Enhanced release of soluble urokinase receptor by endothelial cells in contact with peripheral blood cells

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Received 2 October 2000; revised 17 November 2000; accepted 17 November 2000

First published online 29 November 2000

Edited by Masayuki Miyasaka

Abstract The urokinase receptor (uPAR) on the cell surface plays an important role in extracellular proteolysis, cell migration and adhesion. Soluble uPAR (suPAR) has been recently discovered in plasma, but its origin is unclear. Our results now demonstrate that both unstimulated blood mononuclear and endothelial cells can release suPAR and that the release is enhanced when either mononuclear cells or thrombocytes are cultured together with endothelial cells. Co-culture without cell–cell contacts fails to enhance suPAR release. We also found suPAR fragments, known to be potent inducers of chemotaxis, in co-culture growth medium samples. Taken together, our results suggest that normal plasma suPAR can be produced by endothelial and mononuclear cells and that suPAR release in cell–cell contacts may have a regulatory role in cell adhesion.

Key words: Urokinase receptor; Soluble urokinase receptor; Cell–cell contact; Endothelial cell; Blood cell

1. Introduction

The urokinase-type plasminogen activator (uPA)-mediated plasminogen activation cascade has been shown to be important in extracellular proteolysis in normal physiology and cancer pathophysiology [1]. However, in recent years, growing evidence has suggested that uPA and its high-affinity cell surface receptor (uPAR) are not only important in proteolytic events, but have an equally important role in cell migration, adhesion and chemotaxis [2–4].

uPAR is bound to the cell surface via a glycosylphosphatidylinositol (GPI) anchor and consists of three homologous domains (D1–D3), which have different binding properties for various ligands [5]. With its amino-terminal D1 domain, uPAR binds uPA, whereas domains 2+3 (D2D3) are important in vitronectin binding [6]. The full-size receptor, however, is needed for high-affinity interactions [7,8]. Several enzymes, including uPA and plasmin, are capable of cleaving uPAR between domains 1 and 2+3 [9,10]. Yet, the physiological enzyme responsible for cleavage of the whole receptor from the cell surface has remained unclear, although in vitro results have suggested that GPI-specific phospholipase D may catalyze receptor shedding from the cell surface [11]. Several members of β1-, β2- and β3-integrin families have also been associated with uPAR, indicating its strong involvement in cell adhesion [2,12,13].

In body fluids, such as plasma, urine and ascitic fluid, a soluble form of uPAR (suPAR) has been discovered in recent years [14–18]. In healthy individuals, plasma suPAR levels are quite low [15]. However, in cancer patients, suPAR levels are elevated at diagnosis [18–20] and they are also associated with poor survival prognosis in leukemia [20] and in ovarian and colon cancer [18,19]. Furthermore, our studies in leukemia have shown that plasma suPAR levels decrease rapidly during chemotherapy concomitantly with the disappearance of tumor cells [21], suggesting that tumor cells produce plasma suPAR. However, in healthy individuals, the origin of plasma suPAR and the physiological events leading to its release from the cell surface have remained obscure. We have therefore studied the shedding of uPAR from endothelial and leukocyte cell surfaces under physiological conditions and the presence of suPAR and various uPAR fragments in the growth media of these cells. In addition, we have analyzed the effect of cell–cell interactions on suPAR release in co-culture experiments.

2. Materials and methods

2.1. Materials

Anti-uPAR antibodies (R2, R3 and R4) were kindly provided by Dr. Gunilla Høyer-Hansen (Finsen Laboratory, Copenhagen, Denmark). Cell culture growth medium RPMI 1640, fetal bovine serum (FBS), glutamine, penicillin and streptomycin were purchased from Gibco-BRL (Karlsruhe, Germany). Transwell co-culture plates were from Costar (Corning Inc., Corning, NY, USA) and Ficoll-Paque from Amersham Pharmacia (Uppsala, Sweden). Protease inhibitor tablets (Complete™) and immobilized streptavidin were from Boehringer Mannheim (Mannheim, Germany). BCA protein assay kit and chemiluminescent substrate (SuperSignal Ultra) were from Pierce (Rockford, IL, USA).

2.2. General cell cultures

Human microvascular endothelial cells (HMEC-1) [22], kindly provided by Dr. T.J. Lawley (Emory University School of Medicine, Atlanta, GA, USA), were grown in RPMI 1640 in the presence of 10% FBS containing 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. Peripheral blood mononuclear cells and thrombocytes were separated by Ficoll-Paque centrifugation from EDTA

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Abbreviations: uPA, urokinase-type plasminogen activator; (s)uPAR, (soluble) uPA receptor; D1, domain 1 of uPAR; D2D3, domains 2+3 of uPAR; GPI, glycosylphosphatidylinositol; ECM, extracellular matrix; PMA, phorbol 12-myristate 13-acetate; ELISA, enzyme-linked immunosorbent assay

0014-5793/00/$20.00
PII: S0014-5793(00)02312-7

blood of healthy donors. After separation, the cells were washed twice with phosphate-buffered saline (PBS) and suspended in RPMI 1640. Purified thrombocytes were also obtained from the Finnish Red Cross Blood Transfusion Service (Helsinki, Finland). Only freshly isolated cells were used.

2.3. Co-cultures

Endothelial cells (10^5 cells/0.4 ml of growth medium) were seeded in 48-well plates and left to form monolayers overnight at +37°C in a humidified 5% CO_2 atmosphere. The next morning, the growth medium was removed, and freshly isolated blood mononuclear cells (2.5×10^5 cells/0.4 ml of growth medium) or thrombocytes were added either to the endothelial cells or directly to empty wells. After 4, 8, 12, 24, 48 and 72 h of culture, the growth media were collected, centrifuged and stored frozen until assay.

Cells were also co-cultured in Transwell plates (12-well plate, polycarbonate membrane with 0.4 μm pore size). Endothelial cells (2.5×10^5/ml) were grown in the lower chamber and mononuclear cells (6.3×10^5/ml) or thrombocytes were added either to the lower chamber on the endothelial cells to allow for cell–cell contacts or into the upper chamber of the same wells to share of the growth medium without cell–cell contacts. Growth media were collected at 1–3 days of culture. Cells were counted from different wells after growth medium collection, and the number of cells was found to be equal in the different experimental settings.

Blood mononuclear cells were removed from the co-culture after 3 days. After washings, the endothelial cells were harvested from the culture plates with cell scrapers and lysed in PBS containing 1% Triton X-100 and protease inhibitors (Complete™). The lysates were kept on ice for 1 h, centrifuged for 10 min at $14\,000\times g$ at 4°C, and the supernatants were stored at −70°C until assay. The amount of total protein was determined by the BCA protein assay kit.

2.4. Incubation of peripheral blood

Peripheral citrated or heparinized whole blood was obtained from healthy volunteers. Immediately after the collection and after 1, 2 and 3 days of incubation on a rotator at 4°C or at 37°C, the plasma was separated by centrifugation for 30 min at 4°C at $1800\times g$ and stored frozen in aliquots at −70°C until analysis.

2.5. Assay of soluble uPAR and uPAR in cell lysates

The uPAR enzyme-linked immunosorbent assay (ELISA) has been described previously [15]. It uses a polyclonal rabbit anti-human uPAR as a catching antibody and a mixture of monoclonal antibodies (R2, R3 and R5) as detecting antibodies. 1:2 dilutions of growth media, 1:10 dilutions of plasma samples and a volume equal to 20 μg of protein from the cell lysates were used in the ELISA.

2.6. Immunoprecipitation and immunoblotting

The methods for immunoprecipitation and immunoblotting have been described previously [23]. In brief, growth media and cell lysate samples were immunoprecipitated with biotinylated R2 and R3 monoclonal antibodies prebound to immobilized streptavidin. Immunoprecipitated proteins were fractionated by 12% SDS–PAGE under non-reducing conditions. The proteins were transferred to nitrocellulose membranes and detected with polyclonal rabbit anti-uPAR IgG, with chemiluminescent visualization of the complexes.

2.7. Immunofluorescence staining

Cells were grown on glass coverslips in multi-well culture plates. After culturing, plates were transferred on ice, and cells on the coverslips were washed twice with Dulbecco's balanced salt solution supplemented with 0.5% bovine serum albumin. After washings, monoclonal anti-uPAR antibodies (R4) (10 μg/ml) or control antibodies were added on coverslips and incubated for 30 min on ice, after which cells were washed again. Before secondary antibody addition, cells were fixed with ice-cold methanol for 15 min at −20°C, followed by thorough washings and incubation with secondary FITC-conjugated antibodies. Finally, cells were washed again, and coverslips were mounted on microscope slides.

2.8. Statistical analysis

Results are reported as mean ± S.E.M. Student's unpaired and paired *t*-tests were used for comparison of results. Results were considered significant when $P = 0.05$.

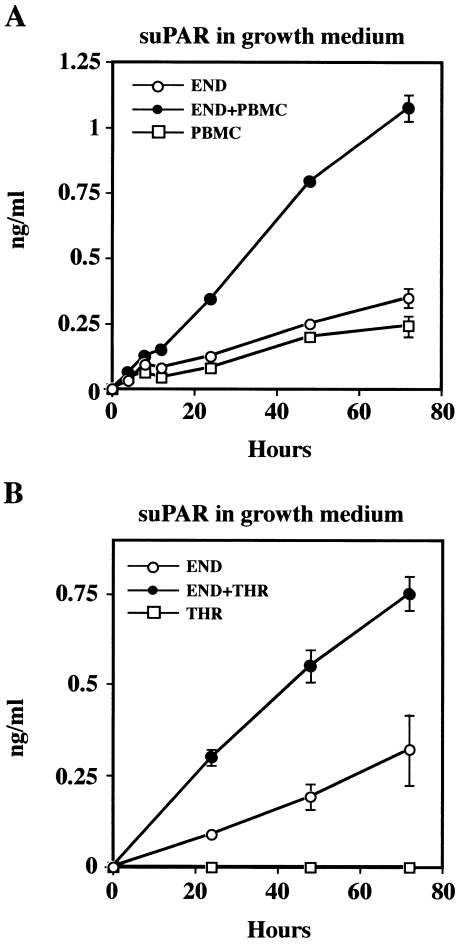

Fig. 1. Secretion of suPAR by endothelial cells and blood cells in culture. Freshly isolated, unstimulated peripheral blood mononuclear cells (PBMC) (A) or thrombocytes (THR) (B) were cultured either alone or with endothelial cells (END) in normal cell culture conditions. Samples of growth media were collected at 4, 8, 12, 24, 48 and 72 h of culture, and suPAR levels were measured with uPAR ELISA. Shown are the mean ± S.E.M. values from three separate experiments.

3. Results

3.1. Secretion of suPAR by cultured endothelial cells and blood mononuclear cells

Both endothelial and blood mononuclear cells are candidate sources of the plasma suPAR, because they are known to express uPAR. Therefore, we wanted to analyze the release of suPAR by these cells and by freshly isolated thrombocytes in normal cell culture conditions. Our results demonstrate that both unstimulated endothelial cells and peripheral blood mononuclear cells are able to secrete small amounts of suPAR (0.48 and 0.13 ng/10^6 cells, respectively, in 24 h) into their growth medium (Fig. 1A). Lysates of these cells also contained uPAR as detected with uPAR ELISA and with immunoprecipitation followed by immunoblotting. In contrast, purified fresh thrombocytes did not contain any uPAR and neither did they secrete suPAR (Fig. 1B), and even after stimulation with phorbol 12-myristate 13-acetate (PMA) or adherence to collagen, no suPAR was detected in their growth medium (results not shown).

We also incubated citrated or heparinized whole blood

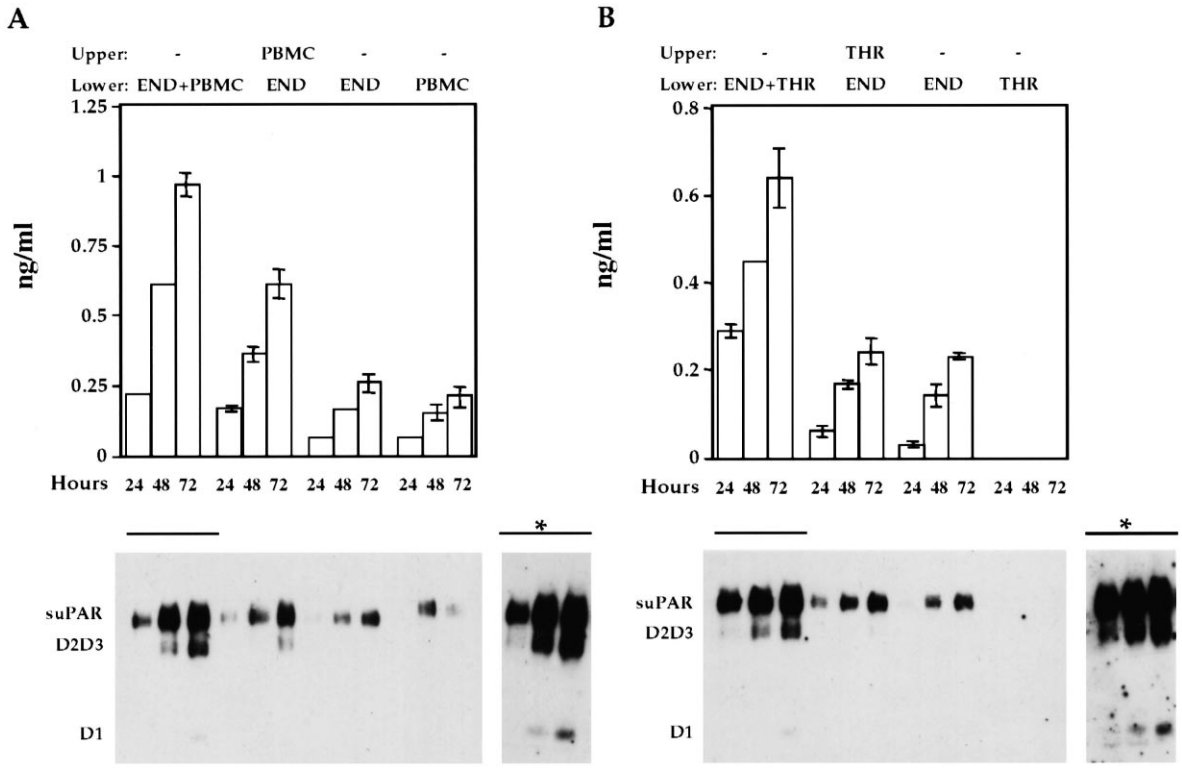

Fig. 2. Secretion of suPAR in co-cultures. Endothelial cells (END) were co-cultured with blood mononuclear cells (PBMC) (A) or with thrombocytes (THR) (B) in Transwell chambers either with or without cell–cell contacts. suPAR levels were measured with uPAR ELISA and the mean ± S.E.M. values from three separate experiments are shown. Samples of growth media were also immunoprecipitated with monoclonal uPAR antibodies; the lower panel shows a representative immunoblotting experiment done with polyclonal uPAR antibodies. The panels marked with an asterisk show longer exposures of co-culture growth medium samples.

from healthy individuals on a rotator at 4 or 37°C to see if blood cells can release suPAR in ex vivo conditions. When blood was incubated at physiological temperature (37°C) allowing normal cell functions, a significant increase in the plasma suPAR level was already seen after 1 day of incubation (1.32 ± 0.21 vs. 0.71 ± 0.12 ng/ml basal level; $P < 0.02$, paired t-test) and a further increase to 1.93 ± 0.19 ng/ml after 3 days of incubation ($P < 0.001$, paired t-test). No significant change in suPAR level was observed in blood incubated at 4°C.

3.2. *Higher amounts of suPAR in the growth media of endothelial cells co-cultured with blood mononuclear cells or with thrombocytes*

In circulation, blood cells are in close contact with endothelial cells, and prior to extravasation, leukocytes need to adhere to endothelial cells. Therefore, we wanted to study the production of suPAR in co-cultures and grew endothelial and blood mononuclear cells either together or alone. From these cultures, growth media were collected at 4, 8, 12, 24, 48 and 72 h. After 24 h, the suPAR level was already significantly increased in endothelial cell+blood mononuclear cell co-culture media as compared with growth media in which either endothelial cells or mononuclear cells had grown alone, and a similar trend was observed in samples collected at 48 and 72 h (Fig. 1A). After 72 h, suPAR levels were still quite low in media in which either endothelial or mononuclear cells had been grown alone (0.35 ± 0.06 and 0.24 ± 0.05 ng/ml, respectively), but in co-culture media, the suPAR level (1.07 ± 0.07 ng/ml) was double the amount expected from addition of endothelial+mononuclear cell values (Fig. 1A). The number of cells in co-cultures was equivalent to that of cultures where either cell type was grown alone.

Purified thrombocytes were similarly co-cultured with endothelial cells. The amount of suPAR was already significantly higher at 24 h (0.30 ± 0.03 ng/ml) in the co-culture growth medium as compared with the situation where endothelial cells were grown alone (0.09 ± 0.01 ng/ml; $P < 0.01$, Student's unpaired t-test) (Fig. 1B). In addition, no suPAR was detected in the growth medium where thrombocytes had been cultured alone. As already observed for mononuclear leukocytes, the difference in suPAR levels continued at 48 and 72 h of culture (Fig. 1B).

3.3. *Cell–cell contacts needed for induced suPAR secretion to the growth medium*

To examine whether cell–cell contacts were required for the enhanced suPAR secretion to the growth medium or whether soluble molecules in a shared growth medium were sufficient to induce this synergistic effect, cells were co-cultured in Transwell chambers. When blood mononuclear cells were grown in the upper Transwell chambers without cell–cell contacts to endothelial cells in the lower chambers, the suPAR levels in the growth medium did not differ notably from combined suPAR values of separately growing cells (Fig. 2A). This was even more evident in thrombocyte–endothelial cell co-culture. When thrombocytes were cultured in the upper chamber without cell–cell contact to endothelial cells, the suPAR values in the growth media were equal to the values of endothelial cells grown alone (0.24 vs. 0.23 ng/ml after 72 h, respectively) (Fig. 2B). In contrast, when both cells were cul-

tured in contact with each other in the lower chamber, the suPAR level was markedly increased (0.64 ng/ml), suggesting the requirement of cell–cell contacts for the synergistic effect (Figs. 1 and 2).

3.4. Different forms of cleaved suPAR in culture medium samples

suPAR fragments have been shown to induce chemotactic effect in vitro [3,4]. To determine the forms of suPAR antigen and the possible presence of uPAR fragments in endothelial/blood cell culture media, the samples were immunoprecipitated with uPAR antibodies and run in SDS–PAGE. Immunoblotting confirmed the results obtained by uPAR ELISA, and more suPAR was seen in culture media of cells in contact with each other in co-cultures (Fig. 2). Notably, both full-length receptor (55 kDa) and D2D3 (35 kDa) fragment were detected. Longer exposure to chemiluminescent visualization showed that the D1 (15 kDa) fragment was also present in growth medium samples of cells cultured together (Fig. 2).

Interestingly, the molecular weight of suPAR in culture medium samples of blood mononuclear cells cultured alone seemed to be slightly higher than that of suPAR in endothelial cell media or in co-culture growth media (Fig. 2A), suggesting that the increased level of suPAR found in co-cultures was derived from endothelial cells and not from mononuclear cells.

3.5. Increased amount of uPAR in endothelial cell lysates and increased intensity of cell surface uPAR staining in endothelial cells co-cultured with peripheral blood cells

Most likely, in thrombocyte–endothelial cell co-cultures, endothelial cells were responsible for the enhanced suPAR release, because thrombocytes failed to express and release suPAR. However, in blood mononuclear–endothelial cell co-cultures this was unclear, because both of these cell types had uPAR on the surface. Therefore, we examined the presence and the amount of uPAR on endothelial cell surfaces and in the lysates of cells cultured for 3 days either with or without blood mononuclear cells or thrombocytes. Before making lysates, the endothelial cells were washed and only a few mononuclear cells or thrombocytes remained attached after washings. Equal amounts of protein of the lysates were analyzed with uPAR ELISA and with immunoprecipitation followed by immunoblotting. Both methods showed that increased amounts of uPAR were present in endothelial cell lysates of cells co-cultured either with mononuclear cells or with thrombocytes as compared with lysates made of endothelial cells cultured alone (Fig. 3). Immunoblotting revealed that both full-size uPAR and D2D3 fragments were present in endothelial cell lysates, and importantly, in endothelial cell lysates co-cultured either with mononuclear cells or with thrombocytes, more fragmented uPAR was found (Fig. 3).

In addition, endothelial cells were cultured on glass coverslips with purified blood mononuclear cells or with thrombocytes for 24 h, after which cells were stained with uPAR antibodies. When endothelial cells were cultured alone, they expressed small amounts of uPAR on the cell surface, and some staining was also seen in stria-like structures (Fig. 4). After co-culturing with mononuclear leukocytes or with thrombocytes, the intensity of uPAR staining was markedly higher in endothelial cells and the localization of uPAR differed in various co-cultures (Fig. 4). In co-cultures with leukocytes, uPAR staining seemed to concentrate in focal adhesion-like structures, whereas in thrombocyte–endothelial cell co-cultures, staining was more diffuse (Fig. 4). Similar differences were also observed after 48 h of co-culturing.

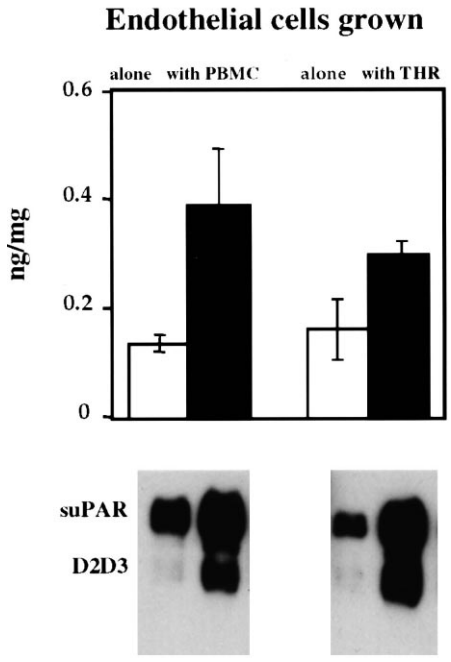

Fig. 3. uPAR in endothelial cell lysates. Endothelial cells were either cultured alone or with blood mononuclear cells (PBMC) or with thrombocytes (THR), and cells were collected and lysed after 3 days of culture. The upper panel shows mean uPAR levels in cell lysates from three different experiments measured with uPAR ELISA, and the lower panel shows a representative experiment of immunoblotting of immunoprecipitated lysate samples. Equal amounts of protein were taken from each sample for ELISA (20 μg) and immunoprecipitation analysis (40 μg).

Taken together, both the increased intensity of uPAR staining on the endothelial cell surfaces and increased amount of uPAR in cell lysates suggest that the source of enhanced suPAR level in co-cultures is endothelial cells.

4. Discussion

This study demonstrates that both unstimulated endothelial cells and blood mononuclear cells are able to produce suPAR in cell culture conditions. We were also able to show that suPAR release is enhanced in co-culture conditions when either blood mononuclear cells or thrombocytes are cultured in cell–cell contact with endothelial cells, while without cell–cell contacts, induced suPAR release was not observed. In addition, the results suggest that in co-cultures the endothelial cells are responsible for this enhanced suPAR release, as in endothelial cell lysates and on the endothelial cell surface uPAR levels were increased after co-culturing, and furthermore, because thrombocytes fail to express uPAR. Different forms of uPAR fragments were also observed in growth medium and cell lysate samples.

suPAR was first discovered in the ascitic fluid of ovarian cancer patients [14], and since then, elevated levels of suPAR have been found in plasma or serum of patients with various types of cancer [15,16,18–20]. The origin of suPAR in cancer

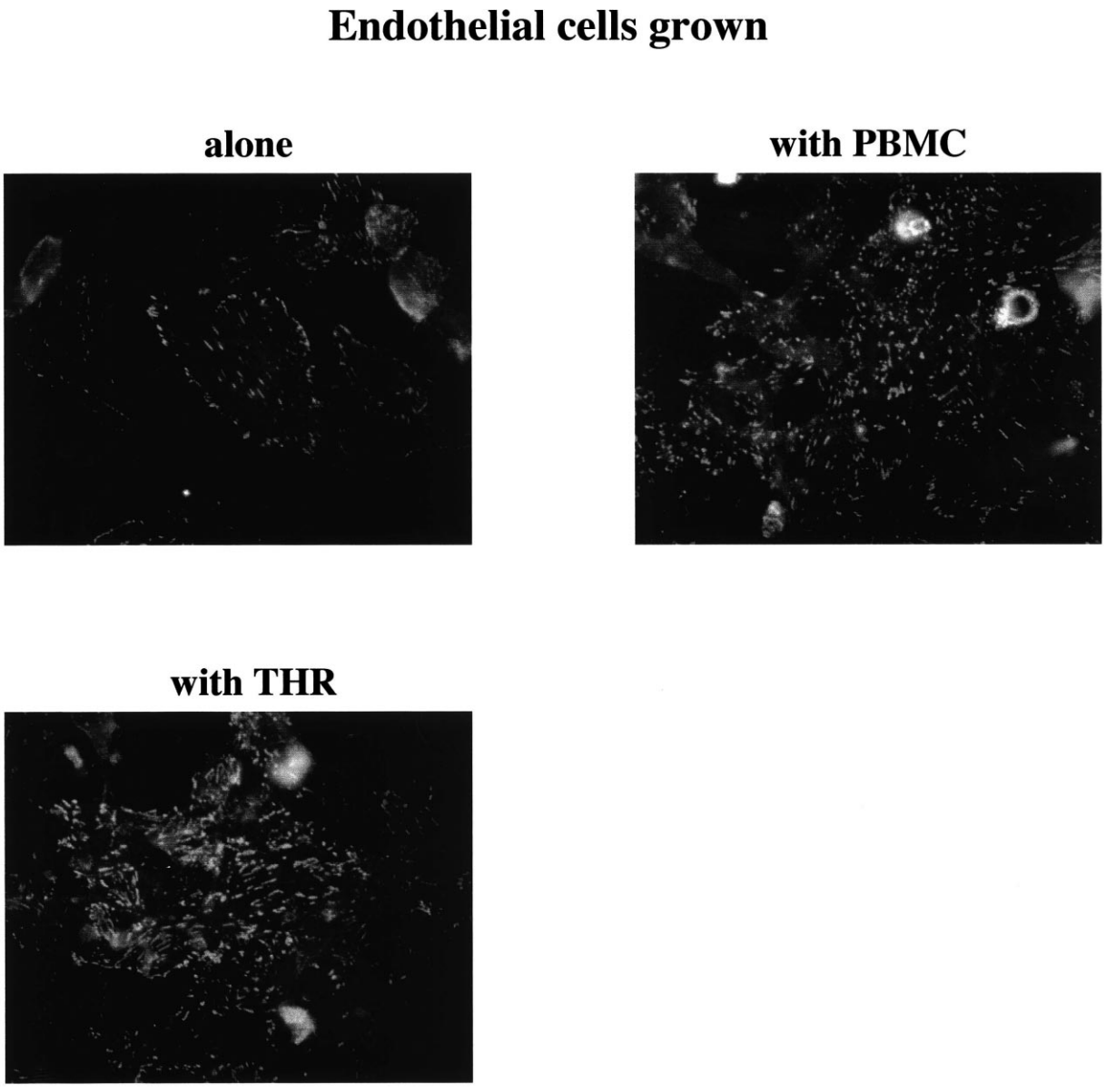

Fig. 4. Expression of uPAR in endothelial cells. Endothelial cells were cultured for 24 h either alone or with peripheral blood mononuclear cells or with thrombocytes on the glass coverslips. Cells were stained with monoclonal anti-uPAR antibodies (R4), and detection was based on immunofluorescence method. Magnification 500×.

patients' plasma has been debated, but recent reports have shown that human suPAR can be found in the serum samples of mice carrying human xenograft tumors [24], indicating that the tumors are, at least partially, the source of suPAR in plasma. In addition, our studies in leukemia patients, showing that plasma suPAR levels correlate closely with the tumor cell load in circulation and decrease rapidly during chemotherapy concomitant with the disappearance of tumor cells [21], support this possibility. In normal blood cells, uPAR is expressed in monocytes and granulocytes, but not in unstimulated lymphocytes or thrombocytes [25,26]. In adherent cells, uPAR is also present in various cell types, e.g. in endothelial cells, smooth muscle cells and keratinocytes [5]. The U937 monocytic cell line has recently been shown to produce suPAR in culture [23,27], but to our knowledge, the present report is the first demonstration that blood cells produce suPAR in the plasma and that in normal cell culture conditions mononuclear cells and endothelial cells release suPAR to their growth medium without stimulation.

Under normal physiological conditions, leukocytes circulate in the blood without adhering to endothelial cells. When inflammation or other stimuli occur, leukocytes start to adhere to endothelial cells and after extravasation, migrate to the site of inflammation. On one hand, they need to adhere to endothelial cells and to the extracellular matrix (ECM), but on the other hand, they must detach to be able to move on. Notably, our results showed that more suPAR is released to the culture medium when cells are in physical contact with each other. This may be a feedback mechanism for cells to control their adherence to ECM, as uPAR has been shown to be an important adhesion receptor for vitronectin and also because uPAR is associated with many ECM integrins [2,12,13]. Furthermore, removal of uPAR from the cell surface has been shown to prevent β2-integrin-mediated leukocyte adhesion to

the endothelium [28]. However, if uPAR is released from the cell surface to the growth medium, the plasminogen activation cascade diminishes on the cell surface and cells lose part of their proteolytic activity.

In vitro experiments have shown that recombinant suPAR fragments and chymotrypsin-cleaved suPAR possess strong chemotactic activity [3,4]. These fragments have now been found in various biological fluids, e.g. urine [17,23] and ascitic and cystic fluids of cancer patients [16]. In normal plasma, only a full-length receptor has been found thus far, but in leukemia patients' plasma samples, we have also observed cleaved suPAR [21]. Our results now indicate that suPAR fragments occur in growth media of endothelial cells cultured together with mononuclear cells or with thrombocytes. Furthermore, fragment D1, which has thus far only been found in urine samples and in culture media of PMA-stimulated U937 cells, was present in co-culture growth medium samples. The reason for cleaved fragments not being observed in plasma samples of healthy individuals may be that the half-life of suPAR fragments is very short in circulation and that mostly full-length receptors are released. Our experimental setting allowing mononuclear cells to adhere to endothelial cells may represent more closely the in vivo situation at the site of injury or inflammation when cells start to adhere to the endothelium and extravasate. Based on this, it can be speculated that suPAR release and especially the release of suPAR fragments in cell–cell contacts may be a chemotactic stimulus of these cells to recruit other cells.

Which type of cells in endothelial cell and blood mononuclear cell co-cultures are responsible for the enhanced suPAR release? Our results show that after co-culturing cell surface expression of uPAR is enhanced in endothelial cells and that their lysates also contain more uPAR, which suggests that endothelial cells may be activated and produce more suPAR in the growth medium. This view is supported by thrombocyte experiments. Earlier reports have shown that thrombocytes do not contain uPAR [25], i.e. they are not able to secrete suPAR either. To confirm that they are unable to release suPAR even when stimulated, we treated thrombocytes with PMA or allowed them to adhere to collagen, which is known to activate thrombocytes. The thrombocytes, however, failed to produce suPAR.

In conclusion, our results show that suPAR can be produced by endothelial cells and blood mononuclear cells in culture, suggesting that normal suPAR found in plasma is, at least in part, produced by these cells. Furthermore, we have demonstrated that cell–cell contacts are needed to enhance suPAR release to the growth medium in endothelial–blood cell co-cultures. However, further studies are needed to clarify which factors influence suPAR shedding from the cell surface and to learn whether suPAR has a biological role in soluble form in the circulation.

Acknowledgements: This work was supported by grants from the Foundation for the Finnish Cancer Institute, the Finnish Cancer Societies, the Finnish Medical Foundation, the Finnish Association of Haematology and the Finnish Society of Haematology. We thank Professor F. Blasi for helpful discussions and Dr. G. Høyer-Hansen for the kind gift of uPAR antibodies.

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