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Regulation of Plasminogen Activator in Rat Endometrial Stromal Cells: The Role of Prostaglandin E_2[1]

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ABSTRACT

The rat endometrium undergoes decidualization, a tissue remodeling process, during embryo implantation. Plasminogen activator (PA), particularly the urokinase-type PA WPA), has been implicated in tissue remodeling. The present study determined whether rat endometrial stromal cells secrete uPA during decidualization in vitro and, if so, whether the secretion is regulated by prostaglandins that are required in decidualization. Endometrial stromal cells were obtained from rats that had been treated with estrogen and progesterone to sensitize their uteri for decidualization, and the cells were cultured for up to 72 h in a serum-free medium. PA activity in the conditioned medium, as determined by a chromogenic assay, increased steadily during the 72-h culture period. PA secretion decreased when endogenous prostaglandin synthesis was inhibited by the addition of indomethacin to the cell cultures. The inhibitory effect of indomethacin on PA secretion was reversed by prostaglandin E_2, and much less effectively by prostaglandin $F_{2\alpha}$. PA activity in the medium was due primarily to uPA because 1) PA activity was inhibited by a uPA-specific inhibitor-amiloride-and by an anti-mouse uPA antibody, and 2) the predominant PA activity in the medium, as identified in zymography, had a molecular mass of approximately 40 kDa, similar to that reported for uPA. Northern blot analyses of RNA from the cultured cells demonstrated that the steady-state levels of mRNA for uPA, but not for tPA and plasminogen activator inhibitor-1, were decreased by inclomethacin; this decrease was reversed by prostaglandin E_2. Taken together, the data indicate that rat endometrial stromal cells secrete uPA during decidualization in vitro, and that prostaglandin E_2 regulates uPA secretion by the decidualizing cells by directly increasing uPA gene transcription and/or stabilizing its transcripts. These findings may help to partially elucidate the mechanism of action of prostaglandin E_2 in decidualization.

INTRODUCTION

In rodents, the fibroblastic endometrial stromal cells proliferate and differentiate into decidual cells in response to implanting blastocysts or artificial stimuli [1]. This process, which begins in the antimesometrial region and subsequently spreads to the mesometrial region, ultimately results in the formation of the maternal component of the placenta in pregnancy. Decidualization can be obtained in response to many different types of artificial stimuli, but only if these are applied during a limited time in pregnancy or pseudopregnancy or when the uterus has been sensitized by an appropriate regimen of hormone injections [1]. If isolated from sensitized uteri, rat endometrial stromal cells also undergo decidualization when cultured in vitro [2, 3], and this culture system serves as a model for the study of responses that occur in these cells during this process. Both in vivo and in vitro, the process of decidualization is mediated, at least in part, by prostaglandins (PGs), particularly of the E series (reviewed in [4]).

Decidual transformation of the endometrium in vivo requires extensive tissue remodeling [5]. Plasminogen activators (PA), including the tissue-type (tPA) and the urokinase-type (uPA), have been implicated in tissue remodeling processes because of their roles in extracellular matrix turnover [6, 7]. Thus, PA may play an important role in decidualization. The objective of this study was to determine whether rat endometrial stromal cells secrete PA during in vitro decidualization and, if they do, to determine whether the secretion is regulated by PGE,

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MATERIALS AND METHODS

Animals

Female Sprague-Dawley rats, obtained from Harlan Sprague-Dawley Inc. (Indianapolis, IN) at 200-225 g BW, were housed in temperature- and light-controlled conditions (lights-on from 0500 to 1900 h) with free access to food and water. The animals were ovariectomized under ether anesthesia and allowed at least 5 days to recover. To obtain rats with uteri sensitized for decidualization, estradiol and progesterone were administered s.c. in sesame oil as described previously Q81; protocol [21).

Endometrial Stromal Cell Isolation and Culture

Endometrial stromal cells were obtained from the sensitized uteri by enzymatic dispersion as described in detail elsewhere [3, 9]. In brief, uterine horns, split longitudinally, were initially incubated in pancreatin (GIBCO-BRL, Burlington, ON, Canada) and dispase II (Boehringer Mann-

heim, Laval, PQ, Canada) to liberate luminal epithelial cells, then in collagenase (type II; Sigma Chemical Company, St. Louis, MO) to liberate endometrial stromal cells. The stromal cells were suspended in Dulbecco's Modified Eagle's medium:F-12 nutrient mixture containing heat-inactivated charcoal-stripped fetal calf serum (10%), penicillin (50 U/ml), streptomycin (50 μg/ml), and fungizone (1.25 μg/ml; all GIBCO-BRL). The cell suspension was filtered through nylon mesh (70 μm) to remove contaminating glands and clumps of epithelial cells, and plated at 5 X 10^5 cells in 0.5 ml medium in 24-well plates (Becton-Dickinson, Lincoln Park, NJ). An initial incubation period of 2 h at 37°C under 5% $C0_2$:95% air was conducted to allow differential attachment of the stromal cells. After this initial incubation, the medium and free-floating cells were removed and replaced with serum-free medium**.** The resultant cultures of attached cells were free of epithelial cells, as determined by the absence of detectable cytokeratin by immunocytochemistry, but contained some (less than 5%) leukocytes, presumably macrophages (unpublished results). The stromal cells were cultured for up to 72 h with indomethacin (Sigma), with indomethacin plus PGE_2, or with indomethacin plus $PGF_{2\alpha}$. (Cayman Chemical Company, Ann Arbor, MI). The treatments were added to the culture medium in ethanol, the final concentration of which did not exceed 1%; cells not receiving a treatment were cultured with an equivalent concentration of ethanol. The culture media were replaced every 24 h, frozen at -20°C, lyophilized, and reconstituted with water just prior to determination of PA activity. To determine the amount of cellular protein in each well, the cells were washed with Dulbecco's PBS (GIBCO-BRL) after the medium was removed, solubilized in 1% SDS-1 mM EDTA, and assayed by the method of Lowry et al. [10] with BSA as the standard.

PA Activity Assays

A chromogenic assay for PA activity [11] was modified and used in this study to quantify PA activity in the conditioned media. Briefly, 25 μl of sample was incubated with 25 ng of plasminogen (Sigma) in a 96-well microtiter plate for 30 min at 37°C to generate plasmin. Chromogenic substrates for plasmin-0.22 mM of 5,5'-dithio-bis(2-nitrobenzoic acid) and thiobenzyl benzyloxycarbonyl-L-lysinate (Sigma)-were then added, and the incubation was continued for a further 15-30 min. The intensity of the yellowish color that resulted from the plasmin action was determined using an automated microplate reader at the 405nm wave length. Human uPA (Calbiochem, San Diego, CA) was included in each assay to construct a standard curve, which was used to convert the color intensity (absorbance) in the sample to the International Unit for PA. All substrates were added at saturating concentrations to the assay to ensure that all sample values fell within the linear range of the standard curve. A uPA-specific inhibitor (amiloride [20 mM, Sigma] [7] and anti-mouse uPA antibody (American Diagnostic Inc., Greenwich, CT) were added to some assays to determine the nature of PA activity present in the samples.

The nature of the PA activity was further characterized in a modified zymographic assay [12]. Aliquots of samples containing the same amount of total protein were subjected to electrophoresis in a 9% polyacrylamide gel containing casein (2 mg/ml) and plasminogen (1 μg/ml). After electrophoresis, the gel was washed in 2.5% Triton X100 for 60 min and incubated in PBS for 12-16 h at 37C. The gel was then fixed in a mixture of methanol:acetic acid:water (45:10:45) and stained with 0.5% Coomassie brilliant blue dye. PA activity was revealed by a cleared zone in the gel, and its molecular size was estimated by prestained molecular size markers (GIBCO-BRL) that were co-electrophoresed with the samples.

Northern Blot Hybridization

Total RNA was extracted from cultured cells by a single-step guanidine thiocyanate procedure [13] and was quantified by absorbance at 260 nm as well as by ethidium bromide staining after electrophoresis through agarose gels. Total RNA (10 μg) was denatured in 6% formaldehyde: 50% formamide in MOPS buffer (20 mM 3-[N-morpholino] propanesulphonic acid [MOPS], 5 mM sodium acetate, 1 mM EDTA, pH 7.2) at 65°C for 15 min, electrophoresed in 1.1% agarose-formaldehyde gels, blotted by capillary transfer onto Hybond-N membranes (Amersham, Oakville, ON, Canada), and then cross-linked to the membranes by UV radiation.

The membranes were prehybridized in 2.2 mM sodium pyrophosphate, 1 M NaCl, 50 mM Tris, 1% SDS, 10-strength Denhardt's reagent [14], pH 7.5, containing 80 μg/ml denatured salmon sperm DNA at 65°C for 2 h. Probes for uPA and tPA were prepared by reverse transcription and polymerase chain reaction (RT-PCR), with total RNA from rat embryos used as templates. The RT-PCR products, 348 bp in length for uPA and 450 bp for tPA, were cloned into pBluescript vectors for sequencing to verify their identity [15]. The probe for PA inhibitor (PAI)-1 was a *Pvu* II fragment (980 bp) of the rat PAI-1 full-length cDNA (kindly provided by Dr. T.D. Gelehrter of University of Michigan, Ann Arbor, MI [16]). The probe for PAI-2 was prepared and verified in a similar way as for uPA and tPA probes, except that total RNA from mouse embryos was used as template for RT-PCR [17]. These DNA probes were labeled by the random priming technique in the presence of [α^{32}P]dCTP (Amersham) using an oligo-labeling kit (Random Primers DNA Labeling System, GIBCO-BRL). Hybridizations were carried out at 65°C for approximately 18 h. The membranes were then washed twice (30 min each time) in double-strength SSC (0-3 M NaCl, 0.03 M sodium citrate, pH 7.0)0.1% SDS at 65°C, and then twice (30 min each time) at 65°C

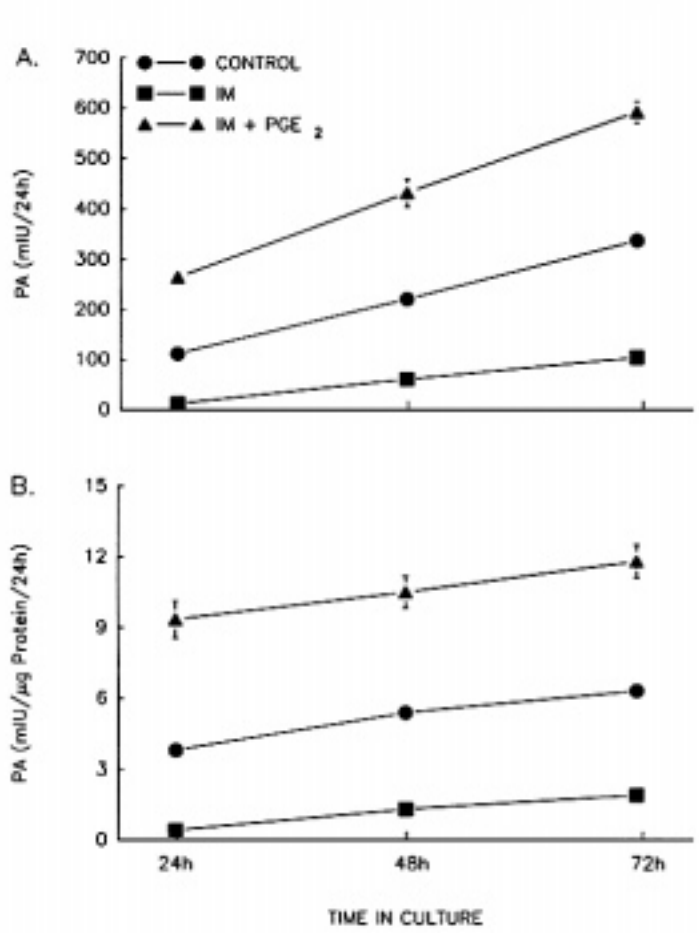

FIG 1. Effects of indomethacin (IM, 10^{-5} M) or indomethacin plus PGE_2 (1 µg/ml) on PA activity in medium of endometrial stromal cells from sensitized rat uteri at 24, 48, and 72 h in culture. PA activity was measured by chromogenic assay. **A)** PA activity per culture well. **B)** PA activity per unit of protein. Each point represents mean ± SEM for 4 wells.

in 0.2-strength SSC-0.1% SDS. The membranes were then subjected to autoradiography at -70°C with intensifying screens. Between hybridizations, the blots were stripped by immersion in 1 mM Tris, 1 mM EDTA, 0.1-strength Denhardt's reagent (pH 8.0), for 2 h at 75° C.

Table 1. Effects of amiloride or anti-uPA antibodies on PA activity as determined in triplicate in the chromogenic assay.

	PA activity (mIU; mean + SID)			
Addition to assay	uPA Standard[a]	tPA Standard[b]	Medium[c] from control cultures	Medium[c] from cultures with IM + PGE_2
None	1.22 ± 0.08	0.92 ± 0.06	8.95 ± 0.44	22.60 ± 0.71
Amiloride, 0.1 mM	0.02 ± 0.00	0.93 ± 0.07	1.20 ± 0.06	2.00 ± 0.08
Anti-uIPA antibody, 0.1 mg/mi	0.19 ± 0.06	0.89 ± 0.12	2.42 ± 0.06	3.22 ± 0.10

[a] 1.0 mIU human uPA.

[b] 2 ng human tPA.

[c] 5 µl of medium conditioned by culturing rat endometrial stromal cells.

The relative amounts of RNA loaded into each lane and transferred to the membranes were determined by probing the blots with a radiolabeled cDNA for mouse 18S ribosomal RNA [18]. The relative levels of the signals for the mRNAs on the autoradiograms were quantified by image analysis (Mocha Image Analysis, Corte Madera, CA), and the results were expressed as the ratios of the mRNA signals to 18S rRNA signals.

Statistical Analysis

The data for PA activity in the media are presented as the mean ± SEM of quadruplicate observations from a single experiment. Each experiment was performed at least three times with different endometrial stromal cell preparations. Because of significant differences between experiments, as determined by analysis of variance, the data from different cell preparations have not been pooled; comparison of experiments indicated that the treatment effects were qualitatively, although not always quantitatively, consistent.

Other data were analyzed by one-way analysis of variance followed by Duncan's New Multiple Range tests [19] when analysis of variance indicated significant treatment effects.

RESULTS

PA Secretion into the Culture Medium

In the first series of experiments, repeated four times with different endometrial stromal cell preparations, the effects of time in culture, and of the manipulation of PGE_2 levels, on PA activity in the conditioned medium were determined by the chromogenic assay. The results of a representative experiment are presented in Figure 1. For control cultures, total PA activity per well increased substantially ($p < 0.001$) over the 72-h culture period (Fig. 1A). The inhibition of endogenous PG synthesis in the cultured cells by the addition of indomethacin (10^{-5} M) significantly ($p < 0.001$) reduced the level of PA activity present in the conditioned medium at all three times. The addition of PGE_2 (1 µg/ml) along with indomethacin resulted in levels of PA activity higher ($p < 0.001$) than those of the corresponding controls at each time. When PA activity was expressed per unit of cellular protein (Fig. 1B), the pattern of changes in activity was similar.

Characterization of PA Activity

PA activity in the conditioned media was almost completely inhibited when a uPA-specific inhibitor (amiloride) or an anti-mouse uPA antibody was added to the chromogenic assay (Table 1). The zymographic assay (Fig. 2) detected a single band of PA activity, at a position corresponding to a molecular mass of approximately 40 kDa. This molecular mass is similar to that of uPA but not tPA (molecular mass = 70-72 kDa). Almost identical results were obtained from three zymographic assays of samples from three different culture experiments.

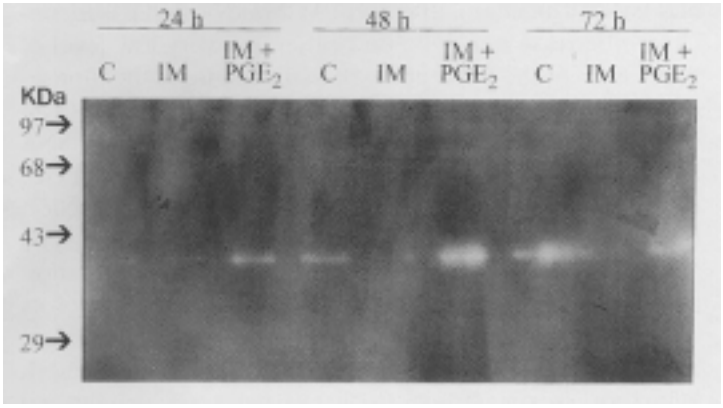

FIG.2. Endometrial stromal cells from rat uteri sensitized for decidualization were cultured in absence (Control, C) or presence of 10^{-5} M indomethacin (IM) or of indomethacin and 1 μg/ml PGE_2 (IM + PGE_2)- Conditioned media were collected at 24, 48, and 72 h in culture and subjected to electrophoresis in 9% polyacrylamide gel containing casein and plasminogen. After electrophoresis, the gel was incubated for 12-16 h in PBS before being fixed and stained with Coomassie brilliant blue. Cleared zone represents PA activity. Proteins with known molecular sizes were coelectrophoresed as standards for estimating molecular masses of PA activities.

Concentration-Response Relationships

In a series of experiments utilizing three different endometrial cell preparations, the concentration-response relationships between PGE_2, $PGF_{2\alpha}$, and total PA activity in the medium of cells in which endogenous PG production was inhibited by indomethacin were investigated. The results of a representative experiment are presented in Figure 3. For both PGE_2 and $PGF_{2\alpha}$, analyses of variance indicated highly significant ($p < 0.0001$) interactions between the effects of time in culture and PG concentration. These interactions arose because the extent to which PA activity was increased by the PGs differed between times, being greatest at 72 h

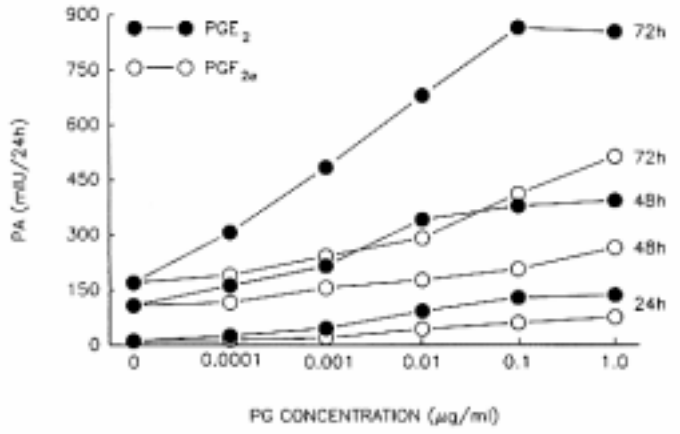

FIG 3. Concentration-response relationships between $PG1E_2$, $PGf_{2\alpha}$, and PA activity at 24, 48, and 72 h in medium of cultured rat endometrial stromal cells. All cultures contained indomethacin (10^{-5} M) to inhibit endogenous PG production during culture period. PGE_2 or $PGF_{2\alpha}$, at specified concentration was added at end of differential attachment period. Medium was replaced every 24 h with fresh medium containing same concentration of PG. Each point represents mean ± SEM for 4 wells.

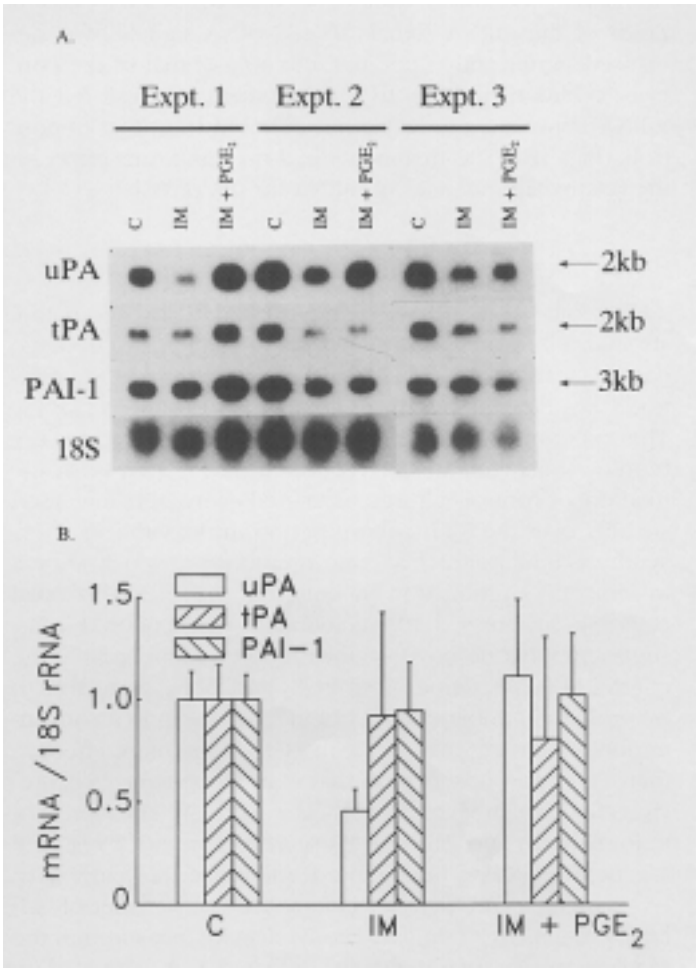

FIG.4. Northern blot analyses of uPA, tPA, and PAH from rat endometrial stromal cells cultured for 72 h under control conditions (C) or with indomethacin (IM, 10^{-5} M), or indomethacin plus PGE_2 (IM + PGE_2, 1 μg/ml). RNA (10 μg) was electrophoresed and transferred to a nylon membrane. **A)** Autoradiographs of membranes from three experiments. Each membrane was hybridized sequentially with ^{32}P-labeled cDNA probes for uPA, tPA, PAH, and 18S ribosomal RNA. **B)** Mean (± SEM, n = 3) ratio of mRNA/18S ribosomal RNA signals, as determined by image analysis with the ratios for controls set at 1, for the three treatment groups.

and least at 24 h. At all times examined, both PGs stimulated the secretion of PA activity into the medium in a concentration-dependent fashion, with PGE_2 being more effective than $PGF_{2\alpha}$. When the data were expressed as PA activity per unit of cellular protein, a similar pattern was observed (data not shown).

Northern Blot Analyses

Northern blot analyses were used to determine the effect of manipulating PGE_2 levels on the steady-state concentrations of mRNAs for PAs and PA inhibitors in the cultured cells. In each case, a single species of mRNA was identified when Northern blots were probed sequentially for uPA, tPA, and PAI-1 (Fig. 4A); no signal was obtained for PAI-2 (data not shown). When cells were cultured with indomethacin, the relative abundance of uPA mRNA, as assessed by the

ratios of the mRNA signals to 18S rRNA signals, was decreased significantly ($p < 0.05$) to 40% of that of the controls; addition of PGE_2 to the indomethacin restored the mRNA abundance to levels not different from that of controls (Fig. 4B). The treatments had no significant effect on the relative abundances of mRNA for tPA or PAI-1.

DISCUSSION

Previous studies have demonstrated that endometrial stromal cells from appropriately sensitized rats can undergo decidualization in vitro [2, 3, 20] and that this decidualization is regulated in part by endogenously produced PGs [3]. This experimental model was employed in our study to determine the regulation of PA secretion. PA activity in the medium of control cultures, measured every 24 h, increased steadily over the 72-h culture period. Indomethacin, a PG synthesis inhibitor [21], at concentrations previously shown to inhibit PG production by cultured endometrial stromal cells [3], suppressed PA secretion by the cultured cells, suggesting that de novo synthesized PGs play a role in the control of PA secretion. Both PGE_2 and $PGF_{2\alpha}$ were able to override the inhibitory effect of indomethacin in a concentration-dependent manner, with PGE_2 being more effective than $PGF_{2\alpha}$ an observation that is in agreement with previous findings in vivo [22, 23] and in vitro [3]. The effects of indomethacin and PGE2 on PA secretion are not a result of a general effect on the cultured cells since the patterns of PA changes, expressed per culture well or per unit of cellular protein, were similar. The PA activity measured in the medium was primarily uPA rather than tPA, as indicated by the inhibitory effects of amiloride and of uPA antibodies. This conclusion was further substantiated by the zymographic assay, which indicated that the activity had a molecular mass of approximately 40 kDa, appropriate for uPA but not for tPA (70-72 kDa; [6, 24]).

Several natural inhibitors inhibit uPA activity in vivo, including PAI-1 and PAI-2 [25]. The chromogenic assay measures net PA activity; changes in activity measured therein may be a consequence of a change in total PA activity or a change in PA inhibitor levels in the sample. Because zymography separates PAs from their inhibitors, it provides an indication of changes in total PA activity. In the present study, the results of the zymographic and chromogenic assays were consistent, and suggest that indomethacin and PGE, act, at least in part, to modulate uPA secretion, rather than secretions of PA inhibitors.

The results of the Northern blot analyses are also consistent with the notion that indomethacin and PGE_2 affect uPA. The steady-state levels of mRNA for uPA were decreased by indomethacin, and this reduction was reversed by PGE_2, although unlike the activity, the levels were not significantly greater than in the controls. Neither indomethacin nor PGE_2 had any significant effects on the levels of mRNAs for tPA and PAI-1. The failure to detect PAI-2 mRNA signal is inconclusive because it may be due either to a very low level of expression of the PAI-2 gene, or to the failure of the mouse-derived probe to hybridize with rat mRNA. To the best of our knowledge, the nucleotide sequence of rat PAI-2 mRNA is not available. Nevertheless, these data suggest that PGE_2 regulates uPA at least in part by increasing the transcription rate of the uPA gene and/or stabilizing its transcripts.

Although the essential role of PGE, in the decidualization process has been well documented, the mechanism of its action during decidualization is not yet fully elucidated**.** Some of its actions in inflammation and other tissue remodeling processes, such as increasing vascular permeability and inducing angiogenesis, are probably required in the induction of decidualization [26, 27]. Increased uPA expression and extracellular matrix turnover have been associated with inflammatory responses including vascularization [7, 28]. The breakdown of some extracellular matrix components in the endometrium has been observed during decidualization. For example, the level of hyaluronan in the mouse endometrium is reduced during decidualization. This reduction is independent of embryonic signals since it occurs in the deciduoma that is induced by an artificial deciduogenic stimulus [29]. Similarly, collagen degradation has also been observed in the decidual tissue, independent of embryonic control [30]. Data from this study suggest that PGE_2 may participate in the control of extracellular matrix turnover by regulating uPA secretion during decidualization.

In the rat and some other rodents, as in the human, the embryos invade the endometrium during implantation. It has been proposed that uPA and other proteinases from the embryos are responsible for breaching the basement membrane in the endometrium during the invasion process [31, 32]. However, histological studies have found that breakdown of extracellular matrix in the endometrium precedes any sign of embryo implantation /invasion in the mouse uterus [33], and this breakdown is presumably mediated by proteinases produced within the endometrium. It is possible that endometrial uPA, in addition to embryonic uPA, is required for successful implantation.

In summary, the present study shows that rat endometrial stromal cells undergoing decidualization in vitro secrete uPA and that this is regulated by PGE_2. Future studies should determine whether uPA is produced by decidual cells in vivo. The effect of in vivo sensitization of the stromal cells on their secretion of uPA should also be determined in order to establish uPA secretion as a possible bio-marker for decidualization.

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