Elevated plasma levels of type 1 plasminogen activator inhibitor (PAI-1) have been implicated in mediating the fibrin deposition and occlusive lesions that occur within the placental vasculature in preeclampsia (PE) and intrauterine growth retardation (IUGR). In this report we identify the cells within the normal-appearing villous tissue that are responsible for the local production of PAI-1 in women with PE and IUGR. Levels for another fibrinolytic inhibitor (ie, type 2 plasminogen activator inhibitor [PAI-2]) were determined for comparative purposes. Elevated levels of PAI-1 were detected in placenta extracts from PE/IUGR patients (121 ± 38 ng/mg, n = 8) when compared with the levels in placenta extracts from normal women (43 ± 17 ng/mg, n = 10) or women with IUGR but not PE (51 ± 22 ng/mg, n = 11). Immunohistochemical analysis of paraffin sections showed an increased immunoreactivity for PAI-1 in the placental villous syncytiotrophoblasts from PE/IUGR women compared with the immunostaining of placental samples from the normal or IUGR group. In contrast, antigen levels and immunostaining for PAI-2 were reduced in the placentas harvested from not only the PE/IUGR women (209 ± 144 ng/mg) but also the IUGR group (169 ± 106 ng/mg) in comparison with the PAI-2 levels in normal placentas (535 ± 98 ng/mg). To document that the increased immunoreactivity for PAI-1 in PE/IUGR syncytiotrophoblasts was mediated by an increased production of PAI-1 within these cells, in situ hybridization analysis was performed. A strong positive signal for PAI-1 mRNA in villous syncytiotrophoblasts from PE patients (n = 5) was obtained after 2 weeks of exposure to the NTB2 emulsion in comparison with the week signal for PAI-1 mRNA that required a 10-week exposure of the normal placenta sections (n = 10). Northern blotting for PAI-1 mRNA showed that both transcripts (ie, 3.2 and 2.3 kb) were elevated in samples of two PE patients in comparison with the PAI-1 mRNA transcripts present in a normal placenta and an IUGR placental sample. These results show increased PAI-1 and mRNA levels in placentas from PE patients and raise the possibility that localized elevated levels of PAI-1 may play a role in the initiation of placental damage, as well as in the thrombotic complications associated with this disease.

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MATERIALS AND METHODS

Clinical groups. Patients and normal pregnant women entered into this study were from the Maternity Center of Hospital La Fe during 1991 and 1992. Informed consent was obtained from the women before blood sampling and collection of placenta. Birth weight was determined after cutting of the umbilical cord within 2 cm of newborn insertion about 15 minutes after delivery. The placental weight was determined after removal of blood clots from the decidual surface, trimming of the membrane, and cutting of the umbilical cord within 2 cm of its insertion.

The control group (group A) consisted of 10 healthy normotensive women in the third trimester of pregnancy (range 33 to 40 weeks of pregnancy) whose newborns had a weight in the 10th percentile of appropriate birth weight for gestational age (range 2,800 to 4,500 g) and a neonatal ponderal index >2.20. No complications were observed during pregnancy and labor. The women did not receive any type of medication except a vitamin complex.

The pure severe preeclamptic group (group B) included eight patients with no previous history of hypertension in whom severe PE developed during the third trimester of pregnancy (range: 32 to 40 weeks of pregnancy). Severe PE was diagnosed in these women when blood pressure was >160/110 mm Hg at bed rest on at least two occasions 6 hours apart.26 In these patients, proteinuria was >1 g/L in at least two random specimens collected 6 hours apart. All of these PE patients had pregnancies complicated with IUGR.

The IUGR group without PE (group C) included 11 pregnant women in the third trimester of pregnancy (range: 32 to 40 weeks of pregnancy) who were diagnosed as having suspected IUGR when serial ultrasonographic measurements of biparietal diameter and femur length were >2 SD below the mean for gestational age two consecutive times in a month.26 After delivery, diagnosis of IUGR was confirmed when newborn weight (700 to 2,400 g) was below the 10th percentile of appropriate birth weight for gestational age and neonatal ponderal index was <2.20 (range: 1.95 to 2.19). This IUGR group shows fetal growth retardation type II or asymmetrical IUGR.25 Although asymmetrical IUGR are often caused by maternal diseases, our IUGR cases without PE are of unknown etiology and only two mothers whose pregnancies were complicated with IUGR without PE were smokers.

Blood sampling. Pregnant women suffering with severe PE (group B) or IUGR without PE (group C) were hospitalized 1 to 3 days before delivery to monitor their pregnancy. A blood sample was obtained from these patients at that time. The women included in the control group had provided a blood sample for clinical analyses when blood pressure was >160/110 mm Hg at bed rest on at least two occasions 6 hours apart. In these patients, proteinuria was >1 g/L in at least two random specimens collected 6 hours apart. All of these PE patients had pregnancies complicated with IUGR.

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Proteins were extracted from the frozen placenta sections by incubating 1 g of a sample in 10 mL of 0.1 mol/L Tris-HCl, pH 7.5, 2 mol/L potassium thiocyanate (KSCN) at 4°C. The cellular debris was removed by centrifugation (10,000g, 30 minutes) and the supernatant was dialyzed against cold PBS. All steps during the extraction of the placenta were performed at 4°C to minimize degradation of PAIs. Total protein in the samples was determined by using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL).

Assay for PAI-1 and PAI-2 antigen. PAI-1 antigen in plasma and placental samples was quantified using a two-site immunoassay according to procedures described previously.27 PAI-2 antigen in samples was quantified by a commercially available enzyme-linked immunosorbent assay (Tint Elize PAI-2; American Diagnostica, Greenwich, CT).

Immunohistochemistry. Immunohistochemical staining was performed using a three-step avidin-biotin-peroxidase method as described in detail previously.28 Briefly, fixed and paraffin-embedded placental tissue blocks were cut into 3- to 5-μm sections using a microtome, placed onto slides coated with polylysine, and air dried. The sections were deparaffinized in xylene, cleared in 95% ethanol, incubated (22°C, 10 minutes) in 3% hydrogen peroxide in methanol, and rehydrated through a graded ethanol. After washing with Tris-buffered saline (TBS; 0.05 mol/L Tris-HCl/0.15 mol/L NaCl, pH 7.4), the sections were treated with an increasing concentration of Triton X-100 (0.1% to 1%) in TBS for permeabilization. To unmask PAI immunoreactivity, the sections were incubated (37°C, 5 minutes) with 0.23% (wt/vol) pepsin (2,830 U/mg; Worthington Biochemical Corp, Freehold, NJ) in 0.01 N HCl. Tissue sections were subsequently blocked with normal serum to prevent nonspecific binding by incubation (1 hour, 22°C) with either 1% normal goat serum in TBS for PAI-1 antigen or 1% normal rabbit serum for PAI-2. Then the sections were incubated with either 10 μg/mL of affinity-purified rabbit antiantihuman PAI-127,28 or 10 μg/mL of preimmune (normal) rabbit IgG or with either 5 μg/mL of affinity-purified goat antiantihuman PAI-2 (American Diagnostica) or 5 μg/mL of preimmune goat IgG (American Diagnostica). After washing with 1% Triton-TBS (3 × 3 minutes), the appropriate second antibody (i.e., biotinylated goat antiantihuman IgG or biotinylated rabbit antiantihuman IgG; Jackson Immunoresearch Laboratories, Inc, West Grove, PA; 1:100 dilution) was added (22°C, 15 minutes). The tissue sections were washed again with 1% Triton-TBS (3 × 3 minutes), incubated (22°C, 10 minutes) with streptavidin-peroxidase conjugate (Jackson Immunoresearch Laboratories), washed with 1% Triton-TBS (3 × 3 minutes), and incubated (22°C, 15 minutes) with the chromogen aminoethylcarbazole/hydrogen peroxide mixture (Jackson Immunoresearch Laboratories) that results in a reddish-brown deposit indicative of positive immunoreactivity. Finally, the sections were counterstained with Mayer’s hematoxylin for 3 minutes at room temperature, rinsed well with tap water, and mounted in GVA-mount (Zymed, San Francisco, CA).

In situ hybridization. A PAI-1 cDNA fragment containing nucleotides 1 to 1085 in pGEM-32 was used for the preparation of antisense and sense riboprobes by in vitro transcription in the presence of [35S]-UTP (specific activity, 1,200 Ci/mmol; Amersham, Arlington Heights, IL) using SP6 RNA polymerase or T7 RNA polymerase, respectively.21,28 The DNA template was removed by digestion using RQ1 DNase for 15 minutes at 37°C and the riboprobe purified by phenol extraction and ethanol precipitation, and the probes were used at a specific activity of 2 × 108 cpm/μg of riboprobe. After in situ hybridization of the 35S-labeled antisense probe with the tissue sections, the slides were coated with Kodak NTB2 emulsion (Eastman Kodak, Rochester, NY) and exposed in the dark at 4°C for 2 to 12 weeks. Slides were developed for 2 minutes in Kodak D19 developer, fixed, washed, and counterstained with hematoxylin/cosin that stains nuclei blue. Parallel sections were analyzed using a sense.
probe as the control for nonspecific hybridization and in no instance was a specific signal detected, even after 12 weeks of exposure. Specimens were analyzed using combined light/epiluminescence microscopy to permit a simultaneous visualization of the sample and exposed silver grains. The latter appear as black or green dots depending on the illumination of the light and indicate the presence of PAI-1 mRNA.

RNA isolation and Northern hybridization. Total cytoplasmatic RNA was isolated from placenta and 30 μg of RNA was analyzed for PAI-1 mRNA by Northern blotting according to published protocols. The probe for this procedure consisted of a cDNA that was radiolabeled by the random primer technique to a specific activity of >5 × 10⁶ cpm/μg using α-32P dGTP (>3,000 Ci/mmol; Amersham). Autoradiography was performed at ~80°C using Kodak XAR-5 film. Equal loading and transfer of the RNA was confirmed not only by visualization of the ethidium bromide-stained RNA in the nylon membrane after transfer, but also by rehybridization of the blot using a commercially available human β-actin antisense RNA probe (ie, a digoxigenin-labeled probe that corresponded to bases 60 to 618 of β-actin (Boehringer Mannheim Biochemica, Mannheim, Germany). A probe concentration of 120 ng/mL was used in hybridization and detection of the bound probe was performed by incubation with alkaline phosphatase-conjugated antidigoxigenin followed by the chemiluminescent substrate, Lumiphos 330, according to the manufacturer’s protocol (Boehringer Mannheim Biochemica). Autoradiography was performed at ~80°C using Kodak XAR-5 film.

Statistical analysis. Because plasma and placental PAI-1 and PAI-2 data showed a normal distribution when analyzed by a Kolmogorov-Smirnov test,3 a Student’s t-test was used to evaluate differences between groups. Correlations between plasma and placental PAI levels were calculated by the Spearman rank test.

RESULTS

Plasma and placental PAI levels in the clinical groups. Table 1 summarizes several pertinent features of the clinical population analyzed in this study. For example, no differences were observed between the three clinical groups in relation to their gestational age and the age of the women, immunologic quantitation of PAI-1 showed a significant correlation (r = 0.68, P < .001) between the levels for this inhibitor in the plasma and the levels in the placental extracts obtained from the women classified into these three groups (Fig 1A). Thus, the higher PAI-1 levels previously detected in the plasma of PE/IUGR patients2,14 correlated with higher levels of PAI-1 antigen in the placenta of these patients (group B: 121 ± 38 ng/mg) when compared with the levels for this inhibitor present in extracts of placentas obtained from normal (43 ± 17 ng/mg) and pregnancies complicated solely with IUGR (51 ± 22 ng/mg) (Table 1). In light of the reduced newborn birth weight of both PE/IUGR patient (group B) and the patients with IUGR (group C), the elevated placenta PAI-1 levels of only group B resulted in a clustering of the individual data points into three distinct populations when the newborn birth weight was compared with the PAI-1 levels in either the plasma (Fig 1B) or in the placenta (Fig 1C).

Table 1. Statistical Comparison of Data Obtained From the Three Clinical Groups

<table>
<thead>
<tr>
<th></th>
<th>Normal Preg</th>
<th>Severe PE With IUGR</th>
<th>IUGR Without PE</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>Group B</td>
<td>Group C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall clinical data</td>
<td>n = 10</td>
<td>n = 8</td>
<td>n = 11</td>
<td></td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>Mean ± SD</td>
<td>27 ± 3</td>
<td>28 ± 7</td>
<td>28 ± 5</td>
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<tr>
<td></td>
<td>Range</td>
<td>22-33</td>
<td>22-43</td>
<td>21-37</td>
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<tr>
<td>Gestational age at sampling (wks)</td>
<td>Mean ± SD</td>
<td>38 ± 2</td>
<td>36 ± 3</td>
<td>37 ± 3</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>33-40</td>
<td>32-40</td>
<td>32-40</td>
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<tr>
<td>Birth weight (g)</td>
<td>Mean ± SD</td>
<td>3,600 ± 650</td>
<td>1,850 ± 380</td>
<td>1,622 ± 625</td>
</tr>
<tr>
<td></td>
<td>Range</td>
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<td>1,400-2,450</td>
<td>700-2,400</td>
</tr>
<tr>
<td>Placental weight (g)</td>
<td>Mean ± SD</td>
<td>761 ± 182</td>
<td>425 ± 85</td>
<td>348 ± 105</td>
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<td></td>
<td>Range</td>
<td>600-1,150</td>
<td>300-500</td>
<td>180-500</td>
</tr>
<tr>
<td>PAI-1 in PPP</td>
<td>Mean ± SD</td>
<td>94 ± 16</td>
<td>187 ± 87</td>
<td>69 ± 21</td>
</tr>
<tr>
<td>(ng/mL)</td>
<td>Range</td>
<td>80-129</td>
<td>140-356</td>
<td>40-105</td>
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<tr>
<td>PAI-2 in PPP</td>
<td>Mean ± SD</td>
<td>221 ± 59</td>
<td>135 ± 78</td>
<td>94 ± 45</td>
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<tr>
<td>(ng/mL)</td>
<td>Range</td>
<td>160-345</td>
<td>60-300</td>
<td>42-184</td>
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<tr>
<td>PAI-1 in placenta extracts</td>
<td>Mean ± SD</td>
<td>43 ± 17</td>
<td>121 ± 38</td>
<td>51 ± 22</td>
</tr>
<tr>
<td>(ng/mg)</td>
<td>Range</td>
<td>23-82</td>
<td>80-200</td>
<td>22-96</td>
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<tr>
<td>PAI-2 in placenta extracts</td>
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<td>536 ± 98</td>
<td>209 ± 144</td>
<td>168 ± 106</td>
</tr>
<tr>
<td>(ng/mg)</td>
<td>Range</td>
<td>413-726</td>
<td>30-388</td>
<td>30-333</td>
</tr>
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</table>

Abbreviation: NS, not significant.

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In addition to alterations in the plasma levels of PAI-1 in PE, reduced levels of PAI-2 in plasma of women whose pregnancies were complicated with IUGR have been reported.\(^1\) An analysis for PAI-2 antigen in the various samples showed a significant correlation between the levels of PAI-2 in plasma and the levels for this inhibitor in placental extract (plasma vs placental PAI-2: \( r = .63; \) Fig 1D). Furthermore, significant positive correlations were observed in all the clinical groups between newborn weight and either plasma PAI-2 concentration (\( r = .75; \) Fig 1E) or placental PAI-2 concentration (\( r = .74, \) Fig 1F). Thus, the individuals who delivered small-for-gestational-age infants were found to have significantly reduced placenta PAI-2 levels (group B: \( 209 \pm 144 \text{ ng/mg}, \ P < .001; \) group C: \( 169 \pm 106 \text{ ng/mg}, \ P < .0003)\) than present in normal placentas (535 ± 98 ng/mg) (Table 1).

Immunohistochemical localization of PAI-1 and PAI-2 in placental tissues. Immunohistochemical staining for PAI-1 and PAI-2 was initially used to investigate the distribution of these inhibitors within the placentas of these three groups. As described by Feinberg et al, weak PAI-1 immunoreactivity was observed in the syncytiotrophoblast cells from the normal placenta (Fig 2A shows a representative syncytiotrophoblast area from a normal placenta). No PAI-1 signal was observed in the endothelial cells (ECs) of the placenta (Fig 2A), whereas these cells could be identified...
Fig 2.
in parallel sections by positive stained using an antibody to von Willebrand factor (data not shown). Weak PAI-1 immunoreactivity was also observed in the syncytiotrophoblasts of the 11 placentas from the IUGR group (Fig 2B), whereas prominent PAI-1 immunostaining was observed in numerous villous syncytiotrophoblast cells from the eight PE placentas (Fig 2C shows a representative syncytiotrophoblast area from one of the eight PE patients). Moreover, extensive infarction areas (infarcts involved >20% of total villous tissue) were observed in five of the eight PE patients studied and an intense staining was observed in every infarction area from the PE placentas (Fig 2, D and E). Furthermore, no PAI-1 signal was detected in ECs of fetal vessels from PE placentas (Fig 2C). No immunostaining was observed when nonimmune rabbit IgG was used (Fig 2, G through K). Extensive infarction areas were not observed in the group of patients suffering from IUGR without PE.

As previously reported, prominent PAI-2 immunostaining was observed in syncytiotrophoblast cells from normal placentas (Fig 2M, n = 10). In contrast to this intense PAI-2 immunostaining, a decrease in PAI-2 immunoreactivity was observed in syncytiotrophoblast cells from IUGR placentas without (Fig 2N, n = 11) or with PE (Fig 2O, n = 8). PAI-2 antigen staining was not detectable within the infarction areas from the PE placentas (Fig 2P, n = 5). No immunostaining was obtained in control experiments using nonimmune goat IgG as the primary antibody (data not shown).

Detection of PAI-1 mRNA in placental samples. The elevated PAI-1 concentration and immunostaining of the PE samples may have occurred as a result of an altered deposition of this inhibitor in the placenta or because of altered PAI-1 production within placenta. To define the levels of PAI-1 mRNA in the placental tissue, we have used in situ hybridization. Analysis of paraffin sections of a placenta obtained from a healthy normotensive woman using 35S-labeled antisense riboprobes for PAI-1 did not show any positive signal after 2 weeks of exposure, and only a weak signal was observed after 10 weeks exposure to the NTB2 emulsion (Fig 2, Q and F, respectively, n = 10). In contrast, a strong positive signal for PAI-1 mRNA in villous syncytiotrophoblast from PE patients was observed after 2 weeks of exposure (Fig 2L, n = 5). A strong positive signal for PAI-1 mRNA was also observed in infarction areas (Fig 2R, n = 5). When a sense probe was used as a control in these experiments, no specific signal was detected in placentas after 12 weeks of exposure.

To confirm that the level of PAI-1 mRNA in the placental tissue was elevated in PE patients, total RNA was extracted from placentas of pregnant women from each of the three groups and PAI-1 mRNA was analyzed by Northern blotting. An increase in PAI-1 mRNA was observed in two placentas from severe PE patients (Fig 3, lanes 1 and 4) in comparison with the PAI-1 mRNA level in the placenta from a normal pregnancy (Fig 3, lane 3) and from an IUGR pregnancy (Fig 3, lane 2).

DISCUSSION

The plasminogen activation system appears to be regulated in the human female during ovulation, blastocyst implantation, and pregnancy. Two distinct fast-acting inhibitors of PAs have been identified, and both immunologic and molecular probes are available for analyzing the cellular expression of these two inhibitors. Immunologic analyses have shown that both PAI-1 and PAI-2 are present in normal human placental tissue and that plasma levels for both inhibitors continuously increase during the course of pregnancy. A number of published reports have raised the possibility that abnormal production of these inhibitors during pregnancy may not only be correlated with but may contribute to certain obstetric pathologies. In this report, we have identified the cells within the normal-appearing villous tissue that are responsible for the local production of PAI-1 in women with PE and IUGR. Initial studies showed that the levels for PAI-1 in placental extracts from PE/IUGR patients were significantly elevated over the levels detected in placentas from normotensive pregnancies (Table 1). To reduce the possibility that our analyses of PAI antigen levels within the placenta extracts would be influenced by contaminating blood, our initial strategy during sample collection was focused on dissecting the placenta tissues into small pieces (≤1 mm3) that were washed extensively before storage in liquid nitrogen. Similar studies on PAI-2 in our three clinical groups demonstrated that a decrease in placenta PAI-2 levels was observed in IUGR pregnancies in comparison with normal pregnancies (Table 1).
To determine the distribution of these inhibitors within the placentas of the different clinical groups, immunohistochemical analysis for PAI-1 and PAI-2 were used. Prominent PAI-1 immunoreactivity was detected within the infarction areas and in numerous syncytiotrophoblast cells from PE/IUGR placentas in comparison with the weak immunoreactivity observed in placentas from the IUGR group (Fig 2) or reported for placentas from normal pregnant women. In contrast, a decrease in PAI-2 immunoreactivity was detected in syncytiotrophoblasts from all IUGR placentas (Fig 2) in comparison with the prominent PAI-2 staining reported for normal placentas. In situ hybridization analysis was subsequently used to document that the increased cellular staining for PAI-1 antigen correlated with an increased PAI-1 mRNA content. A strong positive hybridization signal was obtained in villous syncytiotrophoblasts and in infarction areas from placentas of PE/IUGR patients after 2 weeks of exposure (Fig 2). In comparison, a positive signal for PAI-1 mRNA in normal placentas required a 10-week length of exposure to the photographic emulsion. Because cultured trophoblasts have been reported to contain both the 2.3- and 3.2-kb mRNA transcripts for PAI-1, we examined placenta samples from a representative of all three groups by Northern analysis for these two PAI-1 mRNA species. Our data indicate that both transcripts of PAI-1 mRNA are increased in two placentas from severe PE/IUGR patients in comparison with placenta tissue from a normal pregnancy and from an IUGR pregnancy (Fig 3).

Although data presented in this study document that the expression of PAI-1 is elevated in PE/IUGR placenta, little information is available on the factors that regulate the production of PAI-1 within either normal or PE/IUGR placentas. Recent data indicate that a wide variety of compounds (eg, cytokines, growth factors, etc) are capable of affecting the expression of PAI-1. The observation that normal human and rat placentas are capable of producing several different cytokines, hormones, and growth factors raises the possibility that changes in the levels of one or more of these molecules may modulate the expression of PAI-1 in patients suffering with PE and/or IUGR. For example, the reduced uteroplacental blood flow often present in pregnancies complicated with PE and/or IUGR could increase the local concentration of a particular cytokine or growth factor. Moreover, the changes in the placental vasculature and decidual spiral arteries, commonly observed in PE patients, often result in hypervascularity of the villi, placental infarction, and ischaemia. This latter condition is known to affect vascular PAI-1 production. In light of the high second-order rate constant of PAI-1 (Ki = 10^7 [mol/L]^-1 s^-1) against single-chain t-PA (ie, the predominant form of t-PA in the vascular system) in comparison with the low second-order rate constant of PAI-2 (Ki = 9 × 10^2 [mol/L]^-1 s^-1) against this form of the enzyme, the localized elevated placenta PAI-1 levels observed in PE/IUGR patients would be expected to rapidly neutralize single-chain t-PA and thus play a role in the thrombotic complications associated with this disease. Elucidation of the mechanisms that regulate the altered production of PAI-1 in a patient's placenta should shed light on the processes that underlie these pathologic conditions.

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