $\pm$ 0.014
PDE	0.381 $\pm$ 0.024
PDE (0min) $\rightarrow$ +PPACK	0.195 $\pm$ 0.018
PDE (5min) $\rightarrow$ +PPACK	0.166 $\pm$ 0.021
PDE (10min) $\rightarrow$ +PPACK	0.156 $\pm$ 0.022
PDE (30min) $\rightarrow$ +PPACK	0.122 $\pm$ 0.021

Aliquots of PDE from a representative donor (dialyzed against PBS) or PBS were diluted 1:5 with M199 containing 4% supplemented calf serum and incubated for 0, 5, 10, and 30 minutes at 37°C. At the time points indicated PPACK (5 $\mu\text{mol/l}$  final concentration) was added and the samples were incubated with confluent HUVECs. Samples without addition of this protease inhibitor were used as control. After an incubation time of 4 hours selectin antigen was determined using a cell-ELISA as described in Methods. Data presented are the mean  $\pm$  SEM of quadruplicate determinations.

## Discussion

Our study reveals that peritoneal dialysis effluents stimulate cultured human umbilical vein endothelial cells to express TF procoagulant activity and TF surface antigen. Furthermore the expression of E-selectin was increased in response to these effluents, while ICAM antigen and VCAM antigen were not induced in these cells.

The small cellular fraction of PDEs (0.3 to 45 million cells per liter) consists predominantly of macrophages and lymphocytes (33). Since only low TF inducing activity was detected in pellets obtained by centrifugation of fresh PDEs, it can be excluded that substances contained in these cells play a mayor role in the activation of HUVECs by PDEs. TF and selectin upregulatory activity was not affected by dialysis of PDEs using a dialysis tubing with a molecular weight cut off of 12-14 kD. Therefore it can be ruled out that small molecules such as peptides contribute to the observed effects. The possibility that LPS contributed to the activation of HUVECs is unlikely because heat treatment abrogated TF and selectin upregulatory activity in all PDE sam-

ples analyzed. TF and adhesion molecules are upregulated in EC in response to TNF $\alpha$  and IL-1. Therefore PDEs were screened for the presence of these cytokines. The concentrations of TNF $\alpha$ , IL-1 $\alpha$ , and IL-1 $\beta$  were below the assay detection limits of 5 pg/ml, 2 pg/ml, and 1 pg/ml respectively. These findings are in agreement with cytokine levels described for noninfected PDE samples (34). Other authors described low levels of TNF $\alpha$  antigen in noninfected PDE, but were unable to detect biologic activity presumably because of the excess of inhibitory soluble TNF-receptors (35). These data exclude that TNF $\alpha$ , IL-1 $\alpha$ , or IL-1 $\beta$  were predominantly responsible for the observed activation of endothelial cells by PDEs. On the other hand a mixture of synergistically acting cytokines together with the protease described in this report may be active even if the individual constituents are present in low concentrations.

Since protease inhibitor treatment reduced the upregulation of TF and selectins in response to PDEs, it can be concluded that a protease activity was at least in part responsible for the activation of HUVECs. This assumption is further supported by the finding that TF upregulatory activity could partially be purified by a benzamidine-Sepharose column. It has been shown that the protease thrombin induces expression of TF and endothelial selectins in endothelial cells by cleaving and activating its receptor. Thrombin activity was below 75 mU in native PDEs as well as in the fraction from the benzamidine-Sepharose column that contained peak protease activity and peak TF upregulatory activity. Another protease contained in PDEs is complement factor D. In fact PDEs can be used as starting material for the purification of complement factor D utilizing its ability to bind to Bio Rex 70 (30). Furthermore Bio Rex 70 can also be used to remove thrombin from a complex mixture (31). Purified complement factor D did not induce procoagulant activity and the TF/selectin upregulatory activity could not be removed from PDE by means of Bio Rex 70. These data exclude that thrombin or factor D were responsible for the observed endothelial activation. Another possible candidate is trypsin, a mast cell derived serine protease, able to activate protease-activated receptors (32). Trypsin levels in PDEs were below the assay detection limit of 1 ng/ml indicating that also mast cell trypsin does not contribute predominantly to EC activation by PDEs. Furthermore addition of the protease inhibitor PPACK after preincubation of PDE with cell culture medium (containing 4% SCS) inhibited selectin upregulatory activity of PDEs. Consequently it is unlikely that the activation of HUVECs was caused indirectly by mediators generated from substrates in the cell culture medium by proteases in the PDE.

These results obtained are compatible with protease(s) present in PDE that interact directly with endothelial cells. On the cell surface the protease(s) might then activate a protease-activated receptor (PAR) leading to EC activation. In fact besides the thrombin receptor (PAR-1) also PAR-2 was found on EC (19) and recently a PAR-3 could be cloned (18). While PAR-1 seems to be specific for thrombin, PAR-2 can be activated by trypsin and mast cell trypsin (32).

Mediators that appear in PDE may not merely be transperitoneally transported from the circulation, but can be produced locally in the peritoneum (35, 36). Activated leukocytes might be a source of endothelial activating serine proteases (37).

We therefore conclude that peritoneal dialysis effluents from CAPD patients contain an activity that induces a procoagulatory and proadhesive phenotype in HUVECs. This activity is partially protease dependent and can not be attributed to LPS, TNF $\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$ , active thrombin, complement factor D, or mast cell trypsin. The described protease activity might cause endothelial activation not only in vitro but also in vivo and might consequently contribute to the development of an inflammatory state of peritoneal vasculature during CAPD.

#### Acknowledgments

This work was supported in part by grants from the Austrian Science Foundation to MG (P 10823-Med) and BRB (F 509). We gratefully acknowledge the help of Peter Valent (Department of Medicine I, Division of Hematology, University of Vienna) and his laboratory staff, who performed the determinations of mast cell tryptase.

#### References

- Nolph K, Lindbald AS, Novak JW. Continuous ambulatory peritoneal dialysis. *N Engl J Med* 1988; 318: 1595-600.
- Pollock CA, Ibels LS, Eckstein RP, Graham JC, Caterson RJ, Mahony JF, Sheil AGR. Peritoneal morphology on maintenance dialysis. *Am J Nephrol* 1989; 9: 198-204.
- Medcalf JF, Walls J. New frontiers in continuous ambulatory peritoneal dialysis. *Kidney Int* 1997; 52: S-108-10.
- Goedde M, Sitter T, Schiffel H, Bechtel U, Schramm W, Spannagl M. Coagulation and fibrinolysis-related antigens in plasma and dialysate of CAPD patients. *Perit Dial Int* 1997; 17: 162-6.
- Sitter T, Spannagl M, Schiffel H, Held E, van Hinsbergh VWM, Kooistra T. Imbalance between intraperitoneal coagulation and fibrinolysis during peritonitis of CAPD patients: the role of mesothelial cells. *Nephrol Dial Transplant* 1995; 10: 677-83.
- Dobbie JW. Serositis: comparative analysis of histological findings and pathogenetic mechanisms in nonbacterial serosal inflammation. *Perit Dial Int* 1993; 13: 256-69.
- Honda K, Nitta K, Horita S, Yumura W, Nihei H. Morphological changes in the peritoneal vasculature of patients on CAPD with ultrafiltration failure. *Nephron* 1997; 72: 171-6.
- Osterud B. Tissue factor: a complex biological role. *Thromb Haemost* 1997; 78: 755-8.
- Moll T, Czyn M, Holzmüller H, Hofer-Warbinek R, Wagner E, Winkler H, Bach FH, Hofer E. Regulation of the tissue factor promoter in endothelial cells. *J Biol Chem* 1995; 270: 3849-67.
- Nawroth PP, Stern DM. Modulation of endothelial cell hemostatic properties by tumor necrosis factor. *J Exp Med* 1986; 163: 740-5.
- Bevilacqua MP, Pober JS, Majeau GR, Cotran RS, Gimbrone MA. Interleukin 1 (IL-1) induces biosynthesis and cell surface expression of procoagulant activity in human vascular endothelial cells. *J Exp Med* 1984; 160: 618-23.
- Moore KL, Andreoli SP, Esmon NL, Esmon CT, Bang NU. Endotoxin enhances tissue factor and suppresses thrombomodulin expression of human vascular endothelium in vitro. *J Clin Invest* 1987; 79: 124-30.
- Tedesco F, Pausa M, Nardon E, Introna M, Mantovani A, Dobrina A. The cytolytically inactive terminal complement complex activates endothelial cells to express adhesion molecules and tissue factor procoagulant activity. *J Exp Med* 1997; 185: 1619-27.
- Ikeda K, Nagasawa K, Horiuchi T, Tsuru T, Nishizaka H, Niho Y. C5a induces tissue factor activity on endothelial cells. *Thromb Haemost* 1997; 77: 394-8.
- Clauss M, Gerlach M, Gerlach H, Brett J, Wang F, Familletti PC, Pan Y-CE, Olander JV, Conolly DT, Stern D. Vascular permeability factor: a tumor-derived polypeptide that induces endothelial cell and monocyte procoagulant activity, and promotes monocyte migration. *J Exp Med* 1990; 172: 1535-45.
- Nishimura H, Tsuji H, Masuda H, Nakagawa K, Nakahara Y, Kitamura H, Kasahara T, Sugano T, Yoshizumi M, Sawada S, Nakagawa M. Angiotensin II increases plasminogen activator inhibitor-1 and tissue factor mRNA expression without changing that of tissue type plasminogen activator or tissue factor pathway inhibitor in cultured rat aortic endothelial cells. *Thromb Haemost* 1997; 77: 1189-95.
- Bartha K, Brisson C, Archipoff G, de la Salle C, Lanza F, Cazenave JP, Beretz A. Thrombin regulates tissue factor and thrombomodulin mRNA levels

- and activities in human saphenous vein endothelial cells by distinct mechanisms. *J Biol Chem* 1993; 268: 421-9.
18. Ishihara H, Connolly AJ, Zeng D, Kahn ML, Zheng YW, Timmons C, Tram T, Coughlin SR. Protease-activated receptor 3 is a second thrombin receptor in humans. *Nature* 1997; 386: 502-6.
  19. Molino M, Woolkalis MJ, Reavey-Cantwell J, Pratico D, Andrade-Gordon P, Barnathan ES, Brass LF. Endothelial cell thrombin receptors and PAR-2. *J Biol Chem* 1997; 272: 111333-41.
  20. Wertheimer SJ, Myers CL, Wallace RW, Parks TP. Intercellular adhesion molecule-1 gene expression in human endothelial cells. *J Biol Chem* 1992; 267: 12030-5.
  21. Osborn L, Hession C, Tizard R, Vasallo C, Luhowskyj S, Chi Rosso G, Lobb R. Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. *Cell* 1989; 59: 1203-11.
  22. Kansas GS. Selectins and their ligands: current concepts and controversies. *Blood* 1996; 88: 3259-87.
  23. Smith CW. Leukocyte-endothelial cell interactions. *Semin Hematol* 1993; 30 (4, Suppl. 4): 45-55.
  24. Shankar R, de la Motte CA, Poptic EJ, DiCorleto PE. Thrombin receptor-activating peptides differentially stimulate platelet-derived growth factor production, monocytic cell adhesion, and E-selectin expression in human umbilical vein endothelial cells. *J Biol Chem* 1994; 269: 13936-41.
  25. Harboe N, Ingild A. Immunization, isolation of immunoglobulins, estimation of antibody titre. In: *A manual of quantitative immunoelectrophoresis. Methods and applications.* Axelsen NH, Kroll J, Weeke B (eds). Universitetsforlaget Oslo 1973, pp 161-46.
  26. Zhang JC, Fabry A, Paucz L, Wojta J, Binder BR. Human fibroblasts down-regulate plasminogen activator inhibitor type-1 in cultured human macrovascular and microvascular endothelial cells. *Blood* 1996; 88: 3880-6.
  27. Shankar R, de la Motte CA, DiCorleto PE. 3-deazaadenosine inhibits thrombin-stimulated platelet-derived growth factor production and endothelial-leukocyte adhesion molecule-1-mediated monocytic cell adhesion in human aortic endothelial cells. *J Biol Chem* 1992; 267: 9376-82.
  28. Winkler ME, Blaber M. Purification and characterization of recombinant single-chain urokinase produced in *Escherichia coli*. *Biochemistry* 1986; 25: 4041-5.
  29. Mizuno K, Nakamura T, Takada K, Sakakibara S, Matsuo H. A membrane-bound, calcium-dependent protease in yeast  $\alpha$ -cell cleaving on the carboxyl side of paired basic residues. *Biochem Biophys Res Comm* 1987; 144: 807-14.
  30. Catana E, Schifferli JA. Purification of human complement factor D from the peritoneal fluid of patients on chronic ambulatory peritoneal dialysis. *J Immunol Methods* 1991; 138: 265-71.
  31. Gardiner JE, Griffin H. Studies on human protein C inhibitor in normal and factor V/VIII deficient plasmas. *Thromb Res* 1984; 36: 197-203.
  32. Molino M, Barnathan ES, Numerof R, Clark J, Dreyer M, Cumashi A, Hoxie JA, Schechter N, Woolkalis M, Brass LF. Interactions of mast cell tryptase with thrombin receptors and PAR-2. *J Biol Chem* 1997; 272: 4043-9.
  33. Lewis S, Holmes C. Host defense mechanisms in the peritoneal cavity of continuous ambulatory peritoneal dialysis patients. *Perit Dial Int* 1991; 11: 14-21.
  34. Brauner A, Hylander B, Wretling B. Tumor necrosis factor- $\alpha$ , interleukin-1 beta, and interleukin-1 receptor antagonist in dialysate and serum from patients on continuous ambulatory peritoneal dialysis. *Am J Kidney Dis* 1996; 27: 402-8.
  35. Zemel D, Imholz ALT, Waart DR, Dinkla C, Struijk DG, Krediet RT. Appearance of tumor necrosis factor- $\alpha$  and soluble TNF-receptors I and II in peritoneal effluent of CAPD. *Kidney Int* 1994; 46: 1422-30.
  36. van Hinsbergh VWM, Kooistra T, Scheffer MA, van Bockel JH, Goos NP, van Muijen NP. Characterization and fibrinolytic properties of human omental tissue mesothelial cells. Comparison with endothelial cells. *Blood* 1990; 75: 1490-7.
  37. Totani L, Cumashi A, Piccoli A, Lorenzet R. Polymorphonuclear leukocytes induce PDGF release from IL-1 $\alpha$ -treated endothelial cells. Role of adhesion molecules and serine proteases. *Arterioscler Thromb Vasc Biol* 1998; 18: 1534-40.

Received February 3, 1999 Accepted after revision March 8, 1999