Rapid inactivation of the plasminogen-activator inhibitor upon secretion from cultured human endothelial cells

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In conditioned medium (CM) from cultured human endothelial cells, two forms of plasminogen-activator inhibitor (PA-inhibitor) can be demonstrated: a fast-acting active form and an immunologically related, inactive form. Evidence is presented that endothelial cells produce active PA-inhibitor which is rapidly inactivated upon secretion into the medium. This inactivation can, at least partly, be prevented by culturing cells with excess of tissue-type plasminogen activator (t-PA). This results in the formation of large amounts of t-PA-PA-inhibitor complex at the cost of accumulation of inactive PA-inhibitor. No complex was detectable when inactive PA-inhibitor preparations were incubated with t-PA either in the absence or in the presence of cells. Furthermore, in cell extracts, predominantly functionally active PA-inhibitor was present. PA-inhibitor derived from the t-PA-PA-inhibitor complex showed an M_r approx. 4000 lower by polyacrylamide-gel electrophoresis than that of the inactive form. The rapid inactivation seems to be confined to newly synthesized molecules, since PA-inhibitor molecules in CM are inactivated much more slowly (even with cells or cell homogenates) than necessary to explain the excessive production of inactivated PA-inhibitor by cells. It could not be prevented by inhibitors of oxidative processes, like butylated hydroxytoluene, dithiothreitol, superoxide dismutase and catalase.

INTRODUCTION

The fibrinolytic system plays an important role in the removal of fibrin, formed by coagulation, after its function has been exerted. A pivotal step in fibrinolysis is the conversion of the zymogen plasminogen by tissue-type plasminogen activator (t-PA) into the active enzyme plasmin, which can degrade fibrin. Regulation of t-PA activity in the circulation occurs at various levels, including t-PA release from the vessel wall, clearance by the liver, and specific interaction with fibrin (for recent reviews, see Danø *et al.*, 1985; Emeis *et al.*, 1985; Erickson *et al.*, 1985). In recent years it has become clear that t-PA activity is further controlled by a specific, fast-acting, plasminogen-activator inhibitor (PA-inhibitor), directed against both tissue-type and urokinase-type plasminogen activator (Emeis *et al.*, 1983; Kruithof *et al.*, 1984; Thorsen & Philips, 1984; Verheijen *et al.*, 1984; Wiman *et al.*, 1984).

The origin of the PA-inhibitor in plasma is not known, but an inhibitor closely related with regard to M_r , dissociation constant with t-PA, immunological characteristics, and instability at 37 °C has been demonstrated in platelets, and in the conditioned medium (CM) of cultured endothelial cells and hepatocytes (Erickson *et al.*, 1984a; Sprengers & Jansen, 1985; Sprengers *et al.*, 1985a; Sprengers, 1986). Interestingly, two different forms of this so-called endothelial-cell-type PA-inhibitor have been described: a fast-acting active form, which reacts with plasminogen activators (PAs) and an inactive form with no apparent anti-PA activity (Sprengers *et al.*, 1984; Korninger *et al.*, 1985). However, this latter component can be 'activated' by incubation with protein denaturing agents, such as SDS, urea, guanidine, etc.

(Sprengers *et al.*, 1984; Hekman & Loskutoff, 1985). On the other hand, incubation of the fast-acting PA-inhibitor at 37 °C resulted in a spontaneous decline in PA-inhibitor activity, with a half-life of about 3 h. This inactivation process was found to be accelerated by the presence of activated protein C or thrombin (Van Hinsbergh *et al.*, 1985).

Sprengers *et al.* (1985b) have recently shown that the two forms of PA-inhibitor are immunologically related; moreover, the inactive PA-inhibitor after SDS treatment reacts with t-PA to a complex with the same M_r and dissociation constant as the complex between the fast-acting PA-inhibitor and t-PA. The fast-acting PA-inhibitor and the inactive PA-inhibitor thus probably represent different molecular forms of the same protein. The question arises whether the inactive PA-inhibitor originates from the active form by an inactivation process, or whether the inactive PA-inhibitor represents a pro-form. A second question concerns the molecular form of the PA-inhibitor released by cultured endothelial cells. Endothelial-cell CM contains both forms of PA-inhibitor, which leaves open the possibility of simultaneous production of both forms of PA-inhibitor as well as the possibility of synthesis of only one of the two forms [which must then subsequently be converted into the other one by an (in)activation process to explain the presence of a mixture of PA-inhibitor forms in CM]. We have sought to answer these questions by culturing human endothelial cells in the continuous presence of an excess of t-PA. Such procedure would prevent the putative inactivation process, since the fast-acting PA-inhibitor would be trapped in a complex with t-PA. The amount of complex formed is then a measure for the actual amount of active PA-inhibitor produced. Our

Abbreviations used: PA, plasminogen activator; t-PA, tissue-type plasminogen activator; PA-inhibitor, plasminogen-activator inhibitor; CM, conditioned medium; ECGF, crude preparation of endothelial cell growth factor; HSA, human serum albumin; PBS, phosphate-buffered saline (0.14 M-NaCl/0.01 M-sodium phosphate, pH 7.2); r.f.a., reverse fibrin autography.

results show that at least part of the inactive PA-inhibitor in endothelial-cell CM originates from the fast-acting form and becomes rapidly inactivated during or after leaving the cell. Studies were undertaken to clarify the mechanism of this inactivation process.

MATERIALS AND METHODS

Materials

A crude preparation of endothelial-cell growth factor (ECGF) was prepared from bovine hypothalamus as described by Maciag *et al.* (1979). Culture reagents were purchased from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K., and plastics were obtained from Costar, Cambridge, MA, U.S.A. Sterile, pyrogen-free, human serum albumin (HSA) was from the Centraal Laboratorium van de Bloedtransfusiedienst, Amsterdam, The Netherlands. [³⁵S]Methionine (1084 Ci/mmol) and En³Hance were supplied by New England Nuclear Corp., Boston, MA, U.S.A. t-PA (>99% two-chain) was purified from Bowes-melanoma-cell culture medium as described by Kluft *et al.* (1983). The activity is expressed as international units (i.u.), using the first international standard of the World Health Organisation, code 83/517, as a standard (Gaffney & Curtis, 1985). Our enzyme has a specific activity of 300 000 i.u./mg. The reagents used in SDS/polyacrylamide-gel electrophoresis and fibrin autography have been described in detail elsewhere (Sprengers *et al.*, 1984). Rabbit anti-(t-PA) IgG was prepared as described previously (Rijken *et al.*, 1981). An antiserum against the endothelial-cell PA-inhibitor was raised in rabbits by standard procedures and fulfilled the conventional criteria of specificity (Sprengers, 1986). The IgG fraction was isolated using protein A-Sepharose chromatography. Protein A-Sepharose and concanavalin A-Sepharose were from Pharmacia, Uppsala, Sweden. Protein *M_r* standards for SDS/polyacrylamide-gel electrophoresis were from Bio-Rad, Richmond, CA, U.S.A. All other reagents were of the highest grade available.

Cell culture

Endothelial cells from human umbilical veins were isolated by the method of Jaffe *et al.* (1973), and cultured as previously described (Van Hinsbergh *et al.*, 1983). Cells were grown on fibronectin-coated flasks in M199 medium, supplemented with 20 mM-Hepes, 20% (v/v) human serum (heat-inactivated), 200 µg of ECGF/ml, 100 i.u. of penicillin/ml, and 100 µg of streptomycin/ml under a CO₂ atmosphere. Subcultures were obtained by trypsin/EDTA treatment at a split ratio of 3:1.

Conditioned media (CM) and cell lysates

Confluent cultures were used at second or third passage and were always refed the day before the experiment. CM were obtained by incubating cells at 37 °C for various times with culture medium, or (after two washes with serum-free medium) with medium containing 0.3 mg of human serum albumin (HSA)/ml instead of serum, unless stated otherwise. CM were centrifuged for 2 min in a Beckman Microfuge to remove cells and cellular debris, and samples were frozen at -20 °C until use. Cell lysates were obtained by washing the cells twice with ice-cold phosphate-buffered saline (PBS), and scraping the cells off the dish with a rubber

policeman in the presence of ice-cold buffer, containing 0.5% (w/v) Triton X-100. Cell lysates were also frozen at -20 °C until use.

Radiolabelling of endothelial-cell proteins

Incorporation of [³⁵S]methionine into endothelial-cell proteins took place during incubation of endothelial cells with M199 medium (methionine-free), supplemented with Hepes, HSA, penicillin and streptomycin as described above, and 15–20 µCi of [³⁵S]methionine/ml (immunoprecipitation studies and purification of radiolabelled PA-inhibitor). Radiolabelled CM and cell lysates were collected as described above for unlabelled samples.

Immunoprecipitation

Immunoprecipitation of radiolabelled PA-inhibitor or t-PA-PA-inhibitor complexes in [³⁵S]methionine-labelled endothelial-cell CM was performed essentially by the method of Kessler (1976), with the modifications suggested by Bollen *et al.* (1981). A 100 µl portion of labelled medium was incubated with 10 µg of the appropriate antiserum IgG fraction for 18 h at 4 °C. The complexes formed were precipitated with *Staphylococcus aureus*, and, after washing extensively, dissolved in 50 µl of electrophoresis buffer containing 2% (w/v) SDS and 5% (v/v) β-mercaptoethanol. The solutions were then heated for 3 min in a boiling-water bath. Radioactivity in 5–10 µl samples of the dissolved immunoprecipitates was measured by liquid-scintillation counting. Samples subjected to SDS/polyacrylamide-gel electrophoresis were centrifuged for 5 min in a Beckman Microfuge to remove any insoluble material.

Protein synthesis

Overall protein synthesis was determined by measuring the incorporation of [³⁵S]methionine into the 10%-(w/v)-trichloroacetic-acid-precipitable fraction of radiolabelled CM. Total production of PA-inhibitor was assessed by specific immunoprecipitation of radiolabelled CM.

SDS/polyacrylamide-gel electrophoresis and autoradiography

SDS/polyacrylamide slab-gel electrophoresis was performed by the method of Laemmli (1970), with resolving gels of 10% (w/v) acrylamide and stacking gels of 4% (w/v) acrylamide. Protein *M_r* standards were used for calibration of the gel. For autoradiography, the gel was treated with an autoradiofluorography enhancer (En³Hance) according to the manufacturers' instructions, dried and placed on X-ray film (Kodak AXR) for 1–25 days at -90 °C.

Purification of radiolabelled PA-inhibitor (inactive form)

Radiolabelled inactive PA-inhibitor was purified essentially by the method of Van Mourik *et al.* (1984). Endothelial-cell CM was depleted of inhibitor activity by the addition of an excess of t-PA, followed by immunoabsorption on to immobilized anti-(t-PA). A 10 ml portion of [³⁵S]methionine-labelled endothelial-cell CM was mixed with 80 ml of unlabelled endothelial-cell CM and passed through a concanavalin A-Sepharose column. The adsorbed protein was subsequently eluted with 0.2 M-α-methyl mannopyranoside in 0.02 M-Tris/HCL (pH 7.4)/0.5 M-NaCl/0.1% Tween 80 buffer. The eluate was dialysed against 0.1 M-NaHCO₃, pH 7.7, and subsequently freeze-dried. The freeze-dried material was

subjected to preparative SDS/polyacrylamide-gel electrophoresis. The section containing a protein band corresponding to M_r of approx. 50000 was cut from the gel. Protein was eluted, and excess of SDS was removed by repeated Sephadex G-50 filtration in the presence of 2.5% (v/v) Triton X-100. The protein preparation was subjected to SDS/polyacrylamide-gel electrophoresis and reverse fibrin autography (r.f.a.) to assess its purity.

Assays

t-PA activity was determined by a spectrophotometric assay (Verheijen *et al.*, 1982). In this assay, PA activity is quantified by measuring the enzymic activity of the plasmin formed with a synthetic substrate. In the presence of PA-inhibitor, the amount of t-PA was also assessed by an enzyme immunoassay, as described by Rijken *et al.* (1984), or fibrin autography, essentially as described by Granelli-Piperno & Reich (1978). After SDS/polyacrylamide-gel electrophoresis, gels to be analysed by fibrin autography were soaked for 2 h in 2.5% (v/v) Triton X-100 (with one change) to remove the SDS and applied to the surface of an indicator gel containing 1.7 mg of fibrinogen, 0.05 mg of plasminogen and 0.05 NIH (National Institutes of Health) units of thrombin/ml.

(Production of) PA-inhibitor was measured in several ways. PA-inhibitor activity was quantified by titration of samples with increasing amounts of t-PA, followed by spectrophotometric measurement of the residual t-PA activity, as reported by Verheijen *et al.* (1984). Data were plotted, and an asymptote to the sample curve, parallel with the buffer titration curve, was constructed. The amount of inhibitor activity was then derived from the intersection with the abscissa. One unit of PA-inhibitor activity is defined as the amount of inhibitor that neutralizes one i.u. of t-PA activity. Latent PA-inhibitor activity was assessed by SDS/polyacrylamide-gel-electrophoresis, followed by r.f.a. as described by Erickson *et al.* (1984b) and Sprengers *et al.* (1984). Regions of the SDS/polyacrylamide gel containing inhibitor are indicated by the formation of opaque, lysis-resistant, areas in the indicator film.

In experiments with endothelial cells incubated in the presence of excess of t-PA, production of active PA-inhibitor was calculated by subtracting residual t-PA activity from the amount of t-PA activity added. For analysis of latent PA-inhibitor activity in these samples, preparations were first depleted of free t-PA and t-PA-PA-inhibitor complex by immunoadsorption, using immobilized affino-specific anti-(t-PA) IgG. The

Fig. 1. Determination of PA-inhibitor in CM and cell extracts from endothelial cells

(a) Determination of PA-inhibitor activity was performed by titration with t-PA, as described by Verheijen *et al.* (1984). To microtitre cups containing 10 μ l of CM (○), cell extract (●) or saline (■), 60–480 m-i.u. of t-PA were added; residual t-PA activity was determined spectrophotometrically. The PA-inhibitor activity was determined by graphical extrapolation of the linear part of the curve to the x-axis. In this example, 10 μ l of 24 h CM and 10 μ l of cell extract contained 300 and 135 m-i.u. of PA-inhibitor activity respectively. Appropriate corrections were made for the presence of low concentrations of PA-inhibitor activity in the serum-containing media (about 35 m-i.u./10 μ l). (b) The samples used in Fig. 1(a) were also analysed for the presence of inactive PA-inhibitor by r.f.a. after SDS/polyacrylamide-gel electrophoresis (see the Materials and methods section for details). The region of the SDS/polyacrylamide gel containing inhibitor activity is visible as an opaque, lysis-resistant, zone in the indicator gel. Lane 1, CM; lane 2, cell extract.

Fig. 2. PA-inhibitor production by endothelial cells

(a) Time course of PA-inhibitor activity accumulation in CM from endothelial cells incubated in the presence of 0.03% (w/v) HSA (○). When cells were incubated with an excess of t-PA (●), by adding 30 i.u. of t-PA/h per ml of culture medium, PA-inhibitor production was calculated by subtracting the amount of residual t-PA activity from the amount of t-PA added. (b) Conventional fibrin underlay of SDS/polyacrylamide-gel-electrophoresed samples. Lane 1, CM from cells incubated in the presence of 0.03% (w/v) HSA and an excess of t-PA; lane 2, as lane 1, but t-PA added at the end of the incubation period. The arrows indicate the electrophoretic mobility of t-PA-PA-inhibitor complex (top) and t-PA (bottom); (c) R.f.a. of the CM used in (b) for conventional fibrin autography. The samples had been depleted of t-PA and t-PA-PA-inhibitor complex by specific immunoadsorption. Lane 1, CM from cells incubated in the presence of 0.03% (w/v) HSA and excess of t-PA; lane 2, as lane 1, but t-PA added at the end of the incubation period.

resulting eluants were then analysed for inhibitor activity with r.f.a. after SDS/polyacrylamide-gel electrophoresis.

Stability of the PA-inhibitor activity

Samples were incubated at 37 °C for various times up to 24 h, followed by the determination of the residual PA-inhibitor activity.

RESULTS

Measurement of PA-inhibitor in CM and cell lysates from endothelial cells

Human umbilical-vein endothelial cells in culture produce two different forms of PA-inhibitor, which can be detected by two different techniques. One form, the fast-acting PA-inhibitor, can be demonstrated and quantified (both in CM and cell lysates) by its capacity to neutralize PA in a kinetic assay (Fig. 1a). The other form is inactive, but it can be (partially) activated by denaturing agents such as SDS and guanidine. It can be detected in CM by r.f.a. after SDS/polyacrylamide-gel electrophoresis, with an M_r of approx. 50000 (Fig. 1b). No inactive PA-inhibitor is demonstrable by this technique in the cell lysate analysed in Fig. 1(b). In other experiments, however, cell extracts sometimes contained very small amounts of inactive inhibitor. By specific immunoprecipitation of radiolabelled inhibitor present in CM from endothelial cells incubated in the presence of [35 S]methionine, it can be calculated that the PA-inhibitor represents up to 10% of the total

radiolabelled protein released by the cells in a 24 h period. Comparison of the amount of active PA-inhibitor with the actual amount of PA-inhibitor protein indicates that at least 95% of the PA-inhibitor in 24 h CM is in the inactive form.

Production of PA-inhibitor in the presence of excess of t-PA

To gain more insight in the actual production of fast-acting PA-inhibitor by endothelial cells, cells were incubated in the continuous presence of an excess of t-PA. It was anticipated that, under these conditions, newly synthesized fast-acting PA-inhibitor would be trapped in a complex with t-PA and thus prevented from being inactivated. The amount of t-PA consumed during the incubation would then be a measure of the accumulation of active PA-inhibitor in CM. The amount of endogenously synthesized t-PA was always less than 5% of the amount added and was therefore ignored in the calculations. The validity of this approach was verified in the following ways. It was checked that t-PA added to CM was stable over an incubation period of 8 h at 37 °C. No significant amount of t-PA was bound by plastic or bound/taken up by cells, by showing that the amount of t-PA antigen added to the culture medium was still present at the end of the incubation (recovery: 97 and 105% in a duplicate experiment), as measured by an enzyme immunoassay. It was also verified that the presence of an excess of t-PA in the culture medium had no effect on the overall PA-inhibitor synthesis by endothelial cells, as judged from the amount of

immunoprecipitable radiolabelled PA-inhibitor in [³⁵S]-methionine-containing CM. To verify that t-PA does not form a complex with inactive PA-inhibitor, the following experiments were performed. First, no t-PA was consumed when samples containing only inactive PA-inhibitor were incubated with excess of t-PA. Secondly, a purified, radiolabelled inactive PA-inhibitor preparation was incubated for 3 h at 37 °C with a molar excess of t-PA (both in the absence and in the presence of cells) and analysed by SDS/polyacrylamide-gel electrophoresis and autoradiography. No complex-formation with t-PA or degradation of PA-inhibitor was detectable.

Fig. 2(a) shows the time course of the production of active PA-inhibitor by cells incubated in the absence or presence of an excess of t-PA. In the latter case, accumulation of 'active' PA-inhibitor was calculated from the amount of t-PA consumed during the incubation. As illustrated for serum-free incubation conditions, the amount of active PA-inhibitor in CM containing an excess of t-PA increased linearly with time and at a much higher rate, reaching a 19-fold excess over the active form in the control experiment (i.e. without added t-PA) by 8 h. Comparable results were found when cells were incubated in the presence of serum and an excess of t-PA (results not shown). The presence of t-PA during the incubation resulted in a large amount of t-PA-PA-inhibitor complex, as revealed by fibrin autography (Fig. 2b). When t-PA was added at the end of the 8 h incubation period, a very weak band corresponding to the complex became visible only after prolonged incubation. Since t-PA had no measurable effect on the overall rate of PA-inhibitor synthesis, trapping of newly synthesized active PA-inhibitor by t-PA must be at the expense of inactive PA-inhibitor accumulation. This could not be demonstrated directly by using the semi-quantitative r.f.a. method: no significant change in the intensity of the lysis-resistant zone was seen when CM were analysed by r.f.a. (Fig. 2c). However, other experiments did show a decrease in the amount of 46000-*M_r* protein (see below).

Supporting evidence for the above conclusions came from experiments in which endothelial cells were incubated in the presence of [³⁵S]methionine and an excess of t-PA. Portions of radiolabelled CM were allowed to react with specific antibodies and the immunoprecipitates subjected to SDS/polyacrylamide-gel electrophoresis, followed by autoradiography. As can be seen in Fig. 3 (lanes a and e), reaction of anti-(PA-inhibitor) IgG with control endothelial-cell CM specifically precipitates a 46000-*M_r* protein, the PA-inhibitor. When cells were incubated in the presence of an excess of t-PA (lanes b and f), radioactive bands of 100000 and 42000 *M_r* can be detected in addition to a weaker PA-inhibitor band at 46000-*M_r*. This points to the formation of other forms of PA-inhibitors (the bands at 100000 and 42000 *M_r*) at the expense of the band at 46000 *M_r*. No radioactive band was observed when control endothelial-cell CM was allowed to react with anti-(t-PA) antibodies (lane c), but, with CM from cells incubated with an excess of t-PA, two bands representing 100000 and 42000 *M_r* were visible (lane d). These experiments indicate that, in CM from endothelial cells incubated with [³⁵S]methionine and excess of t-PA, an SDS-stable complex between (unlabelled) t-PA and (radiolabelled) PA-inhibitor is formed. This complex can be specifically immunoprecipitated with both anti-(t-PA) and anti-(PA-inhibitor) antibodies. The appearance of a

band at 42000 *M_r* (lanes d and f) suggests that the PA-inhibitor molecule is apparently cleaved during, or after, complex-formation with t-PA. The complex then partly dissociates during SDS/polyacrylamide-gel electrophoresis.

Studies on the (rate of) inactivation of PA-inhibitor

From the foregoing findings it is clear that, under standard culture conditions, the amount of active PA-inhibitor found in CM is an underestimate of the actual amount of active PA-inhibitor secreted from the endothelial cell. We have to assume an inactivation process, and subsequently we propose that the amount of PA-inhibitor activity detectable in the medium is the result of two opposing processes: the rate of synthesis and the rate of inactivation of active PA-inhibitor. A steady state is reached when these two processes balance each other (Fig. 2a). If we assume that the inactivation process follows first-order kinetics, the steady-state conditions can be described by the equation:

$$v = k \cdot C_s$$

where *v* is the rate of synthesis of active PA-inhibitor (i.u./h per ml), *k* is the rate constant of inactivation (h⁻¹) and *C_s* is the steady-state concentration of active PA-inhibitor (i.u./ml). By introducing the values for *v* (20.4 i.u./h per ml, as derived from the consumption of t-PA) and *C_s* (8.4 i.u./ml), as presented in Fig. 2(a) for an experiment in serum-free medium, it can be calculated that, under these conditions, *k* has a value of 2.43 h⁻¹. By using the equation:

$$t_{\frac{1}{2}} = \frac{\ln 2}{k}$$

an apparent half-life of 0.29 h can be calculated for the active PA-inhibitor molecule. If the assumptions are correct, it shows that this half-life for the fast-acting PA-inhibitor is much shorter than the other one calculated from the disappearance of PA-inhibitor activity in CM upon incubation at 37 °C; in both serum-free and serum-containing CM, half-lives of active PA-inhibitor molecules ranged from 2 to 4 h in various experiments. We conclude that apparently a more rapid inactivation process occurs upon secretion of newly synthesized (active) PA-inhibitor. To test the possibility that intact endothelial cells or cellular components are required for the rapid inactivation of PA-inhibitor, serum-free media containing high PA-inhibitor activity (96 i.u./ml; prepared by incubating endothelial cells for 24 h in the presence of 10 μg of endotoxin/ml) were incubated at 37 °C for various times up to 2.5 h with confluent cells, scraped cells in suspension or a cell homogenate (prepared by freeze-thawing of a cell suspension). No evidence was found for accelerated inactivation of PA-inhibitor in the presence of cells or cell fragments as compared with a control experiment without cells.

Lawrence *et al.* (1985) have presented evidence that rapid inactivation of PA-inhibitor (*t_{1/2}* < 1 min) occurs by selective oxidation of methionine residues. We have incubated cells and CM in the presence of 40 μM-butylated hydroxytoluene, an anti-oxidant, 10 mM-dithiothreitol or 0.4 mg of superoxide dismutase and 200 units of catalase/ml. No marked effect on PA-inhibitor activity levels was found.

Fig. 3. Autoradiography of immunoprecipitation of radiolabelled CM after SDS/polyacrylamide-gel electrophoresis

The Figure shows SDS/polyacrylamide-gel electrophoresis and autoradiography of immunoprecipitates obtained by mixing [35 S]methionine-labelled CM from endothelial cells with anti-(t-PA) or anti-(PA-inhibitor) antibodies. The absence or presence of excess of t-PA during the cell incubations is indicated by - and + respectively. Lanes a and b, CM not subjected to immunoprecipitation; lanes c and d, immunoprecipitates of radiolabelled CM with anti-(t-PA); lanes e and f, immunoprecipitates of radiolabelled CM with anti-(PA-inhibitor); lanes g and h, immunoprecipitates of radiolabelled CM with non-specific rabbit serum. The arrows indicate M_r values deduced from the electrophoretic mobility of the protein M_r markers when the gel was stained for protein. The autoradiograph was developed after 3 days at -90°C .

DISCUSSION

In CM from human umbilical-vein endothelial cells two different forms of PA-inhibitor can be found: an active, fast-acting inhibitor and an immunologically related, inactive, form. The latter component can be converted by protein-denaturing agents into an active form with functional characteristics very similar to those of the fast-acting inhibitor (Sprengers *et al.*, 1984, 1985b; Hekman & Loskutoff, 1985). The present results provide additional evidence for the relationship between the two forms. We propose that at least part of the inactive PA-inhibitor molecules found in CM are released by endothelial cells in an active form, but become rapidly inactivated during, or after, secretion into the medium. This hypothesis is based on the following observations. First, in cell extracts predominantly active PA-inhibitor is detectable. Secondly, accumulation of inactive PA-inhibitor in CM can be partly prevented by incubating cells in the presence of an excess of t-PA. Under these conditions large amounts of t-PA-PA-inhibitor complex are formed at the expense of inactive PA-inhibitor (Figs. 2 and 3). No t-PA-PA-inhibitor complex was formed when samples containing only the inactive form of PA-inhibitor were incubated with t-PA.

During our studies we noted that complex-formation between t-PA and PA-inhibitor is accompanied by a decrease in the M_r of the PA-inhibitor molecule (Fig. 3, lanes c and e). The consequent PA-inhibitor has an M_r of 42000 (as against 46000 for the intact protein) by SDS/polyacrylamide-gel-electrophoretic analysis, and shows no activity by r.f.a. analysis. Further experiments are required to evaluate the (possible) physiological significance of this finding.

It has been reported that the r.f.a. technique can be used as a (semi-) quantitative assay for the detection of inactive PA-inhibitor (Erickson *et al.*, 1984b; Korninger *et al.*, 1985). In our hands, however, an approx. 2-fold decrease in inactive PA-inhibitor production by cells incubated in the presence of t-PA could not be detected by r.f.a. analysis. Only by specific immunoprecipitation of radiolabelled CM, followed by SDS/polyacrylamide-gel electrophoresis, could such a decrease in the PA-inhibitor band at 46000 M_r be shown (Fig. 3, lanes e and f).

It can be deduced that, under standard culture conditions, the accumulation of the inactive PA-inhibitor in CM is much more rapid than that of the active form, reaching at least a 19-fold excess by 8 h in serum-free medium (Fig. 2a). Similar findings were reported by Levin (1985). By using a quantitative assay for measuring both active and inactive PA-inhibitor in CM, based on the denaturant-inducible activity of the latter component, Levin (1985) reported a 14-fold excess of the inactive form over the active form by 8 h, and a 40-80-fold excess by 24 h. Hekman & Loskutoff (1985), using bovine aortic endothelial cells, showed that SDS-treated CM (24 h) contained at least 80 times more detectable PA-inhibitor activity than untreated CM.

As shown in Figs. 2c and 3 (lane e), the addition of an excess of t-PA can only partly prevent the appearance of inactive PA-inhibitor in the medium, despite the reported rapid complex-formation between t-PA and PA-inhibitor (with a rate constant in the order of 10^7 mol/s per litre; Kruithof *et al.*, 1984; Wiman *et al.*, 1984) and the very high affinity of PA-inhibitor for t-PA (with an apparent dissociation constant of about 5×10^{-12} M; Verheijen *et al.*, 1985). A possible explanation for this finding might be that PA-inhibitor reaching the culture

medium via the subendothelial matrix is not accessible at all stages to t-PA, and thus not prevented completely from being inactivated.

Inactivation of PA-inhibitor can occur in two different ways: a rapid inactivation of newly synthesized PA-inhibitor upon leaving the cell, with a calculated half-life of 0.29 h, and a much slower decline in PA-inhibitor activity in the medium, with a half-life of 2–4 h. The molecular mechanisms whereby these inactivation processes occur are at present unknown, but probably do not involve oxidation reactions, since inactivation of PA-inhibitor could not be prevented by inhibitors of oxidative processes. Further experiments need to be directed at unravelling this mechanism. Insight in the inactivation process may permit one to block the accumulation of inactive PA-inhibitor in CM completely, and thus exclude the possibility that the PA-inhibitor fraction that could not be trapped by t-PA represents a pro-form rather than an inactivated form of the fast-acting PA-inhibitor.

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