# Clot Lysis Mediated by Cultured Human Microvascular Endothelial Cells

Wolfgang Speiser, Elisabeth Anders, Bernd R. Binder\*, and Gert Müller-Berghaus

From the Clinical Research Unit for Blood Coagulation and Thrombosis of the Max-Planck-Gesellschaft, Justus-Liebig-Universität, Giessen, FRG, and the Laboratory for Clinical and Experimental Physiology, Department of Medical Physiology\*, University of Vienna, Austria

## **Key words**

Endothelial cells – Clot lysis – Tissue plasminogen activator – Plasminogen activator inhibitor

## Summary

The lysis of fibrin clots on the surface of cultured human omental tissue microvascular endothelial cells (HOTMEC) and cultured human umbilical vein endothelial cells (HUVEC) was studied. Fibrin clots were made by mixing fibrinogen, plasminogen and thrombin on the surface of both cell types. Clot lysis was seen only on the surface of HOTMEC, which were found to synthesize about 100-fold more tissue plasminogen activator (tPA) antigen than HUVEC. Clot lysis of HOTMEC could be blocked by anti-tPA IgG but was not affected by the incorporation of exogenous plasminogen activator (PAI) into the clot in concentrations (75 arbitrary units) exceeding the tPA activity  $(21 \pm 2.5 \text{ IU})$  of the cells. Thus, it is likely that tPA secreted by HOTMEC is protected from inhibition by PAI in the presence of fibrin and endothelial cells. The stimulation of EC to release an excess of tPA over PAI, in contrast to the secretion of an excess of PAI over tPA found in unstimulated cells in the absence of fibrin, is obviously no prerequisite for the initiation of fibrinolysis on the surface of HOTMEC. As thrombin was used for clot formation, its influence on tPA and PAI synthesis of both cell types was investigated. In contrast to HOTMEC, which were not affected by α-thrombin, HUVEC revealed a dose-dependent increase in tPA and PAI synthesis upon incubation with the enzyme. This increase in tPA production by HUVEC was not sufficient to lyse the clots within 48 hours. Furthermore, HUVEC behaved differently towards thrombin as these cells in contrast to HOTMEC revealed the typical shape change reaction upon incubation with the enzyme.

## Introduction

The vascular endothelium plays a central role in the regulation of fibrinolysis by synthesizing and secreting both plasminogen activators (tissue-type plasminogen activator, tPA, and single-chain urokinase-type plasminogen activator, scuPA) and plasminogen activator inhibitor type 1 (PAI-1) (1). Endothelial cells (EC) release an excess of PAI over tPA so that no free tPA activity but free plasminogen activator inhibitor capacity (PAI cap) is found in blood of individuals at rest (2, 3) as well as in conditioned media of unstimulated cultured human endothelial cells (EC) (4). Several stimuli are thought to cause tPA release

Presented at the XIth International Congress on Thrombosis and Haemostasis, Brussels, Belgium, July 6-10, 1987.

Correspondence to: Gert Müller-Berghaus, Clinical Research Unit for Blood Coagulation and Thrombosis, Max-Planck-Gesellschaft, Gaffkystraße 11, D-6300 Gießen, FRG

from vascular endothelial cells in vivo (5), such as venous occlusion (6, 7), physical exercise (8), infusion of vasoactive peptides (9) or extracorporal circulation (10, 11). If high amounts of tPA accumulate in plasma under these conditions, the PAI capacity in the blood is overcome, and free tPA activity can be measured (12). Such a release of an excess of tPA over PAI by the vascular endothelium has been assumed to be a prerequisite for effective plasminogen activation and consecutive fibrinolytic activity in blood. In experiments with cultured human EC, however, considerable amounts of free tPA activity have so far not been detected in the conditioned media or within the cells.

Recently it was shown by Wun et al. (13) that purified tPA added to tPA depleted plasma immediately before clotting activates plasminogen even in the presence of free PAI. Fibrin formation obviously renders tPA relatively inaccessible to inhibition by PAI. Similar observations were first made by Kruithof et al. (2). As fibrin formation is dependent on the action of thrombin on fibrinogen, the effect of this enzyme on the fibrinolytic activities of EC is of major interest. These interactions were studied by several groups and it was shown that thrombin enhances tPA and PAI synthesis by cultured human EC (14–16). It is, however, unclear whether this change in the production rate of tPA and PAI increases or decreases the fibrinolytic capacity of EC.

In the present study fibrin clots were made of fibrinogen, plasminogen and  $\alpha$ -thrombin on the surface of macrovascular human umbilical vein endothelial cells (HUVEC) and human omental tissue microvascular endothelial cells (HOTMEC) 1) to compare the fibrinolytic activities of HUVEC and HOTMEC, 2) to investigate the protective effect of fibrin and cell surface structures on tPA secreted by EC directly on the EC surface and 3) to compare the influence of  $\alpha$ -thrombin on the fibrinolytic activities of HUVEC and HOTMEC.

## **Materials and Methods**

#### Materials

Waymouth's MB 752-1 cell culture medium, fetal calf serum (FCS), penicillin, streptomycin, glutamine and collagenase were obtained from Gibco, Karlsruhe, FRG. Bovine serum albumin (BSA), D(+)-glucose, endothelial cell growth supplement (ECGS) and hirudin were purchased from Sigma, München, FRG. Plastic petri dishes were obtained from Becton Dickinson, Heidelberg, FRG. Fibronectin was from Behringwerke, Marburg, FRG. Human plasminogen, human fibrinogen and melanoma cell derived tPA were from KabiVitrum, Stockholm, Sweden. Reptilase was from Pentapharm, Basle, Switzerland. Anti-tPA and anti-uPA IgG antibodies were from Biopool, Umea, Sweden. Sodium dodecyl sulfate was from Pierce Chemical Company, Illinois, USA.

## Cultivation of Endothelial Cells

Human umbilical vein endothelial cells (HUVEC) were isolated according to Jaffe et al. (17). Human omental tissue microvascular endothelial cells (HOTMEC) were isolated as described previously (18).

The final cell suspensions of HOTMEC as well as of HUVEC were seeded on petri dishes (35  $\times$  10 mm), precoated with fibronectin and were grown to confluency in standard medium (SCM: Waymouth's MB 752-1 medium containing 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine) supplemented with 20% (vol/vol) fetal calf serum (FCS) and 100 µg/ml endothelial cell growth supplement (ECGS). ECGS was used until HOTMEC and HUVEC had grown to confluent monolayers after 4 to 6 days. At confluence, the cells obtained from at least 3 umbilical cords and the omental tissue of 3 patients were trypsinized (incubation with 1 ml 150 mM NaCl, 0.05% (wt/vol) trypsin, 1 mM EDTA for 3 minutes at 37 °C) from the dishes, were pooled and mixed with 40 ml SCM containing 20% (vol/vol) FCS and were then centrifuged at 180×g for 10 minutes. Afterwards the supernatants were discharged, the cells were resuspended in SCM supplemented with 20% (vol/vol) FCS and 100  $\mu g/ml$ ECGS and were seeded on fibronectin-coated petri dishes. Confluent monolayers of pooled and once passaged cells were used for the experiments. FCS used in this study was heat inactivated at 56 °C for

#### Fibrin Formation on the Surface of Endothelial Cells

The following reaction mixture was put on top of HUVEC and HOTMEC: 755 µl of SCM supplemented with 2% (wt/vol) BSA and 2 mM D(+)-glucose, 25 µl of 400 mM CaCl<sub>2</sub>, 20 µl of 10 µM human plasminogen and 100 µl of 20 mg/ml human fibrinogen. Fibrin was formed by the addition of 100 μl serum-free SCM containing 10 NIH U/ml αthrombin [purified as previously described (19)] or 25 units/ml reptilase. Clots formed within 2-3 minutes. Clot lysis was verified macroscopically by the observation of complete liquefaction of the cell supernatant and microscopically by the complete disappearance of the fibrin network from the surface of HOTMEC with residual fibrin clumps left in the liquified supernatant. In control experiments 25 μg/ml anti-tPa IgG or 50 μg/ml anti-uPA IgG were added to the clots. Clots were also formed in the absence of plasminogen. To increase PAI capacity in experiments with HOTMEC, exogenous PAI was incorporated into the clots by adding 150 μl melanoma cell-derived PAI (20) (mPAI, 1,000 arbitrary units per ml) to the reaction mixture before clot formation. mPAI was shown to be of the endothelial cell type, PAI-1 (20). When mPAI was added, 605 µl serum-free SCM were used in the reaction mixture.

#### Secretion Rate and Intracellular Levels of tPA

Confluent monolayers of identically cultivated endothelial cells of the same population as used for the clot lysis experiments  $(9.8\pm1.1\times10^5~\text{HOTMEC},~n=3)$ , and  $6.9\pm1.1\times10^5~\text{HUVEC},~n=3)$  were either incubated in SCM supplemented with 10% (vol/vol) FCS to determine the amount of tPA antigen released by the cells in the absence of a fibrin clot or were trypsinized from the dishes and lysed to determine intracellular tPA antigen levels at the time of clot formation (4).

## Incubation of Endothelial Cells with a-Thrombin

Confluent monolayers of endothelial cells were incubated for 1 hour at 37 °C with 0.0, 0.0001, 0.001, 0.01, 0.1, 1 and 10 NIH U/ml of purified

Table 1 Lysis of fibrin clots generated on the surface of human omental tissue microvascular endothelial cells (HOTMEC) and human umbilical vein endothelial cells (HUVEC)

Incubation mixtures	Lysis time range (n=3) (h)
HOTMEC + fibrin clot	2-3
HUVEC + fibrin clot	no lysis <sup>+</sup>
HOTMEC + fibrin clot + anti-tPA IgG	no lysis <sup>+</sup>
HOTMEC + fibrin clot + anti-uPA IgG	2–3
HOTMEC + fibrin clot + mPAI <sup>++</sup>	2–3

mPAI: melanoma cell-derived plasminogen inhibitor. tPA: tissue plasminogen activator. uPA: urokinase type plasminogen activator. <sup>+</sup> No lysis within 48 hours. <sup>++</sup> mPAI added in excess over the tPA activity of HOTMEC.

human  $\alpha$ -thrombin contained in 1,000  $\mu$ l SCM which was supplemented with 2% (wt/vol) BSA and 20 mM D(+)-glucose. During this period HUVEC and HOTMEC were observed whether they revealed a shape change reaction or not (21). Afterwards, the cells were washed twice with SCM and incubated in SCM containing 5% (vol/vol) heat inactivated FCS. After 6, 12 and 24 hours, the conditioned media were removed from the respective wells, and residual thrombin activity was neutralized by the addition of a five-fold excess of hirudin over the amount of thrombin initially used for incubation. Thereafter, ECCM was centrifuged at  $180 \times g$  for 10 minutes, and the supernatants were separated into aliquots and kept frozen at -30 °C up to 8 weeks until assessment.

#### Stability of PAI Activity in ECCM in the Absence of Cells

Studies were performed with ECCM of HOTMEC obtained after a 3 hours incubation period of the cells in SCM supplemented with 2% (wt/vol) BSA and 2 mM D(+)-glucose. At the end of the incubation period the conditioned media were centrifuged at  $180 \times g$  for 10 minutes and the supernatants were kept frozen at -30 °C up to 8 weeks before they were used. To study the stability of PAI in ECCM of HOTMEC 150 AU/ml of purified mPAI were added to these 3 hours ECCM initially containing  $35.6 \pm 3.4$  AU/ml PAI, n = 3. ECCM were then incubated at 37 °C for 3, 6, 9, 12 and 24 hours in the absence and in the presence of 1 NIH U/ml of  $\alpha$ -thrombin. At time points indicated, aliquots were taken from ECCM, and hirudin was added for thrombin neutralization as described above. These samples were kept frozen at -30 °C up to 1 week until assessment.

#### Assay Systems

tPA antigen was measured by an ELISA according to Korninger et al. (6). PAI cap was measured as described earlier (3). Melanoma cell-derived tPA, presenting a specific activity of 500,000 IU/mg, was used for titraton of PAI in the samples. One AU of active PAI represents the amount of PAI inhibiting 1 IU of tPA. For the determination of latent PAI, 100  $\mu$ l ECCM were incubated with 10  $\mu$ l 5% (wt/vol) sodium dodecyl sulfate at 37 °C for 15 minutes. The samples were then highly diluted for assessment to reduce the SDS concentration in the samples to values not influencing the assay system for PAI activity.

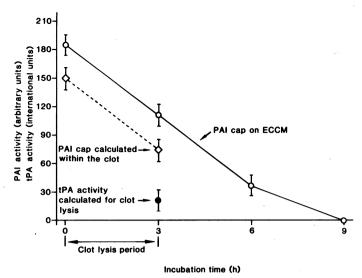
#### Results

## Clot Lysis by Endothelial Cells

The lysis of fibrin clots generated on the surface of HOTMEC and HUVEC was studied. Fibrin clots were made by mixing fibrinogen, plasminogen and thrombin on the surface of both cell types. Clot lysis was seen only on the surface of HOTMEC within 3 hours, whereas HUVEC did not lyse the clots but deteriorated under the fibrin films after 48 hours. Identical results were obtained when fibrinogen was clotted by reptilase. Clot lysis seen in experiments with HOTMEC was blocked by incorporating 25  $\mu g/ml$  anti-tPA IgG into the clot, but was not affected by 50  $\mu g/ml$  anti-uPA IgG. Incorporation of 150 AU PAI (representing an inhibitory activity towards 150 IU tPA) into the clots did not influence clot lysis (Table 1). Clot lysis activity of HOTMEC was found to be dependent on the incorporation of purified plasminogen into fibrin clots.

## tPA and PAI Activities During Clot Lysis

ECCM containing 35.6  $\pm$  3.4 AU/ml PAI cap (n = 3) were harvested from HOTMEC monolayers after an incubation period of 3 hours in serum-free medium, were supplemented with 150 AU/ml melanoma cell derived PAI and were incubated at 37 °C in the absence of cells to determine the stability of PAI activity in ECCM. A decrease in PAI activity over time was detected. In ECCM of HOTMEC containing 185.6  $\pm$  10.4 AU/ml PAI activity at the beginning of the incubation period a complete loss of PAI activity was found within 9 hours (Fig. 1). A decrease



Stability of PAI capacity in ECCM in the absence of HOTMEC and calculation of PAI activity and tPA activity during clot lysis. (O) 150 AU mPAI were added to 1 ml of ECCM of HOTMEC initially containing  $35.6 \pm 3.4$  AU PAI cap (mean  $\pm$  SD, n = 3). A decrease of 25 AU PAI activity per hour upon incubation at 37° C in the absence of cells was found. (()) Calculated decrease of 150 AU PAI cap incubated in a fibrin clot on the surface of HOTMEC during a 3 hours incubation period. A PAI activity of at least 75 AU can be expected within a fibrin clot during the clot lysis period. ( $\bullet$ ) tPA activity:  $32.5 \pm 6.8$  ng tPA (n = 3) were measured intracellulary and  $11.2 \pm 2.0$  ng tPA (n = 3) were released by the cells within 3 hours of incubation in the absence of fibrin. Thus, a maximal tPA antigen of  $43.7 \pm 5.2$  ng corresponding to  $21.9 \pm 2.6$ IU tPA activity was calculated for HOTMEC assuming a specific activity of 500,000 IU per mg. Therefore, an excess of PAI activity within the fibrin clot over tPA activity of HOTMEC was present throughout the clot lysis period

of about 25 AU/ml PAI cap per hour in ECCM of HOTMEC incubated at 37 °C was calculated. The stability of active PAI was not affected by 1 NIH U/ml  $\alpha$ -thrombin (data not shown). If fibrin clots containing 150 AU PAI cap were incubated on the surface of HOTMEC for 3 hours, at least 75 AU [150 -(3  $\times$  25)] PAI cap should be present during the entire 3 hours incubation time. Total tPA antigen of HOTMEC during this 3 hours clot lysis period was calculated by summing up the amount of tPA

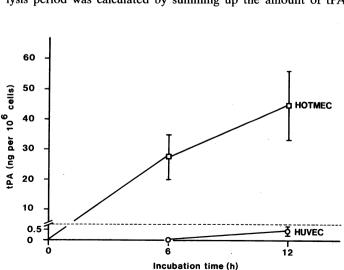
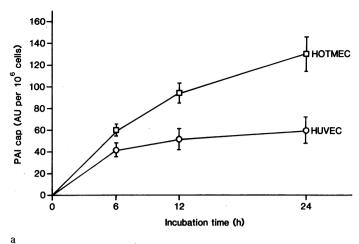


Fig. 2 Increase in tissue plasminogen activator (tPA) antigen in conditioned media of HUVEC ( $\bigcirc$ ) and HOTMEC ( $\square$ ) during a 12 hours incubation period. Mean  $\pm$  SD (n = 3) are given



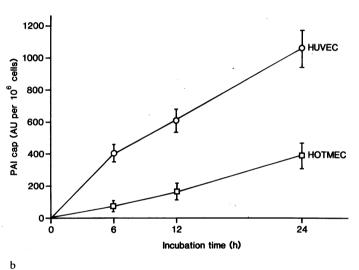


Fig. 3 Increase of plasminogen activator inhibitor capacity (PAI cap) in conditioned media of HUVEC ( $\bigcirc$ ) and HOTMEC ( $\square$ ) during a 24 hours incubation period. a) Changes in active PAI cap were measured in the absence of SDS. b) Changes in the combined active and latent PAI cap. The latent form of PAI cap was activated by the addition of SDS. Mean  $\pm$  SD (n = 3) are given

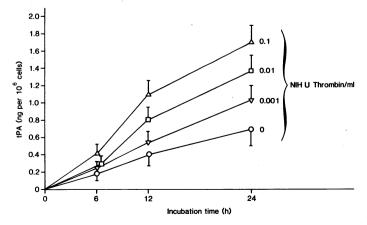


Fig. 4 The effect of  $\alpha$ -thrombin (0.0, 0.001, 0.01, 0.1 NIH U/ml) on the release of tPA antigen by HUVEC. Mean  $\pm$  SD (n = 3) are given

antigen measured intracellularly at the time of clot formation  $(32.5 \pm 6.8 \text{ ng}, \text{n} = 3)$  and the amount of tPA secreted into the culture medium within 3 hours in the absence of fibrin  $(11.2 \pm 2.0 \text{ ng}, \text{n} = 3)$ . Thus, a maximal tPA antigen of  $43.7 \pm 5.2 \text{ ng}$  (n = 3) corresponding to  $21.9 \pm 2.6$  IU tPA activity was calculated for HOTMEC assuming a specific activity of 500,000 IU per mg (22) (Fig. 1).

tPA and PAI Synthesis of HUVEC and HOTMEC in the Absence of Fibrin

In the absence of fibrin, HOTMEC released about 100-fold more tPA antigen than HUVEC into the conditioned media during a 12 hours incubation period (Fig. 2). In conditioned media of both cell types, tPA was inactive; both active and latent PAI could be measured (Fig. 3a, 3b).

Influence of Thrombin on tPA and PAI Synthesis and on the Morphology of HUVEC and HOTMEC

Control experiments were performed to measure the influence of thrombin on the release of tPA and PAI. At concentrations between 0.001, and 0.1 NIH U/ml a dose-dependent maximal 2.5-fold increase in tPA release was found in HUVEC (Fig. 4), whereas tPA secretion by HOTMEC and the levels of free active and latent PAI in conditioned media of both cell types were not affected by thrombin (data not shown). The typical shape change reaction (21) upon incubation with thrombin was detected in HUVEC at thrombin concentrations between 0.001 and 10 NIH U/ml, whereas the morphology of HOTMEC was not altered by the same concentrations of thrombin.

## **Discussion**

Endothelial cells (EC) are known to synthesize both tissue plasminogen activator (tPA) and plasminogen activator inhibitor (PAI). As inhibition of tPA by PAI is very fast and effective, and as unstimulated EC are known to release an excess of PAI over tPA, it has been assumed that fluid phase tPA activity is only generated if EC are stimulated to release an excess of tPA over PAI and thus overcome blood PAI capacity.

In the present study the fibrinolytic activities of cultured human umbilical vein endothelial cells (HUVEC) and cultured omental tissue microvascular endothelial (HOTMEC) were studied. For this fibrin clots containing plasminogen were formed on the surface of both cell types. HOTMEC lysed the clots within 2 to 3 hours, whereas HUVEC deteriorated under the fibrin films after 48 hours. It was surprising to find plasminogen activator activity associated with HOTMEC, as these cells secrete an excess of PAI over tPA in the absence of fibrin. To investigate the influence of fluid phase PAI activity at the cell surface on the fibrinolytic activity of HOT-MEC, purified melanoma cell derived PAI (mPAI) was incorporated into the fibrin clots. According to the determinations of the stability of mPAI in ECCM of HOTMEC in the presence of  $\alpha$ thrombin the inhibitor was used in concentrations two to three times higher than the maximal calculated tPA activity available by the cells during the clot lysis period, to assure an excess of PAI activity during clot lysis. Fibrinolysis mediated by HOTMEC was not affected by such an incorporation of PAI into the fibrin films.

HOTMEC contain an excess of PAI over tPA intracellulary and release an excess of PAI over tPA into the culture medium in the absence of a fibrin clot (4). Therefore, maximal tPA activity of the cells could not be measured but was calculated by summing up intracellular tPA antigen levels measured at the time of clot

formation and the amount of tPA antigen released by the cells during a 3 hours incubation period in the absence of a fibrin clot. Antigen values were converted into activity values taking into account the specific activities known from highly purified tPA preparations. Similar to the observations of others with HUVEC (23), PAI-1 (mPAI) was found to be unstable in HOTMEC ECCM. The fact the stability of PAI in ECCM of HOTMEC was not further decreased by  $\alpha$ -thrombin in our study in contrast to results obtained by others (23) may be due to the lower concentrations of  $\alpha$ -thrombin used in our experiments. Furthermore, different types of EC were used in the two studies.

We conclude from our experiments that the activation of plasminogen by tPA on the surface of HOTMEC can occur even in the presence of free PAI activity. A shift in the secretion rate of tPA and PAI in favour of free tPA is obviously no prerequisite for the generation of fibrinolytic activity on the surface of HOTMEC. although such a change in the ratio of tPA and PAI release in the presence of a fibrin clot could not be excluded in our experiments. The susceptibility of tPA to inhibition by PAI seems to be low in the presence of fibrin and HOTMEC. Similar observations were also made by other investigators who found that tPA is protected from inhibition by PAI in the presence of fibrin (2, 13). This could be due to the high affinity of tPA for fibrin or to an inhibitory effect of fibrin on PAI activity, which has not been clarified until now. It was also reported that tPA bound to EC surfaces is less susceptible to inhibition by PAI (24). Our experiments demonstrate the physiological importance of these phenomena, as HOTMEC are the only cultured human endothelial cells so far described which are capable of lysing fibrin clots formed on their

Binding and stabilization of PAI by the extracellular matrix was described recently (25, 26). Such antifibrinolytic effects of the matrix could not prevent the generation of free tPA activity on the surface of intact monolayers of cultured HOTMEC.

The finding that clot lysis mediated by HOTMEC was dependent on the incorporation of purified plasminogen into the clots and could be blocked by anti-tPA IgG but not by anti-uPA IgG demonstrates the major importance of tPA for fibrinolysis induced by HOTMEC. The high amounts of tPA synthesized by HOTMEC, 100-fold more than produced by HUVEC, may explain why cell-associated clot lysis was detected on the surface of microvascular but not of macrovascular endothelial cells.

As thrombin was used for clotting fibringen on the cells, the influence of this enzyme on tPA and PAI synthesis of HUVEC and HOTMEC was investigated. Despite quantitative differences, the findings of other groups (14-16) were basically confirmed that thrombin enhances tPA and PAI production by HUVEC. A dose-dependent maximal 2.5-fold increase in tPA synthesis was found in HUVEC at a thrombin concentration of 0.1 NIH U/ml. The levels of free active and latent PAI in ECCM of both cell types did not change upon incubation of the cells with thrombin. As, on the other hand, a thrombin mediated increase in tPA production by HUVEC was found, it is likely that this enhanced tPA synthesis was accompanied by an equivalent increase in PAI synthesis. In the present study, endothelial cells were incubated with thrombin only for 1 hour in contrast to other studies (14-16) in which the cells were incubated with thrombin for the entire incubation period; this may account for the quantitative differences in the thrombin-induced increase in tPA and PAI synthesis by HUVEC, reported here in comparison to other publications. Most interestingly, tPA and PAI production of HOTMEC were not affected by thrombin. Furthermore, in contrast to HUVEC, HOTMEC did not show the typical shape change reaction (21) upon incubation with thrombin although HOTMEC can equally mediate protein C activation as HUVEC (4). The shape change reaction is thought to enhance the

procoagulant activities of the vessel wall by exposing intercellular subendothelial structures for further activation of coagulation in case of thrombin formation. The observation that HOTMEC lack this reactivity towards thrombin may be of physiological importance for the limitation of procoagulant activities in the microcirculation as thrombin formed within the vasculature mainly binds to endothelial cells of capillary beds (27). The different reactivities of HUVEC and HOTMEC towards thrombin may be due to a different pattern of thrombin receptors on the cell surface.

Our results provide evidence that in certain parts of the microcirculation the high base line tPA production and the protective effect of fibrin and the endothelial cell surface on tPA against inhibition by PAI ensure immediate plasminogen activation and fibrinolysis in the case of fibrin generation within microvessels although differences in the fibrinolytic properties of various capillary beds cannot be excluded (28). Macrovascular endothelial cells were on the other hand found to synthesize only limited amounts of tPA and fibrinolytic activity could not be detected on the cell surface, even if tPA production was stimulated by a-thrombin. These findings obtained with cultured human endothelial cells are in agreement with observations of Pandolfi et al. (29) who found plasminogen activator activity associated with endothelial cells of microvessels (Vasa vasorum) but not with macrovascular endothelial cells (Vena saphena magna) using Todd's fibrin overlay technique. The high fibrinolytic activity of microvascular EC may be one of the reasons for the rare incidence of microvessel thrombosis compared to macrovessel thrombosis.

#### Acknowledgements

The authors are grateful to Gilda Kierok and Beate Schuster for skilful technical assistance.

#### References

- Loskutoff D J, Levin E. Properties of plasminogen activators produced by endothelial cells. In: Biology of Endothelial Cells. Jaffe E A (ed). Martinus Nijhoff Publishers, Boston The Hague 1984; pp 200–8.
- 2 Kruithof E K O, Tran-Thang C, Ransijn A, Bachmann F. Demonstration of a fast-acting inhibitor of plasminogen activators in human plasma. Blood 1984; 64: 907-13.
- 3 Speiser W, Bowry S, Anders E, Binder B R, Müller-Berghaus G. Method for the determination of fast acting plasminogen activator inhibitor capacity (PAI-cap) in plasma, platelets and endothelial cells. Thromb Res 1986; 44: 503-15.
- 4 Speiser W, Anders E, Preissner K T, Wagner O, Müller-Berghaus G. Differences in coagulant and fibrinolytic activities of cultured human endothelial cells derived from omental tissue microvessels and umbilical veins. Blood 1987; 69: 964-7.
- 5 Bachmann F, Kruithof E K O. Tissue plasminogen activator: chemical and physiological aspects. Sem Thromb Haemostas 1984; 10: 6-17.
- 6 Korninger C, Speiser W, Wojta J, Binder B R. Sandwich ELISA for t-PA antigen employing a monoclonal antibody. Thromb Res 1984; 41: 527-35.
- 7 Clarke R L, Orandi A, Cliffton E E. Induction of fibrinolysis by venous obstruction. Angiology 1960; 11: 367–70.
- 8 Marsh N, Gaffney P. Some observations on the release of extrinsic and intrinsic plasminogen activators during exercise in man. Haemostasis 1980; 9: 238–47.
- 9 Manucci P M, Rota L. Plasminogen activator response after DDAVP: a clinico-pharmacological study. Thromb Res 1980; 20: 69-76.
- 10 Stibbe J, Kluft C, Brommer E J P, Gomes M, De Jong D S, Nauta J. Enhanced fibrinolytic activity during cardiopulmonary bypass in open

- heart surgery in man is caused by extrinsic (tissue-type) plasminogen activator. Eur J Clin Invest 1984; 14: 375–82.
- 11 Speiser W, Wojta J, Korninger C, Kirchheimer J C, Zazgornik J, Binder B R. Enhanced fibrinolysis caused by tissue plasminogen activator release in hemodialysis. Kidney Int 1987; 32: 280-3.
- 12 Wiman B, Mellbring G, Ranby M. Plasminogen activator release during venous stasis and exercise determined by a new specific assay. Clin Chim Acta 1983; 127: 279–83.
- 13 Wun T C, Capuano A. Initiation and regulation of fibrinolysis in human plasma at the plasminogen activator level. Blood 1987; 69: 1354-62.
- 14 Levin E G, Marzec U, Anderson J, Harker L A. Thrombin stimulates tissue plasminogen activator release from cultured endothelial cells. J Clin Invest 1984; 74: 1955–8.
- 15 Levin E G, Stern M D, Nawroth P P, Marlar R A, Fair D S, Fenton J W, Harker L A. Specificity of the thrombin-induced release of tissue plasminogen activator from cultured human endothelial cells. Thromb Haemostas 1986; 56: 115-9.
- 16 Gelehrter T D, Sznycer-Laszuk R. Thrombin induction of plasminogen activator-inhibitor in cultured human endothelial cells. J Clin Invest 1986; 77: 165-9.
- 17 Jaffe E A, Nachman R L, Becker C G, Minick C R. Culture of human endothelial cells derived from umbilical veins. J Clin Invest 1973; 52: 2745-6.
- 18 Anders E, Alles J U, Delvos U, Pötzsch B, Preissner K T, Müller-Berghaus G. Microvascular endothelial cells from human omental tissue: modified method for long-term cultivation and new aspects of characterization. Microvasc Res 1987; 34: 239-49.
- 19 Preissner K T, Wassmuth R, Müller-Berghaus G. Physicochemical characterization of human S-protein and its function in blood coagulation system. Biochem J 1985; 231: 349-55.
- 20 Wagner O F, Binder B R. Purification of an active plasminogen activator inhibitor immunologically related to the endothelial type plasminogen activator inhibitor from the conditioned media of a human melanoma cell line. J Biol Chem 1986; 261: 14474–9.
- 21 Laposata M, Dovnarsky D K, Shin H S. Thrombin-induced gap formation in confluent endothelial cell monolayers in vitro. Blood 1983; 62: 549-56.
- 22 Gaffney P J, Curtis A D. A collaborative study of a proposed international standard for tissue plasminogen activator (t-PA). Thromb Haemostas 1985; 53: 134-6.
- 23 De Fouw N J, van Hinsbergh V W M, de Jong Y F, Haverkate F, Bertina R M. The interaction of activated protein C and thrombin with the plasminogen activator inhibitor released from human endothelial cells. Thromb Haemostas 1987; 57: 176-82.
- 24 Hajjar K A, Hamel N M, Harpel P C, Nachman R L. Binding of tissue plasminogen activator to cultured human endothelial cells. J Clin Invest 1987; 80: 1712–9.
- 25 Levin E G, Santell L. Association of a plasminogen activator inhibitor (PAI-1) with the growth substratum and membrane of human endothelial cells. J Cell Biol 1987; 105: 2543-9.
- 26 Mimuro J, Schleef R R, Loskutoff D J. Extracellular matrix of cultured bovine aortic endothelial cells contains functionally active type 1 plasminogen activator inhibitor. Blood 1987; 70: 721-8.
- 27 Owen W G. Interactions of thrombin, antithrombin III, and protein C with endothelium. In: Biology of Endothelial Cells. Jaffe E A (ed). Martinus Nijhoff Publishers, Boston The Hague 1984; pp 259-67.
- 28 Van Hinsbergh V W M, Sprengers E D, Kooistra T. Effect of thrombin on the production of plasminogen activators and PA inhibitor-1 by human foreskin microvascular endothelial cells. Thromb Haemostas 1987; 57: 148-53.
- 29 Pandolfi M, Robertson B, Isacson S, Nilsson I M. Fibrinolytic activity of human veins in arms and legs. Thromb Diath Haemorrh 1968; 20: 247–56.

Received February 16, 1988 Accepted after revision August 5, 1988