Presence of Urokinase Plasminogen Activator and Plasminogen Activator Inhibitor-1 Messenger Ribonucleic Acids in Rat Endometrium during Decidualization In Vivo

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ABSTRACT

Rat endometrial stromal cells undergoing decidualization in vitro secrete urokinase-type plasminogen activator (uPA), and this secretion is regulated by prostaglandin E2. The present study was undertaken to determine whether uPA and plasminogen activator inhibitor-1 (PAI-1) mRNAs are expressed in vivo in the decidua of pregnant rats and in the decidualuoma of pseudopregnant rats. Total RNA was prepared from nondecidualized and decidualized endometrial tissues at various stages of early pregnancy and examined by Northern blot analysis using specific cDNA probes for rat uPA and PAI-1. There was little uPA mRNA in the endometrium during the first 5 days of pregnancy (Day 1 = the presence of sperm in the vagina). A high level of uPA mRNA was detected on Day 7, and it declined thereafter. There was a gradual increase in PAI-1 mRNA in the decidua from Day 7 of pregnancy, reaching a peak level on Day 15 when the decidua was transformed into the maternal placenta. (RNA from Day 7 of pregnancy, reaching a peak level on Day 15 when the decidua was transformed into the maternal placenta. (RNA from Day 7 of pregnancy, reaching a peak level on Day 15 when the decidua was transformed into the maternal placenta. (RNA from Day 7 of pregnancy, reaching a peak level on Day 15 when the decidua was transformed into the maternal pla... -  )

INTRODUCTION

In rodents, the endometrial stromal cells proliferate and differentiate into decidual cells during embryo implantation. This process is controlled by the endocrine milieu during early pregnancy and by signals from the implanting embryos [1]. As the endometrium undergoes decidualization, the implanting embryos invade the endometrium. In order to establish an intimate contact with the maternal blood supply [2, 3]. Although the implanting embryos produce matrix-degrading enzymes for invasion, some evidence indicates that degradation and turnover of extracellular matrix (ECM) in the endometrium are, at least partially, independent of the implanting embryos [4-6]. Various types of ECM-degrading enzymes are produced in the human endometrium under the control of steroid hormones [7, 8]. These enzymes are probably involved in many tissue remodeling events during decidualization, such as the invasion of trophoblast cells, angiogenesis within the decidua, and migration of blood-borne cells into the decidua.

Plasminogen activators (PAs), particularly the urokinase type PA (uPA), have been implicated in the control of tissue remodeling because of their roles in regulating ECM turnover [9]. Recent studies have demonstrated that rat endometrial stromal cells from uteri sensitized for the decidual cell reaction secrete uPA in vitro when cultured under conditions that allow the cells to decidualize. Prostaglandin E2, a mediator of decidualization, regulates the in vitro secretion of uPA, at least partially, by regulating the steady-state level of uPA mRNA [10]. The present study was designed to determine whether the levels of uPA mRNA in the rat endometrium change during decidualization.

MATERIALS AND METHODS

Preparation of Animals

Female Sprague-Dawley rats, at 200-250 g BW, were purchased from Charles River Canada (St. Constant, PQ, Canada) or Harlan Sprague-Dawley Inc. (Indianapolis, IN) and housed in temperature- and light-controlled conditions (lights-on from 0500 to 1900 h) with free access to food and water. To produce natural pregnancies, female rats were paired with fertile males and checked every day for the presence of sperm in vaginal smears as an indication of mating. The day on which sperm were first found in the vaginal smear was designated Day 1 of pregnancy. To induce artificial decidualization, a state equivalent to pseudopregnancy was produced in ovariectornized rats by the administration of estradiol and progesterone [11]. On the equivalent of Day 5 of pseudopregnancy at approximately noon, decidualization was induced by the bilateral intrauterine injection of 100 μl sesame oil.

Collection of Tissues

To collect endometrial tissue on Days 3 and 5 of pregnancy or "pseudopregnancy," each uterine horn was cut longitudinally and the endometrial tissue was scraped off the myometrium with a spatula as described by Martel and Psychoyos [12]. Decidual tissue on Day 7 of pregnancy was similarly separated from myometrium after the embryonic tissues were removed under a stereomicroscope. On Day...
14 000 tially added to the homogenate. The mixture was centrifuged treated phenol, chloroform, and isoamyl alcohol were sequenc- 0.5% Sarkosyl, and 0.1 M 2-mercaptoethanol. Water-satu- cyanate solution containing 25 mM sodium citrate (pH 7.5), imme- diately homogenized in cooled, 4 M guanidinium thio- procedure [13]. The tissues were minced on ice and process daughter for 1 h at 65 o C using the process autoriadiographs. The ratio of mRNA to 18s rRNA is normalized to Day 3 = 1. * Compared with values for uPA on other days, p < 0.05; ** compared with values for PAI-1 on other days, p < 0.05.

10, decidua containing the embryo was gently squeezed out of the uterus through an incision in the myometrium at the implantation site. The embryonic tissue was removed from the decidua under a stereo microscope. By Day 15 of pregnancy, the decidua has developed into the maternal placenta, which was collected by peeling away the fetal component with fine forceps.

**RNA Isolation and Northern Blot Hybridization**

Total RNA was isolated according to a previously described procedure [13]. The tissues were minced on ice and immediately homogenized in cooled, 4 M guanidinium thiocyanate solution containing 25 mM sodium citrate (pH 7.5), 0.5% Sarkosyl, and 0.1 M 2-mercaptoethanol. Water-satured phenol, chloroform, and isoamyl alcohol were sequential- added to the homogenate. The mixture was centrifuged at 14 000 x g, and the aqueous phase containing the RNA was collected after centrifugation. The RNA was precipitated with isopropanol. and resuspended in water. The RNA samples were electrophoresed in a 1% agarose gel containing formamide and 6% formaldehyde in the presence of MOPS buffer (20 mM 3-[N-morpholinolpropanesulphonic acid, 5 mM sodium acetate, 1 mM EDTA, pH 7.2). After electro- phoresis, the RNA was blotted onto positively charged nylon membranes (Hybond N+; Amersham Canada, Oakville, ON) by capillary transfer and cross-linked by exposing the membranes to a UV light (Stratalinker TM 1800; Stratagene, La Jolla, CA) for 5 min at an intensity of 12 000 joules/cm². The membranes were prehybridized for 15 min and hybrid- ized with P32-labeled cDNA probes for uPA, PAW, and 18S rRNA cDNA. a) Autoradiograph of Northern blots. b) Densitometric analysis of three replicate autoradiographs. The ratio of mRNA to 18s rRNA is normalized to Day 3 = 1. * Compared with values for uPA on other days, p < 0.05; ** compared with values for PAI-1 on other days, p < 0.05.

**Histology and In Situ Hybridization**

Implantation sites from various days of pregnancy were fixed in 4% paraformaldehyde and 0.2% glutaraldehyde at 4°C for 18 to 20 h. After fixation, the tissues were washed in PBS for 48 h at 4°C with changes of fresh PBS every 8 to 10 h, dehydrated, and embedded in paraffin. All the reagents used for in situ hybridization were purchased from Bohringer-Mannheim Canada (Laval, PQ, Canada) except as specified otherwise. The procedures were carried out ac- cording to the instructions of Bohringer-Mannheim with some modifications [14]. Paraffin sections 5 μm thick were dewaxed in two changes of xylene and rehydrated in a 100%, 95%, 80%, and 70% ethanol series for 5 min each and rinsed with 4-strength SSC. The sections were digested with proteinase K and hybridized with digoxigenin (DIG)labeled riboprobes for 16 h at 50°C. After hybridization, nonhybridized RNA was removed by incubating the sections with RNase A. The sections were washed with double-strength SSC and then incubated with anti-DIG antibody conjugated with intestinal alkaline phosphatase. To detect the intestinal alkaline phosphatase activity in the sections, sections were incubated with a mixture of colored substrates (nitro blue tetrazolium and 5-bromo-4-chloro-3indoyl phosphate) in the presence of 1 mM levamisole (Sigma, St. Louis, MO) to block endogenous alkaline phosphatase. Sections were incubated with the mixture for 6 to 16 h in complete darkness to develop a blue-colored precipitate as an indication of hybridization of the probe with target mRNA.

**Preparation of Probes**

A 348-bp cDNA fragment for uPA was obtained by reverse transcription and polymerase chain reaction, with total RNA from rat embryos used as templates, and was cloned into pBluescript vectors (Promega, Madison, WI) for sequencing to verify its identity [15]. For the PAI-1 probe, a Pvu II fragment (980 bp) was excised out of a full-length, rat PAI-1 cDNA (kindly provided by Dr. Gelehrter of University of Michigan, Ann Arbor, MI [16]) and subcloned into pBluescript plasmid. Complementary DNA for mouse 18S rRNA was a gift from Dr. J. Hammond of University of Western Ontario, Ontario, Canada. To prepare probes for Northern blot hybridization, the template cDNA fragments were excised out of the plasmid vectors and labeled with 32P-dCTP (Amersham Canada) by random priming reactions using a random priming kit from Gibco-BRL Canada (Burlington, ON). For in situ hybridization, riboprobes were synthesized by appropriate DNA-dependent RNA polymerases to incorporate DIG-labeled UTP, using a DIG labeling kit from Bohringer-Mannheim. The size of the labeled riboprobes was reduced to approximately 150 nucleotides by limited alkaline hydrolysis as described by Cox et al. [17].

**Densitometry and Data Analysis**

All the Northern blot analyses were repeated three times on RNA samples collected on different occasions. The mRNA signals on autoradiograms were partially quantified by scanning densitometry using a Desk-Top Scanner and ImageMaster Software (Pharmacia-LKI3 Biotechnology, Uppsala, Sweden). The relative density of each target mRNA was expressed as the ratio to the relative density of the 18S rRNA in the same sample, and the ratios were pooled between the three Northern blot analyses to calcu
FIG. 2. In situ hybridization of rat implantation sites. Tissue sections were hybridized with DIG-labeled riboprobes; to produce a bluish-colored precipitate as the hybridization signal. The antisense probe for uPA hybridized with primary decidual cells surrounding the implantation chamber (IC), particularly in the antimesometrial region (A), on a section from Day 7 of pregnancy (a, X25), while the sense probe produced no hybridization signal on an adjacent section (b). Plasminogen activator inhibitor-1 mRNA was detected by antisense probes in decidual cells (M) and, more profoundly, in trophoblast cells (T) and fetal tissues (F) on a section from Day 10 of pregnancy (c, x 100). An adjacent section (d) showed no significant hybridization with the sense probe for PAI-1.

late the mean and SD. The level of significance of differences between the means was determined by Duncan’s Multiple Range test.

RESULTS

Abundance of uPA and PAI-1 mRNAs in Uterine Tissues of Pregnancy

Total RNA from uterine tissues of pregnant rats was examined by Northern blot analysis. Hybridization with uPA and PAI-1 probes each detected a single band corresponding to the target mRNAs, approximately 2 kb for uPA and 3 kb for PAI-1 (Fig. la). The steady-state level of uPA mRNA was low during the first 5 days of pregnancy, was highest on Day 7 of pregnancy, and declined thereafter (Fig. lb). The ratio of uPA mRNA to 18S rRNA was higher on Day 7 than on other days examined (p < 0.05). The abundance of PAI-1 mRNA was low during the first 5 days of pregnancy, gradually increased from Day 7 of pregnancy, and was highest in the maternal placenta on Day 15 of pregnancy (p < 0.05).

Localization of uPA and PAI-1 mRNAs

The uPA and PAI-1 mRNAs were localized by in situ hybridization. Antisense riboprobe for uPA detected positive signals (blue precipitate) in the primary decidual cells surrounding the implantation chamber in tissue sections prepared from Day 7 of pregnancy (Fig. 2a). Positive signals for PAI-1 mRNA were very weak in the decidual cells in tissue sections from Day 7 of pregnancy (data not shown). By Day 10 of pregnancy, PAI-1 signals were easily detected in decidual cells surrounding the fetus, although they were most abundant in all the fetal tissues (Fig. 2c). The sense probes for uPA and PAI-1 did not generate any hybridization signals (Fig. 2, b and d).

Abundance of uPA mRNA in Uterine Tissues of Pseudopregnancy

To determine whether or not expression of uPA and PAI-1 mRNAs in the decidualized tissues is dependent on the presence of live conceptuses, Northern blot analysis was carried out on total RNA from artificially induced deciduo
Plasminogen activator inhibitor-1 mRNA was not detected in on other days of pseudopregnancy (p < 0.05, Fig. 3b). mRNA to 18S rRNA was significantly higher on Day 7 than low on Days 3 and 5 of pseudopregnancy, reached a peak on during natural pregnancy; i.e., the level of uPA mRNA was

The decidualization process involves extensive tissue remod-
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The breakdown of ECM in the endometrium has been observed during decidualization in the mouse and rat [10]. Messenger RNA encoding a 72-kDa metallopro-
teinase (gelatinase A) is present in the mouse decidua around the time of embryo implantation [23]. Studies of human endo-
drometrium have demonstrated the presence of many types of ECM-degrading enzymes, in addition to uPA, and these en-
zymes are controlled by estrogen and progesterone [7, 8, 24]. The presence of high levels of uPA mRNA on Day 7 corre-
sponds to the phase of active trophoblast invasion [2, 3]. Tro-
phoblast cells at this stage of pregnancy begin to express many ECM-degrading enzymes [15,25-27]. In addition to uPA, a 105-kDa metalloproteinase (gelatinase B or MMP-9) has been localized to trophoblast cells in the ectoplacental cone region from mice [27]. Localization of other metalloproteinases in the implantation site has not been investigated. The decidual, uPA, along with other ECM-degrading enzymes, probably plays an important role in tissue remodeling events, including the invasion of trophoblast cells, the infiltration of blood-borne cells into the decidua, and angiogenesis within the decidua [21]. However, uPA does not have an obligatory role in embryo implantation, since inactivation of the uPA gene in the mouse fails to prevent embryo implantation [28].

One of the possible functions of the decidua is to limit the invasive activity of the trophoblast [29]. Inhibitors of ECM-degrading enzymes may mediate this decidual function. Urokinase-type PA activity is controlled by many natural inhibitors, including PAI-1 [9, 30]. In the present study the level of PAI-1 mRNA in the decidua began to increase from Day 7, when the trophoblast begins to invade [3], and reached the highest level on Day 15 of pregnancy, when the decidua is transformed into the maternal placenta. Similar patterns of change in the levels of tissue inhibitors of metalloproteinases-1 and -2 have been found within the implantation sites of mice [23]. These findings suggest that the decidua production of inhibitors of ECM-degrading enzymes increases as trophoblast invasion progresses. Endometrial cells at the beginning of decidualization probably lack the ability to resist trophoblast invasion. Instead, the endometrial cells that have just begun decidualization are capable of altering certain types of ECM in vitro [31]. This activity is probably needed for reorganization of the endometrium as decidualization progresses. In addition to controlling invasion of the trophoblast cells, PAI-1 may also control angiogenesis within the decidua and maternal placenta [19]. In the present study, PAI-1 mRNA was localized in the decidual cells by in situ hybridization, consistent with previous findings [19]. However, more PAI-1 transcripts were found in the fetal tissues and trophoblast cells. Plasminogen activator inhibitor-I of trophoblast origin may also be required for the control of invasive activity. Plasminogen activator inhibitor-I antigen is localized by immunocytochemistry in the human invading trophoblast cells and fetal tissues [32]. Moreover, PAI-1 production is increased in the less invasive trophoblast cells from patients suffering preeclampsia, further suggesting its role in controlling trophoblast invasion [33].

The presence of high levels of uPA mRNA on Day 7 of pregnancy is consistent with the increase in uPA secretion in vitro by stromal cells at the corresponding stage of decidualization [10]. In situ hybridization confirmed the localization of uPA mRNA to the decidual cells on Day 7, consistent with findings from in situ hybridization studies of the mouse decidua at similar stages of pregnancy [18, 19].

The increase in the abundance of uPA mRNA on Day 7 of pregnancy is independent of the presence of implanting embryos, since similar increases were observed in oil-induced deciduoma tissues. This agrees well with the increase in uPA secretion in endometrial stromal cells undergoing decidualization in vitro in the absence of embryos [10]. Taken together, these findings suggest that the uPA secretion is controlled by one or more endogenous factors in the decidual cells and that prostaglandin E2 is probably one such factor.

Urokinase-type PA expression has been associated with tissues that undergo the tissue remodeling process, as seen during tissue invasion, inflammation, and angiogenesis [9, 20]. The decidualization process involves extensive tissue remodeling [21]. The breakdown of ECM in the endometrium has

![FIG. 3. Northern blot analysis of total RNA from rat endometrial/decidual tissues on various days of pseudopregnancy. Kidney RNA from a female nonpermanent rat was included as a control. Ten micrograms total RNA was loaded to each lane. The same membrane was sequentially hybridized with [32P]-labeled cDNA probes for uPA, PAI-1 (data not shown owing to undetectable hybridization), and 18S rRNA cDNA. a) Autoradiograph of Northern blots. b) Densitometric analysis of three re-

Tissues and cells that undergo the tissue remodeling process, as seen during pregnancy, inflammation, and angiogenesis [9, 20]. The decidualization process involves extensive tissue remodeling [21]. The breakdown of ECM in the endometrium has
duomal tissues and 2) its increase at later stages of pregnancy coincides with the cessation of trophoblast invasion. However, this possibility cannot explain the detection of PAI-1 mRNA in stromal cells undergoing decidualization in vitro, which could be a result of an artifact of in vitro cultures [9]. The conceptus may regulate PAI-1 through various pathways. The trophoblast cells may induce PAI-1 expression directly by secretion of transforming growth factor-beta [34, 35], which is known to specifically up-regulate PAI-1 [36, 37]. Since PAI-1 expression is associated with angiogenesis [19, 38], the presence of the conceptus may induce PAI-1 expression indirectly by increasing angiogenic activity within the decidua/maternal placenta.

In summary, the present study demonstrated that uPA and PAI-1 mRNAs are present in the rat decidual cells at different stages of pregnancy, and their expression is probably differentially controlled by factors from different tissues. It is important to note that the PA/plasmin system is only a part of the biochemical mechanism of the control of ECM turnover. Various types of metalloproteinases are also implicated in this process. Further studies are needed for an understanding of the control and function of uPA, in conjunction with metalloproteinases, in the uterine tissue during embryo implantation.

REFERENCES

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