Fibrinolysis in Pregnancy: A Study of Plasminogen Activator Inhibitors

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During pregnancy the plasma concentration of two different inhibitors of plasminogen activators (PAls) increases. The only one found in the plasma of nonpregnant women (PAI1) is immunologically related to a PAl of endothelial cells; its plasma activity, as deduced from the inhibition of single-chain tissue-type plasminogen activator (t-PA), increased from 3.4 ± 2.3 U/mL (mean ± 95% confidence limits) in the plasma of nonpregnant women to 29 ± 7 U/mL at term, and its antigen level, measured by a radioimmunoassay, increased from 54 ± 17 ng/mL to 144 ± 25 ng/mL. In pregnancy plasma a second PAl (PAI 2) related to a PAl found in placenta extracts was observed. Its level, quantified with a radioimmunoassay, increased from below the detection limit (~10 ng/mL) in normal plasma to 260 ng/mL at term. One hour after delivery, PAl 1 activities and antigen decreased sharply, but the PAl 2 antigen levels remained constant. Three days later, the PAl 1 antigen levels had fallen to normal levels, but the PAl 2 antigen levels were still at least eightfold above the nonpregnant values. During pregnancy, the t-PA and pro-urokinase (u-PA) antigen concentrations increased 50% and 200%, respectively, whereas the plasminogen and α2-antiplasmin levels remained constant. Despite the large variations in the levels of PAls and PAl, the overall fibrinolytic activity as measured in diluted plasma by a radioiodinated fibrin plate assay did not change significantly. Just after delivery, a great increase in the t-PA antigen levels was observed. Three to five days after delivery the parameters of the fibrinolytic system were normal again. Our results demonstrate that during pregnancy and in the puerperium profound alterations of the fibrinolytic system occur that are characterized by increases in PAls and their inhibitors, but these alterations do not affect the overall fibrinolytic activity.

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conditioned medium of hydrocortisone (10⁻¹ mol/L) stimulated cells of the human fibrosarcoma cell line HT 1080 by affinity chromatography on ConA-Sepharose, ion-exchange chromatography on diethylaminoethyl Sephalac, and gel filtration on Sephadex G-100. The final product showed one band on reduced and nonreduced SDS-PAGE and was immunoprecipitable by antibodies prepared against a PAI purified from the conditioned medium of bovine and human endothelial cells (gifts from Drs Loskutoff, La Jolla, CA, and Sprengers, Leiden, The Netherlands) respectively.

PAI 2 was purified from the conditioned medium of phorbol myristate acetate (30 ng/mL)-stimulated cells of the human histiocytic lymphoma cell line U-937, and anti–PAI 2 antibodies were raised in rabbits as previously described. PAI 1 did not react with antibodies to PAI 2 and vice versa. PAI 2 was found to be indistinguishable from a PAI partially purified from human placenta by the following criteria: (1) a line of complete identity was obtained in double immunodiffusion, (2) identical molecular weights were obtained after SDS-PAGE followed by electrophoresis and immunologic detection using immunopurified anti–PAI 2 IgG and peroxidase-conjugated goat-IgG IgG, and (3) the second-order rate constant of the reaction of placental PAI and PAI 2 with u-PA and t-PA were similar. PAI 1 and PAI 2 were radioiodinated using the method of Fraken and Speck. Radioiodinated inhibitor was separated from free by 125I by passage over a 12-ML column of Sephadex G-25. The specific radioactivity of the radiolabeled PAI preparations was between 15 and 50 nCi/ng (corresponding to 15 to 50 molecules of 125I incorporated per 100 molecules of PAI 1 or PAI 2).

Plasmas. Blood was obtained by venipuncture, anticoagulated with buffered citrate (nine parts of blood were mixed with one part of 0.1 mol/L sodium citrate/citric acid, pH 4.5), and centrifugated for 15 minutes at 3,600 g at 4 °C. The plasmas were stored at -70 °C. A normal plasma pool was made from the blood of 50 healthy blood donors.

Subjects. The subjects studied were (1) 90 pregnant women whose pregnancy proceeded normally. For each 4-week period of pregnancy, except the first, ten women were admitted. The last period comprised the 37th week until term. Also studied were (2) ten women postpartum (mean time since delivery, 77 ± 54 minutes [mean ± SD]), (3) ten women three to five days after delivery, and (4) ten apparently healthy, nonpregnant women of the same age group (27 ± 4 years) as the pregnant women (27 ± 5 years).

Immunoradiometric assay for t-PA in plasma. The t-PA antigen levels in human plasma were determined by using an immunoradiometric assay essentially as described by Rijken et al. Standard curves were made using a t-PA preparation standardized against the International References Preparation of t-PA. This preparation has a specific activity of 500,000 IU/mg protein (1 IU = 2 ng).

Radioimmunoassay for (pro) u-PA in plasma. One hundred microliters of plasma diluted five times with immunodilution buffer (50 mmol/L Tris-HCl, pH 7.4; 200 mmol/L NaCl, 10 mmol/L EDTA, 0.1% bovine serum albumin, and 0.05% NaN₃) was mixed with 100 µL anti–u-PA IgG that had been diluted such (1:100,000) that in the absence of added cold u-PA half of the 125I-labeled u-PA bound to the antibodies. The sample was incubated four hours at room temperature with IgG. Thereafter, 50 µL of 125I-u-PA (containing 2,000 cpm radiolabel) was added and incubated overnight at 4 °C. The following day, 100 µL of a cellulose suspension to which donkey antibodies to rabbit IgG had been coupled (Sac-Cel, Wellcome Reagents, Beckenham, UK) was added. The suspension was incubated 30 minutes at room temperature, diluted by the addition of 2 mL of 0.15 mol/L NaCl and centrifuged for five minutes at 200 g. The pellet was twice washed with 2 mL of 0.15 mol/L NaCl and counted in a gamma counter. The radioactivity after deduction of the nonspecific binding of 125I-u-PA to Sac-Cel in the absence of added IgG were compared in a logit-log plot with a standard curve of u-PA that had been calibrated against the international reference preparation of u-PA (specific activity, 100,000 U/mg).

Radioimmunoassay for PAI 1 and PAI 2 in plasma. The methodology used for the determination of PAI 1 and PAI 2 antigen was identical to that described for u-PA. Rabbit antihuman PAI 1 antiserum (a gift from Dr Loskutoff, La Jolla, CA) was used in a dilution of 1:1,000, and immunopurified rabbit anti–PAI 2 IgG was used in the concentration of 60 ng/mL. The antigen levels were measured by comparison in a logit-log plot with standard curves of pure PAI 1 or PAI 2. The protein concentrations of the PAI 1 or PAI 2 stock solutions were determined using the protein assay of Bradford and bovine serum albumin as the standard.

Assay for plasminogen and α₂-antiplasmin. The plasminogen and α₂-antiplasmin levels were determined by rocket immunoelectrophoresis using commercially available antisera (Behringwerke, Marburg an der Lahn, Federal Republic of Germany). Rocket heights were compared with those of various dilutions of a normal human plasma pool and the results expressed in percent with respect to the plasma pool.

Assay for the total PA activity in plasma. The PA activity was measured using a 125I-fibrin plate method. Plasminogen-free bovine fibrinogen was converted to fibrin monomers and further purified by three steps of polymerization and dissolution in acetic acid as described by Haverkate and Timan. Human 125I-fibrinogen of clinical grade was rendered plasminogen free by affinity adsorption to lysine-Sepharose. The 125I-fibrin plate was prepared using these two fibrinogen solutions exactly as described by Sharoni et al. No thrombin was detectable in these plates with a chromogenic substrate assay (detection limit of 5 pg/mL). Prior to use, the wells were incubated for 15 minutes with sterile phosphate-buffered saline. The buffer was replaced with a mixture of 250 µL of a solution of plasminogen (5 µg/mL) and 100 µL of a 50- or 100-fold dilution of plasma in 0.1 mol/L Tris-HCl, pH 7.5, containing 1% bovine serum albumin. The plates were incubated at 37 °C; after three and six hours, 50-µL aliquots were removed to determine the degree of 125I-fibrinolysis. The percentage of 125I-fibrinolysis was compared with that of standard curves using a laboratory reference preparation of human t-PA that had been calibrated against the international t-PA reference preparation.

PAI 1 activity assay. Because the reaction rate between PAI 2 and sc t-PA is quite slow but fast between PAI 1 and sc t-PA, we have adapted the previously described assay for PAI activity in plasma, taking care to add to the plasma a t-PA preparation (final concentration, 42.5 U/mL) that contained less than 10% of the tc form.

Preparation of immobilized antibodies. IgG was prepared by affinity chromatography on protein A-Sepharose from 0.5 mL of rabbit preimmune serum, antisem directed against PAI 1, and antisem directed against PAI 2. The IgG was dialyzed overnight against two changes of 0.1 mol/L sodium bicarbonate, pH 8.1, and coupled to 1 mL of CNBr-activated Sepharose 4B.

Immunologic comparison of PAl s in normal and pregnancy plasma. Radioiodinated u-PA (10 µL, 200 ng/mL, 4 × 10⁶ cpm/mL) was incubated for ten minutes at 37 °C with 200 µL of human plasma. Forty microliters of the mixture was added to 200 µL of a 25% suspension of immobilized preimmune IgG, anti–PAI 1 or anti–PAI 2 125I in 50 mmol/L imidazole and 140 mmol/L NaCl, pH 7.35. After a further 20 minutes' incubation at 25 °C, the gel was spun down (five minutes, 1,000 g) and the supernatant diluted twofold with SDS sample buffer. SDS-PAGE in 7.5% gel of a 50-µL sample and autoradiography was performed as described previously.

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RESULTS

PAI 1 and PAI 2 levels in pregnancy plasma. The PAI 1 activity in the plasma of nonpregnant women was extremely variable (3.4 ± 2.3 U/mL, mean ± 95% confidence limits of the error of the mean). In the first 3 months of pregnancy, the PAI 1 activity was slightly, but nonsignificantly higher. Thereafter an almost linear increase in the PAI 1 activity to 29 ± 7 U/mL at term was observed (Fig 1). One hour after delivery, the PAI 1 activity decreased approximately 50% to 15 ± 9 U/mL, and the levels were normal three to five days after delivery.

As observed for the PAI 1 activity, the PAI 1 antigen levels in nonpregnant women were quite variable (55 ± 17 ng/mL). During the first 2 months of pregnancy, the PAI 1 antigen levels appeared to decrease slightly; thereafter they increased steadily to a maximum of 147 ± 32 ng/mL, attained between 32 and 36 weeks of pregnancy (Fig 2). After delivery the PAI 1 antigen levels decreased sharply within one hour and were normal three to five days later.

The PAI 2 antigen levels in nonpregnant women were below the detection limit (< 10 ng/mL). From 4 to 8 weeks of pregnancy the PAI 2 was measurable in about half of the subjects. After that it was detectable in all plasmas, and an almost linear increase of PAI 2 antigen concentration was observed until the 32nd week of pregnancy when the mean levels stabilized at around 240 ng/mL (Fig 3). In contrast to the PAI 1 activity and antigen, the PAI 2 antigen did not decrease within one hour postpartum. Three to five days after delivery the mean level of 77 ± 27 ng/mL of PAI 2 was still at least eightfold higher than in nonpregnant women.

Complex formation of u-PA with PAI 1 and PAI 2 in normal and pregnancy plasma. To verify whether PAIs

other than PAI 1 and PAI 2 are present in normal and in pregnancy plasma, 125I-u-PA was added to a plasma pool obtained from nonpregnant women or from women in the last trimester of pregnancy and the immunologic identity of the complexes formed established. For this experiment u-PA was chosen rather than t-PA because u-PA reacts rapidly with both PAIs and sc t-PA only with PAI 1. The incubation of 125I-u-PA in normal plasma resulted mainly in the formation of a 95 kDa complex (Fig 4). This complex bound to immobilized anti–PAI 1 IgG but not to anti–PAI 2 IgG. In pregnancy plasma, three complexes in the molecular weight region of 85,000 to 110,000 were formed. The largest and the smallest ones were retained by immobilized anti–PAI 2 IgG and the middle one by anti–PAI 1 IgG. These results,
therefore, provide evidence that in pregnancy plasma PAI 1 exists in one form, whereas two different forms of PAI 2 might exist.

**PA activity in plasma during pregnancy.** Figure 5 shows that the PA activity in nonfractionated diluted plasma as measured on radioactive fibrin plates remained essentially constant during pregnancy and shortly after delivery. The slight changes observed were not significant. However, three to five days after delivery the PA activity levels were significantly lower than during the last period of pregnancy ($P < .001$, Mann-Whitney test) or in nonpregnant control women ($P < .005$).

**Antigen levels of t-PA, (pro)-u-PA, plasminogen and $\alpha_2$-antiplasmin in pregnancy plasma.** The surprising observation of an unchanged fibrinolytic activity in the presence of strongly increased levels of two different PAIs prompted us to study also the t-PA, (pro)-u-PA, plasminogen, and $\alpha_2$-antiplasmin antigen levels. During pregnancy the t-PA antigen increased from $2.2 \pm 0.8$ ng/mL (mean $\pm$ 95% confidence limits) in nonpregnant women to a plateau value between 3.0 and 3.6 ng/mL in the last two trimesters of pregnancy (Fig 6). One hour after delivery a doubling of the t-PA levels ($6.5 \pm 1.1$ ng/mL) with respect to late pregnancy values was observed. Three to five days after delivery they were slightly, but significantly higher than those in nonpregnant women.

Additionally, the behavior of (pro)-u-PA antigen during pregnancy was quite different. An almost linear increase from $19 \pm 3$ ng/mL in nonpregnant women to a plateau level of $46 \pm 3$ ng/mL at 32 weeks of pregnancy was observed (Fig 7). Thereafter the mean (pro)-u-PA levels remained near this value until delivery. Just after delivery the u-PA levels increased slightly, but significantly ($P < .05$) to $52 \pm 4$ ng/mL. Three to five days postpartum the mean u-PA level was still greatly increased with respect to that in nonpregnant women ($35 \pm 3 \text{ vs } 19 \pm 3$ ng/mL; $P < .01$).
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Antibodies directed against PAI are often termed placental PAI. It is a very efficient inhibitor of t-PA, with a half-life of approximately two hours. Antibodies directed against PAI are in an active and a latent form. The active form of PAI is synthesized by the endothelial cells and is present in plasma and in blood platelets. PAI exists in an active and a latent form. The active form of PAI readily inhibits t-PA and also sc t-PA but inhibits tc t-PA poorly.

We observed striking increases in the level of PAI 1 and PAI 2 as well as moderate increases in the t-PA and u-PA antigen levels. Our quantitative measurements confirm the recent qualitative observations of Nilsson et al who found that pregnancy plasma contains two different PAIs and those of Lecander and Åstedt who reported increased antigen levels of a placenta-derived PAI with characteristics similar to those of PAI 2. The PAI 1 levels increased steadily after the 20th week of pregnancy and, at term, were threefold higher than in nonpregnant women. The PAI 2 levels were below the detection limit in normal plasma and, at term, were more than 25-fold increased. The PAI 1 activity levels in plasma, as determined from the inhibition of sc t-PA, correlated better with PAI 1 than with PAI 2 antigen concentration. This was particularly noteworthy just after delivery when the PAI activity and PAI 1 antigen levels decreased sharply and PAI 2 remained high. We may therefore conclude that the PAI 1 activity assay is relatively insensitive to PAI 2. We observed, however, a quantitative discrepancy between the PAI 1 activity and PAI 1 antigen levels. In the last trimester, the PAI activity was on average 28 U/mL, corresponding to 60 ng of PAI per milliliter of plasma, whereas the PAI 1 antigen levels were approximately twofold higher. Two possible explanations for this discrepancy exist. In normal plasma virtually all t-PA is bound to PAI 1; consequently, about 3 to 4 ng/mL of PAI 1 are bound to t-PA and not measured by our activity assay. However, because the observed discrepancy is one order of magnitude greater, it can only partially be explained by the inactivation of PAI 1 by t-PA. We must therefore assume that part of the plasmatic PAI 1 is present in the latent, inactive form.

When added to pregnancy plasma, tc u-PA formed two different complexes with PAI 2 of approximately 95 and 115 kDa. The two forms of PAI 2 probably correspond to a heavier glycosylated and a lighter nonglycosylated form of PAI 2 or may be due to cleavage of the PAI 2 molecule after its interaction with u-PA.

Within one hour after delivery, the concentration of some components of the fibrinolytic system changed dramatically: the doubling of t-PA levels is probably due to release from the vessel walls induced by the stress of childbirth and may be responsible for the drop of the PAI 1 activity and antigen levels to about half the levels found at term. No differences were observed for u-PA and PAI 2 levels and for PA activity. Three to five days after delivery the PAI activity and t-PA and PAI 1 antigen levels were not significantly different from control values, but the u-PA and PAI 2 levels were still significantly elevated, whereas the PA activity was significantly decreased. Because u-PA half-lives are relatively short, we have to assume that the increased production and release and/or the decreased clearance of u-PA persisted for several days after delivery. In previous work the rapid decline in PAI activity after delivery was explained by the arrest of PAI release from placental tissue into the circulation after the expulsion of the placenta. The persistence of PAI 2 suggests either that its main site of synthesis in pregnancy and after delivery is not the placenta or that its in vivo half-life is on the order of several days. In contrast, the rapid decrease in PAI activity and PAI 1 antigen levels after delivery might suggest that PAI 1 is produced by the placenta and that it has a short in vivo half-life. The latter is in accordance with the observations of Colucci et al on the short PAI half-life in rabbits.

We found that the PA activity remained essentially constant during pregnancy but was slightly decreased three to five days after delivery. Our results differ from those of Arias et al who observed a significant increase in the whole blood fibrinolytic activity in pregnancy and post partum and from most other studies reporting a significant decrease in the
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Fibrinolytic activity of euglobulins.4-12 Measurement of the fibrinolytic activity in euglobulin precipitates, however, has several drawbacks: (1) u-PA precipitates only partially in the euglobulins; (2) during pregnancy, the fibrinogen levels increase almost twofold and thus prolong the euglobulin clot lysis time; and (3) PAIs precipitate partially in the euglobulins (unpublished observations) and thereby affect the fibrinolytic assay. It is therefore evident that the results of fibrinolytic activity measurements in pregnancy are critically dependent on methodology, particularly on whether the activity is measured in whole blood, plasma, or euglobulins. The increased levels of fibrin degradation products during pregnancy measured by Fletcher et al.14 indicate that the +fibrinolytic system remains functionally active and do not support the hypothesis that its activity decreases significantly during pregnancy.

Our study demonstrates that the changes in the fibrinolytic system during pregnancy are much more complex than hitherto assumed. The physiological meaning of and the mechanisms inducing these changes are at present obscure.

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