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EXPRESSION AND LOCALIZATION OF THE UROKINASE-TYPE PLASMINOGEN ACTIVATOR RECEPTOR (uPAR) IN THE HUMAN PLACENTA

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INDEXING WORDS

urokinase-type plasminogen activator (uPA); urokinase-type plasminogen activator receptor (uPAR); plasminogen activator inhibitor-2 (PAI-2);

SYNOPSIS

In pregnancy the decrease in fibrinolytic activity has been described to date by many researchers. The placenta is a source of both plasminogen activator inhibitor type 2 (PAI-2) and type 1 (PAI-1). The PAI-2 was originally detected in the human placenta, which inhibits urokinase-type plasminogen activator (uPA) while it is unable to inhibit tissue type plasminogen activator (tPA). The urokinase-type plasminogen (uP) is activated by u-PA and urokinase-type plasminogen activator receptor (uPAR). We studied the expression and the localization of u-PAR in the human placenta by in situ hybridization, immunohistochemically and by use of Northern blot analysis. These data show that the decidualized endometorium have the cells with the greatest uPAR mRNA expression in the placenta, and syncytiotrophoblasts express lower level of uPAR mRNA.

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INTRODUCTION

Recent studies have suggested that the fibrinolytic system is associated closely with several processes, including thrombic formation, tumor invasion, inflammation, wound healing, neovascularization and the fetal development. Its activity influences the maintenance of the placenta and various biological behaviors of gestation. In the placenta, tPA is thought to modulate vascular fibrinolysis, but uPA is an important one in the fibrinolytic system. On the other hand, PAI-2 was at first detected from the human placenta and was isolated from its extract[2,6,15,16,20] but PAI-1 had been found out in the liver. PAI-2 mRNA is mainly expressed in the skin, bone marrow, spleen, lung, thymus and urinary bladder.[17] Expression of PAI-2 in the embryo is high in the early and middle stage of gestation, but shows the low titer in the late stage of gestation.[21] PAI-2 mRNA in the placenta rises gradually at the late stage of gestation. PAI-2 may play a role in protecting the embryo from the protease attack in the amniotic fluid and maintaining and guarding the skin and the epithelial surface of embryos.[17] The blood level of PAI-2 increases in women during normal pregnancies. However, the titer of blood PAI-2 decreases in those with pre-eclampsia and intra-uterine growth retardation.[6,8-10,12,14,22,27] PAI-1 inhibits both tPA and uPA efficiently, while PAI-2 is unable to inhibit tPA and is currently thought to be same as uPA inhibitor. uP is activated by uPA and uPAR.[1] Exactly, in our examination, immunohistochemistry data of uPAR in the placenta showed the high positivity in decidual cells. In situ hybridization of uPAR mRNA revealed positive stain in the villous surface and decidual cells. Expression of uPAR mRNA by Northern blotting showed significant positive signals in decidual tissue, however, in the villous placental tissue demonstrated no any obvious uPAR mRNA signals. These data show that the decidualized endometorium have the cells with the greatest positive uPAR mRNA expression in the placenta, but syncytiotrophoblasts express lower level of positive uPAR mRNA. In the gestation, the fibrinolytic system is summarized as the state "Hypercoagulation and hypofibrinolysis in general." Higher occurrence of the formation of thrombi in the placenta is closely correlated to stronger activity of fibrinolytic system in the placenta. These results from our analysis suggest that uPAR may play a role in fibrinolytic system of the placenta and its development.

MATERIALS AND METHODS

Materials

Fifty cases of human placental tissues, ranging in age from 16 to 40 weeks of gestation, were studied pathologically , immunohistochemically and by use of molecular biology procedures. Tissue samples of 18 cases were obtained from pregnancies after Caesarean section delivery, 3 cases were from artificial abortion and 29 cases were from normal deliveries. Furthermore, at the Caesarean section, samples were obtained from two part of the placenta, one part was placental villi, the other was basal decidual tissue. Using these samples, we performed immunohistochemistory, in situ hybridization and Northern blot analysis.

Immunohistochemistry

The expression and localization of uPAR, uPA, PAI-1 and PAI-2 proteins in the tissue sections were examined immunohistochemically using monoclonal antibodies against human uPAR (#3936, American Diagnosis Inc., Greenwich, CT), uPA (#389, American Diagnosis Inc., Greenwich, CT), PAI-1 (MAI11 IgG, Biopool research biochemical) and PAI-2 (#375G, American Diagnosis Inc., Greenwich, CT) Immunohistochemical staining was carried out with the Large Volume DAKO LSAB® Kit, Peroxidase (Dakopatts, Copenhagen, Denmark) according to the manufacturer's instructions. The samples were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, PH 7.0, at 4℃ overnight, and then they dehydrated with a series of ethanol and defatted with chloroform and embedded in paraffin. Each sections were cut at 4 μm in thickness and mounted on glass slides. Those slides were deparaffinized in xylene (5 minutes, three times), dehydrated in ethanol, and incubated with 3% hydrogen peroxide for 15 minutes, then with blocking solution for 30 minutes and finally incubated with the monoclonal antibodies against uPA, PAI-1 and PAI-2, diluted 1:50 in 0.01% PBS for 2 h at room temperature. On the other hand, for the uPAR immunostaining, fresh materials were frozen immediately after their resection in liquid nitrogen with O.C.T.compound and were cut 4 μm in thickness and mounted on glass slides, and dehydrated in ethanol routinely, and then incubated with the monoclonal antibody against uPAR. After washing with Tris buffered solution, expression of staining color was achieved with aminoethylcarbazole chromogen containing hydrogen peroxide. Negative

controls were incubated without the monoclonal antibodies against uPAR, uPA, PAI-1 and PAI-2 proteins. Those sections were counter-stained with hematoxylin and mounted.

Preparation of RNA and cDNA probes

Digoxigenin-labeled single-strand RNA probes were prepared with a DIG RNA Labeling Kit (Boehringer Mannheim GmbH Biochemica, Mannheim, Germany) according to the manufacturer's instructions. To generate the human uPAR probe, the 585bp BamHI and 268bp PstI fragments of the uPAR cDNA clone were subcloned into Vector pGEM®-3Z (Promega, WI, USA). These plasmids were linearized with Hind Ⅲ and transcribed with T7 RNA polymerase to generate an antisense (cRNA) probe, or with EcoRI (for the 585bp BamHI fragment) or AccI (for the 268bp PstI fragment) and transcribed with SP6 RNA polymerase to generate a sense probe. To acquire Dig-labeled c-DNA fragments as probes for Northern blot analysis, the Vector pGEM®-3Z subcloned by the 585 bp BamH1 fragment was amplified by polymerase chain reaction (PCR)with PCR DIG Probe Synthesis Kit (Boehringer Mannheim), using RNA Polymerase Promoter Sequencing Primers, T7 and SP6 (Promega).

In situ hybridization

uPAR mRNA expression in the tissue section was localized by in situ hybridization techniques. We selected 10 samples, and these were frozen in liquid nitrogen with O.C.T.compound and 4 μm sections in thickness were cut and mounted on slides coated with 3-(triethoxysilyl) propylamine (Merck, Darmstadt, Germany). In situ hybridization techniques have been described previously.[1,9,10,12,25] Hybridization of uPAR mRNA was carried out at 50℃ for 16h, and the signals were detected with a nucleic acid detection kit (Boehringer Mannheim Biochemica). The slides were counterstained with hematoxylin. The control was hybridization with the sense probes. This experiment yielded no detectable signals.

Northern blot analysis

Northern blot analysis was carried out by a modified non-radiographic method as described.[19] We selected samples, that were obtained from pregnancies after Caesarean section delivery or from artificial abortion to

get fresh placental tissues. Total RNA was purified from frozen samples with ISOGEN (Nippon Gene, Tokyo, Japan). 20 μg total RNA per lane was electrophorased on 1% agarose gels containing 7.5% formaldehyde and transferred to nylon membranes (Nylon membranes positively charged, Boehringer Mannheim) by capillary action in 20 x SSC. The membranes were baked for 30 min. at 120℃ and prehybridized in prehybridization buffer (0.5% SDS, 5 x SSC, 10 x Denhardt's solution, 10 mM Na_2PO_4, 50% formamide, 0.1 mg/ml sonicated salmon sperm DNA) for 3 h at 50℃. The RNA was probed by hybridization at 50℃ with DIG-labeled DNA probe under prehybridization conditions overnight. The membranes were washed with 0.2 x SSC containing 0.1% SDS (20 min. at 68℃ , twice), rinsed in DIG-buffer 1, incubated with 1% Blocking Reagent (Boehringer Mannheim) in DIG buffer 1 for 60 Min. at room temperature, incubated with 0.5 ml anti-digoxigenin alkaline phosphate-conjugated anti-body diluted 1:10000 in DIG-buffer 1 with 0.2% polyoxethylen(20)solution monolaurate (Wako Pure Chemical Industries Ltd.,Osaka, Japan) for 30 Min. at room temperature, then washed with DIG-buffer 1 (15 min., twice) and rinsed with DIG buffer 3 for 3 min. For detection of signals, the membranes were incubated at 37℃ with CSPD (disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricloro[3.3.1.13,7]decan}-4-ly)phenyl phosphate) (Boehringer Mannheim) diluted 1:100 in assay solution (100 mM diethanolamine, 2 mM $MgCl_2$, 0.02% NaN_3) for 10 min. Hybridization signals were exposed to X-ray film (HyperfilmTM-ECL,Amersham) for 3 h at room temperature. After hybridization, the blots were stripped and rehybridized with ß-actin cDNA probe.

RESULTS

Immunohistochemistry

Immunohistochemical studies with antibodies against human uPAR, uPA, PAI-1 and PAI-2, were performed in all samples to evaluate the expression and localization of their antigens respectively. The immunoreactivities were observed in syncytiotrophoblast and decidual cells. Mild immunoreactivity for uPAR and PAI-1 expression was observed in the cytoplasm of the cells (Figs. 1A, 1B, 1E and 1F), but their immunoreactivity was weak compared with uPA and PAI-2. Marked uPA and PAI-2 immunostaining were observed in cytoplasms of

syncytiotrophoblasts and decidual cells (Figs. 1C, 1D, 1G and 1H). No any expressions of immunostaining were obtained in control experiments and then not involved in this paper.

In situ hybridization of uPAR mRNA

In order to examine the localization and the distribution of uPAR mRNA, in situ hybridization was performed in 10 samples. Weak uPAR mRNA expression was seen in villous syncytiotrophoblasts and stromal cells (Fig. 2A), and strong hybridization signals were detected in cytoplasms of decidual cells (Fig. 2C). When sense probe was used as control in these experiments, no signals were detected in placentae (Figs. 2B and 2D).

uPAR mRNA expression by Northern blot analysis

In the villous placental tissue demonstrated no any obvious uPAR mRNA signals, however, decidual tissue demonstrated significant positive signals (Fig. 3). The intensity of uPAR bands was 30% of that of ß-actin. These results confirmed those of in situ hybridization. In addition we can't find any relations between uPAR expression and clinical complication of pregnancies.

DISCUSSION

It has been known for many years that various modifications in coagulative and fibrinolytic system occur as pregnancy advances. During

Figure 1. Immunohistochemistry of uPAR (A and B), uPA (C and D), PAI-1 (E and F), and PAI-2 (G and H) in placenta samples. Positive staining is shown by the brown color. Frozen sections (A and B) and paraffin sections (C-H) of placentas from normal pregnant women were analyzed by immunohistochemical techniques. (A), (C), (E) and (G) are villous tissues and positive immunoreactivity are detected in syncytiotrophoblasts. (B), (D), (F) and (H) are decidual tissue and positive immunoreactivity are detected in decidual cells. Immunoreactivity of uPAR and PAI-1 were weakly positive but prominently positive observed in uPA and PAI-2. Original magnification X200.

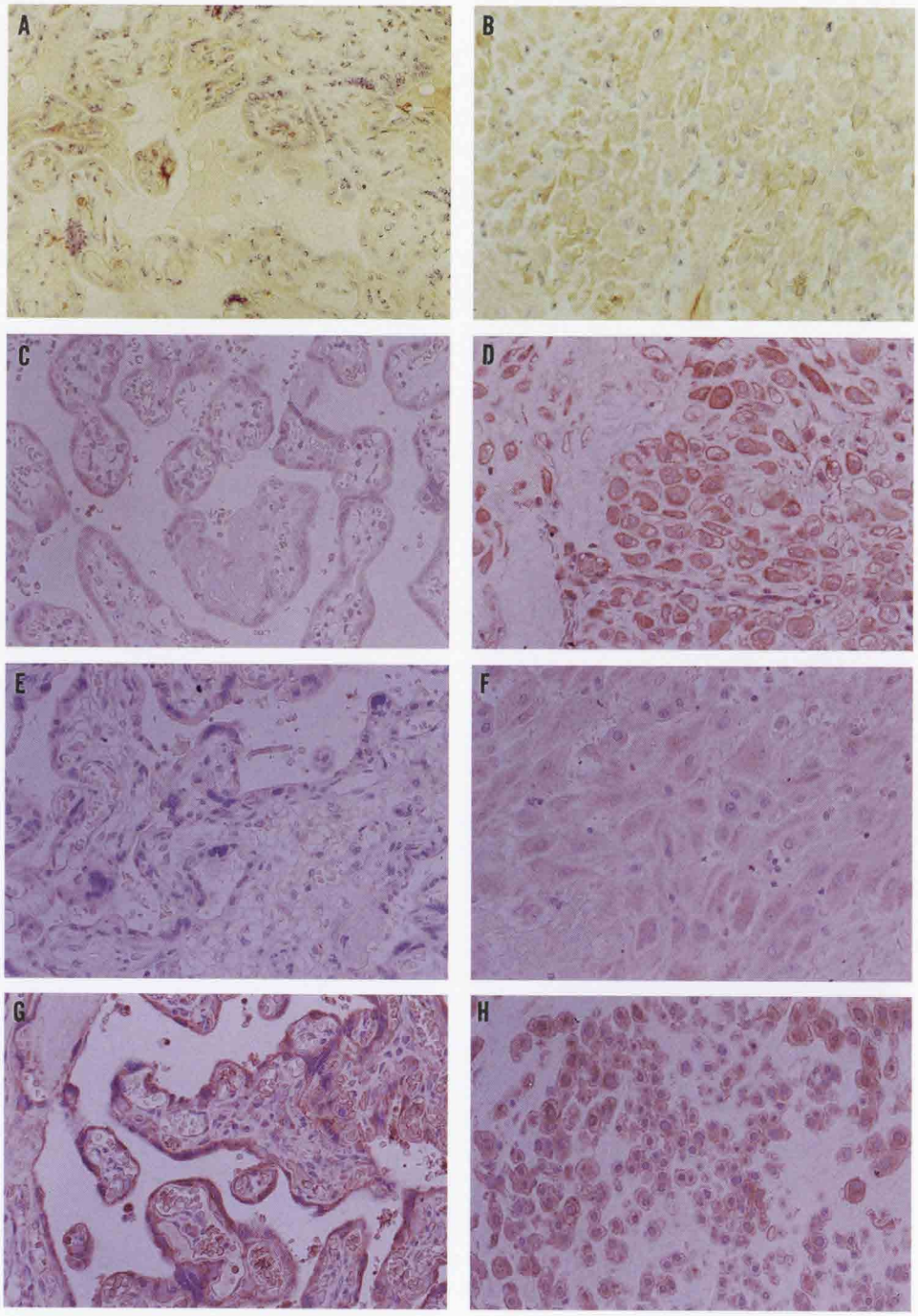

Fig. 1.

pregnancy, the increase of PAI-1, PAI-2, tPA and uPA have been described in plasma levels.[3,26] These reports suggested that the plasminogen activation system may also play a critical role in ovulation, implantation, embryonic tissue remodeling and placental function and so forth. In this study we reported the distribution and expression of uPAR mRNA in the placenta with in situ hybridization and Northern blot analysis, and these data showed that the decidualized endometrium have the cells with the greatest positive uPAR mRNA expression in the

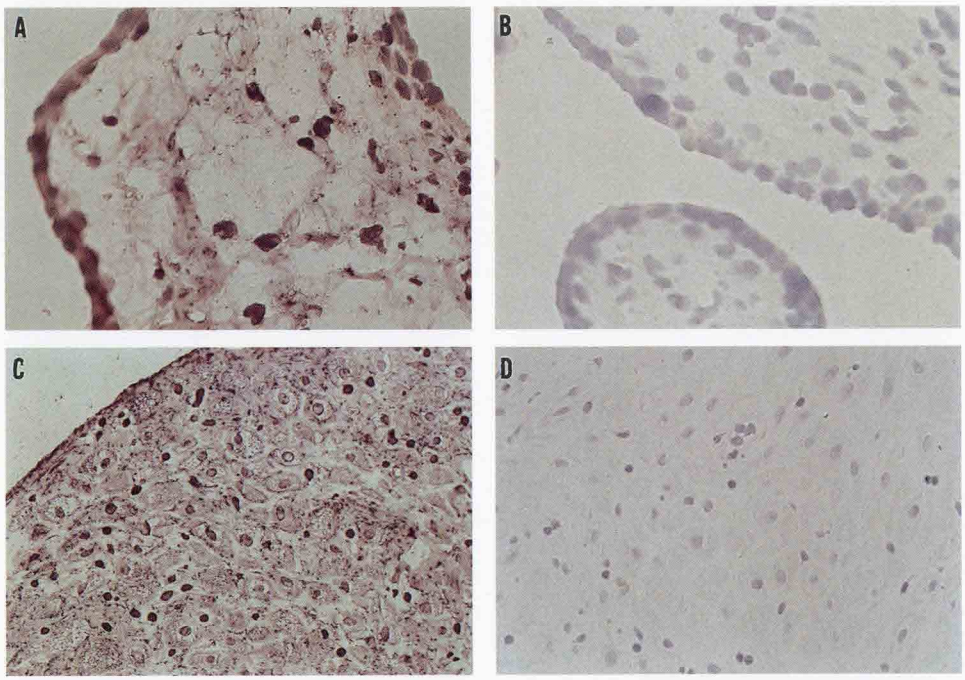

Fig. 2.

Figure 2. Localization of uPAR gene transcript in human placental samples. Frozen sections of placentas from normal pregnant women were analyzed. The sections were hybridized with antisense (A and C) or sense (B and E) riboprobes. (A) and (B) are villous tissues, (C) and (D) .are decidual tissue, and uPAR mRNA is positive in syncytiotrophoblasts and decidual cells. In situ hybridization of uPAR with sense probe (B and D), reactivity is not seen. Original magnification, X400 (A and B), X200 (C and D).

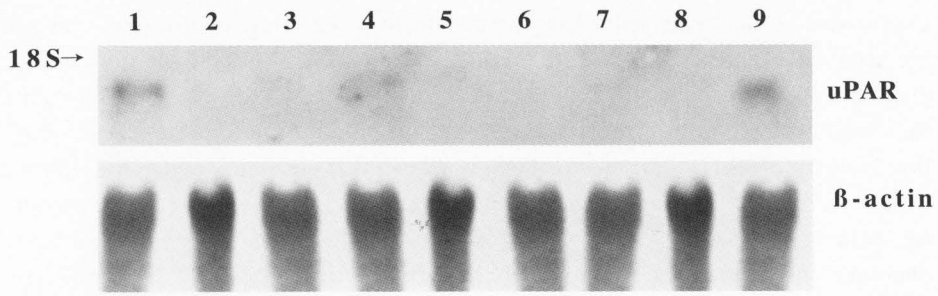

Figure 3. Representative Northern blot analysis of uPAR gene transcript in human placenta. Lane 1: 16 Gestational Week (GW) Intrauterin fetal death, Lane 2: 34 GW toxemia, Lane 3 and 4 (same patient): 25 GW premature delivery, Lane 5: 29 GW C/S, Lane 6: 24 GW toxemia, Lane 7: 36 GW polyhydramnios, Lane 8 and 9 (same patient): 29 GW hydrocephaly. And Lane 2, 3, 5-8 are villous tissue, Lane 4 and 9 are decidual tissue, Lane 1 is mixed tissue. The position of 18S rRNA (186Kb) is isolated. The villous placental tissues demonstrated no obvious uPAR mRNA signals (Lane2, 3, 5 - 8), however, mixed and one of the decidual tissue demonstrated significant positive signals (Lane1 and 9), and another decidual tissue demonstrated weakly positive signal (Lane 4).

placenta, but syncytiotrophoblasts express lower level of positive uPAR mRNA. From the data of Feinberg et al.,[11] using immunohistochemical analysis, PAI-1 demonstrated weakly positive staining of villous syncytiotrophoblasts but prominently positive staining of trophoblasts' cytoplasms which invade in the decidua and myometrium, but PAI-2 stained positively cytoplasms of villous syncytiotrophoblasts, and no stains were detected in invading trophoblasts. This distinctive pattern of PAIs expression may show that the plasminogen activation system has some relationship to trophoblastic invasion.[4,7,18] On the other hand, some other reports said that the significance of uPAR expression in cancer cells is closely associated with cancer invasion and metastasis.[23-25] In the placenta, it is not the same as cancer invasion but has some similarities to the trophoblastic migration.[1,7,13] Our data shows that weakly positive uPAR mRNA expression was seen in villous tissue, but strongly positive signals were detected in decidual cells. This data suggests that uPAR

expression in decidual cells has some role in trophoblastic invasion. From the data of Bugge et al.,[5] using the uPAR-deficient mice, uPAR deficiency dose not compromise fertility, development, or hemostasis. Although they summarized that uPAR is not essential for mouse development, but the human placental tissue is not same as that of mice with the great difference in the size and complicated functions, therefore we can't accept the results so simply. We would like to conclude that trophoblasts and decidual cells produce proteins of the fibrinolytic system, that may keep the normal implantation and regulate normal placental development throughout pregnancy.

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