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Secretion of Plasminogen Activator by Cultured Rat Endometrial Stromal Cells From Uteri Differentially Sensitized for the Decidual Cell Reaction

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ABSTRACT Endometrial stromal cells from rat uteri differentially sensitized for the decidual cell reaction in vivo and which undergo differing degrees of decidualization in vitro were cultured and plasminogen activator (PA) in the medium determined. The cells were obtained by enzymatic dispersion from the uteri of ovariectomized, steroid-treated rats at the equivalent of day 4, 5, or 6 of pseudopregnancy or on day 5 from rats treated on day 4 with 0, 0.3, or 1 .O μg estradiol (low, intermediate, or high dose of estradiol, respectively) and cultured for 24, 48, or 72 hr. For cells from day 4, 5, and 6 uteri cultured under control conditions, PA activity in the medium was greatest for day 5 cells, which were from uteri maximally sensitized for decidualization both in vivo and in vitro. By contrast, for cells from low-, intermediate-, and high-estradiol uteri, PA activity in the medium was greatest for the high-estradiol cells; these cells do not undergo decidualization in vivo or in vitro to the same extent as intermediate-estradiol cells. Indomethacin, an inhibitor of prostaglandin (PG) synthesis, reduced PGE_2 accumulation to nondetectable amounts and for most cultures decreased PA activity in the medium, suggesting that endogenous PG production regulated in part PA secretion under control conditions. The addition of PGE_2 with indomethacin increased PA activities above those under control conditions, but activities were still lower for day 4 and 6 cells compared with day 5 cells, and for low- and intermediate-estradiol cells compared with high-estradiol cells. This indicates that the differences in PA secretion are not explainable by differences in PGE_2 production. Northern blot analysis of RNA from cells cultured for 72 hr under control conditions did not reveal significant differences in steady-state concentrations of mRNA for urokinase-type PA or plasminogen activator inhibitor 1, but those for tissue-type PA were lower in day 6 cells compared with day 4 and 5 cells. It is concluded that PA activity secreted by the cultured endometrial stromal cells, although controlled in part by the endocrine milieu to which they were exposed prior to culture, does not simulate decidualization in vitro and, therefore, that PA activity is not a marker for decidualization in vitro. Mol. Reprod. Dev. 49:268-276, 1998.

Key Words: decidualization; estradiol; prostaglandin E_2; pseudopregnancy

INTRODUCTION

The differentiation of endometrial stromal cells to decidual cells in rodents occurs in vivo during blastocyst implantation or in response to artificial stimuli (Psychoyos, 1973). Decidualization in response to an artificial stimulus is time and hormone dependent. It can be obtained only during a limited time in pregnancy, during pseudopregnancy, or when the animal has received appropriate hormonal treatment (Finn and Porter, 1975; Kennedy and Ross, 1997; Psychoyos, 1973). Furthermore, estrogen in low but not high doses acts synergistically with progesterone to sensitize the rat and mouse uterus (Finn and Porter, 1975; Kennedy and Ross, 1997; Yochim and De Feo, 1963). Decidualization is mediated, at least in part, by prostaglandins (PGs), particularly of the E series (Kennedy, 1994).

Endometrial stromal cells from sensitized rat uteri undergo decidualization when cultured as indicated by morphology (Sananes et al., 1978; Vladimirsky et al., 1977), expression of desmin (Glasser and Julian, 1986; Mani et al., 1992) and laminin (Mani et al., 1992), and changes in alkaline phosphatase (ALP) activity (Kennedy and Ross, 1997; Yee and Kennedy, 1991, 1993). Unlike decidualization in vivo, progesterone is not required for decidualization in vitro (Sananes et al., 1980). However, decidualization in vitro is similar to that occurring in vivo in that it is mediated in part by PGs (Kennedy and Ross, 1997; Yee and Kennedy, 1991, 1993) and is dependent on uterine sensitization (Kennedy and Ross, 1997).

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Recently, Zhang et al. (1996) have reported that endometrial stromal cells from sensitized rat uteri secrete plasminogen activator (PA) when cultured under conditions that allow the cells to decidualize. Two forms of PA, urokinase type (uPA) and tissue type (tPA), have been characterized in mammals (Dano et al., 1985), and there are at least two natural inhibitors of uPA activity in vivo, plasminogen activator inhibitor 1 (PAI-1) and PAI-2 (Blasi et al., 1987). Cultured endometrial stromal cells secrete primarily uPA (Zhang et al., 1996), which is also expressed in vivo in the decidual cells of pregnancy as well as in those induced by an artificial deciduogenic stimulus (Wang et al., 1996). The secretion of uPA in vitro is controlled in part by PGE_2, which regulates the steady-state concentrations of mRNA for uPA. These data suggest that PA secretion may be a marker for the process of decidualization in vitro.

Because uterine sensitization influences subsequent decidualization in vitro (Kennedy and Ross, 1997), we hypothesized that if PA secretion is a marker for decidualization in vitro, its secretion would be greater for cultured endometrial cells obtained from sensitized uteri than for cells obtained from nonsensitized uteri. To test this hypothesis, we used an established protocol that produces differentially sensitized uteri in rats (Kennedy and Ross, 1997). With this protocol, rats treated with an intermediate dose of estradiol have maximally sensitized uteri on the equivalent of day 5 of pseudopregnancy. Because PGs modulate decidualization (Yee and Kennedy, 1991, 1993) and PA secretion (Zhang et al., 1996), the effects of manipulating PG levels during the culture period were determined.

MATERIALS AND METHODS

Animals

Female Sprague-Dawley rats were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN) at 200-225 g body mass and housed in temperature- and light-controlled conditions (lights on from 05:00-19:00 hr) with free access to food and water.

Treatment of Animals

The rats were ovariectomized under ether anesthesia and allowed at least 5 days to recover from the surgery.

Uteri differentially sensitized for the decidual cell reaction were obtained by administering estradiol and progesterone subcutaneously in sesame oil to the rats according to the protocol illustrated (Fig. 1) (Kennedy and Ross, 1997). The effects of temporal changes in uterine sensitization on subsequent performance in vitro of isolated endometrial stromal cells was investigated by administering the same sequence of estradiol and progesterone to all rats and killing them on the morning of the equivalent of day 4, day **5,** or day **6** of pseudopregnancy. In these experiments, the initiation of hormone treatments was staggered so that all animals in an experiment were killed on the same day.

To investigate the effects of estradiol-induced changes in uterine sensitization on subsequent performance in vitro of isolated endometrial stromal cells, different amounts of estradiol (0, 0.3, or 1.0 µg) with a constant amount of progesterone (4 mg) were given on the afternoon of the equivalent of day 4 of pseudopregnancy, and the animals were killed on the morning of the next day. At all times other than day 4, the rats received identical steroid treatments. For brevity, these treatments are referred to as low, intermediate, or high doses of estradiol, respectively

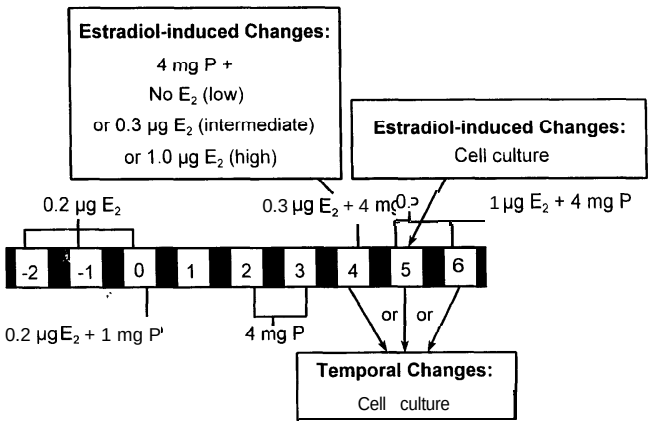

Fig. 1. Schematic representation of the treatment protocols administered to ovariectomized rats to obtain uteri differentially sensitized for the decidual cell reaction. (■ > Periods when the lights were off; (□) when the lights were on. Numbers give the equivalent day of pseudopregnancy. E_2, estradiol; P, progesterone. To investigate temporal-dependent changes, rats were killed on day 4, 5, or 6 to obtain endometrial stromal cells for culture. Except for those killed on day 4, all rats received 0.3 µg E_2 plus 4 mg P on the afternoon of day 4. To investigate estradiol-dependent changes, rats treated with no E_2 (low), 0.3 µg E_2 (intermediate), or 1.0 µg E_2 (high) on the afternoon of day 4 were killed on day 5 to obtain endometrial stromal cells for culture.

Endometrial Stromal Cell Isolation and Culture

The method of McCormack and Glasser (1980), as modified by Yee and Kennedy (1991), was used to isolate endometrial stromal cells from the differentially sensitized uteri. In summary, uterine horns, slit longitudinally, were initially incubated in pancreatin (GIBCO-BRL, Burlington, Ontario) and dispase II (Boehringer Mannheim, Laval, Quebec) to liberate epithelial cells and 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 10% heat-inactivated charcoal-stripped fetal calf serum, 50 units/ml penicillin, 50 µg/ml streptomycin, and 1.25µg/ml fungizone (all GIBCO-BRL). The cell suspensions were filtered through nylon mesh (70 µm) to remove contaminating glands and plated at 5×10^5 cells in 0.5 ml medium in 24-well plates (Becton-Dickinson, Lincoln Park, NJ). An initial incubation period of 2 hr at 37°C under 5% CO_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 resulting cultures were free of

epithelial cells, as indicated by the absence of detectable cytokeratin by immunocytochemistry, but contained some (less than 5%) leukocytes, presumably macrophages (Orlando-Mathur et al., 1996; Zhang et al., 1996).

The stromal cells were cultured for up to 72 hr with 10^{-5} M indomethacin (Sigma), an inhibitor of prostaglandin synthesis (Vane, 1971), or with indomethacin plus 1 µg/ml PGE_2 (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 equal concentration of ethanol. The culture medium was replaced every 24 hr and frozen at -20°C prior to PGE_2 radioimmunoassay; medium in which PA activity was to be measured was lyophilized. To determine the amount of cellular protein in each well, the cells were washed with Dulbecco's phosphate-buffered saline (GIBCO-BRL) after the medium had been removed, solubilized in 1% SDS and 1 mM EDTA, and stored at -70°C until assayed.

Assays

The chromogenic assay of Coleman and Green (1981) as modified by Zhang et al. (1996) was used to quantify PA activity in the conditioned media. In brief, the lyophylized samples were reconstituted with water, and 25 pl of each 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 5,5'-dithio-bis(2-nitrobenzoic acid) and 0.18 mM N-a-carbobenzoxy-L-lysine thiobenzyl ester (Sigma)-were then added, and the incubation was continued for an additional 15-30 min. The absorbance at 405 nm was determined using an automated microplate reader. Human uPA (Calbiochem, San Diego, CA) was included in each assay to construct a standard curve that was used to convert absorbance in the samples to the international units of PA. Amiloride (0.1 mM; Sigma), a uPA specific inhibitor (Vassalli and Belin, 1987), was added to some assays to determine the nature of the PA activity present in the samples.

The method of Lowry et al. (1951) was used for the protein assay, with bovine serum albumin (Sigma) as the standard. PGE_2 was measured in the media by radioimmunoassay as described by Bany and Kennedy (1995). The sensitivity of the assay was approximately 10 pg per tube, and the intra- and interassay coefficients of variation were approximately 8% and 12%, respectively.

Northern Blot Hybridization

Total RNA was extracted from cultured cells by a single-step guanidine thiocyanate procedure (Chomczynski and Sacchi, 1987) and 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 and 50% formamide in MOPS buffer (20 mM 3-[N-morpholinol-propanesulphonic acid, 5 mM sodium acetate, 1 mM EDTA, pH 7.2) at 65°C for 15 min, subjected to electrophoresis in 1.1% agarose-formaldehyde gels, blotted by capillary transfer onto Hybond-N membranes (Amersham, Oakville, Ontario), and then cross-linked to the membranes by ultraviolet (UV) radiation.

The preparation of the ^{32}P-labelled hybridization probes and the prehybridization and hybridization conditions have been described (Zhang et al., 1996). After hybridization at 65°C for approximately 18 hr, the membranes were washed twice (30 min each time) in double-strength SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) plus 0.1% SDS at 65°C and then twice (30 min each time) at 65°C in 0.2-strength SSC plus 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, and O.l-strength Denhardt's reagent (Sambrook et al., 1989), pH 8.0, for 2 hr at 75°C.

The relative amounts of RNA loaded into each lane and transferred to the membranes were determined by probing the blots with a radiolabelled cDNA for mouse 18S ribosomal RNA (rRNA) (Smith and Hammond, 1991). The relative levels of the signals for the mRNAs on the autoradiograms were quantified by image analysis (Mocha Image Analysis, Jandel Scientific Corp., San Rafael, CA), and the results are expressed as the ratios of the mRNA signals to the 18S rRNA signals.

Statistical Analyses

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 effects of treatments were qualitatively, although not always quantitatively, consistent. Within an experiment, treatment effects were determined by analysis of variance; when significant interactions were found, Duncan's new multiple-range tests (Steel and Torrie, 1960) were used for group comparisons. The data for PGE_2 were log-transformed before analysis to remove heterogeneity of variance (Snedecor, 1956). The data from the Northern blot analyses were analyzed by one-way analysis of variance followed by Duncan's new multiple-range tests when analysis of variance indicated significant treatment effects.

RESULTS

Temporal Changes in Uterine Sensitization and Secretion of Plasminogen Activator In Vitro

In a series of experiments, the effects of time in culture and of manipulation of PGE_2 concentrations on total PA activity in the medium and on PA activity per unit of cellular protein were determined for endometrial stromal cells obtained from uteri at the equivalent

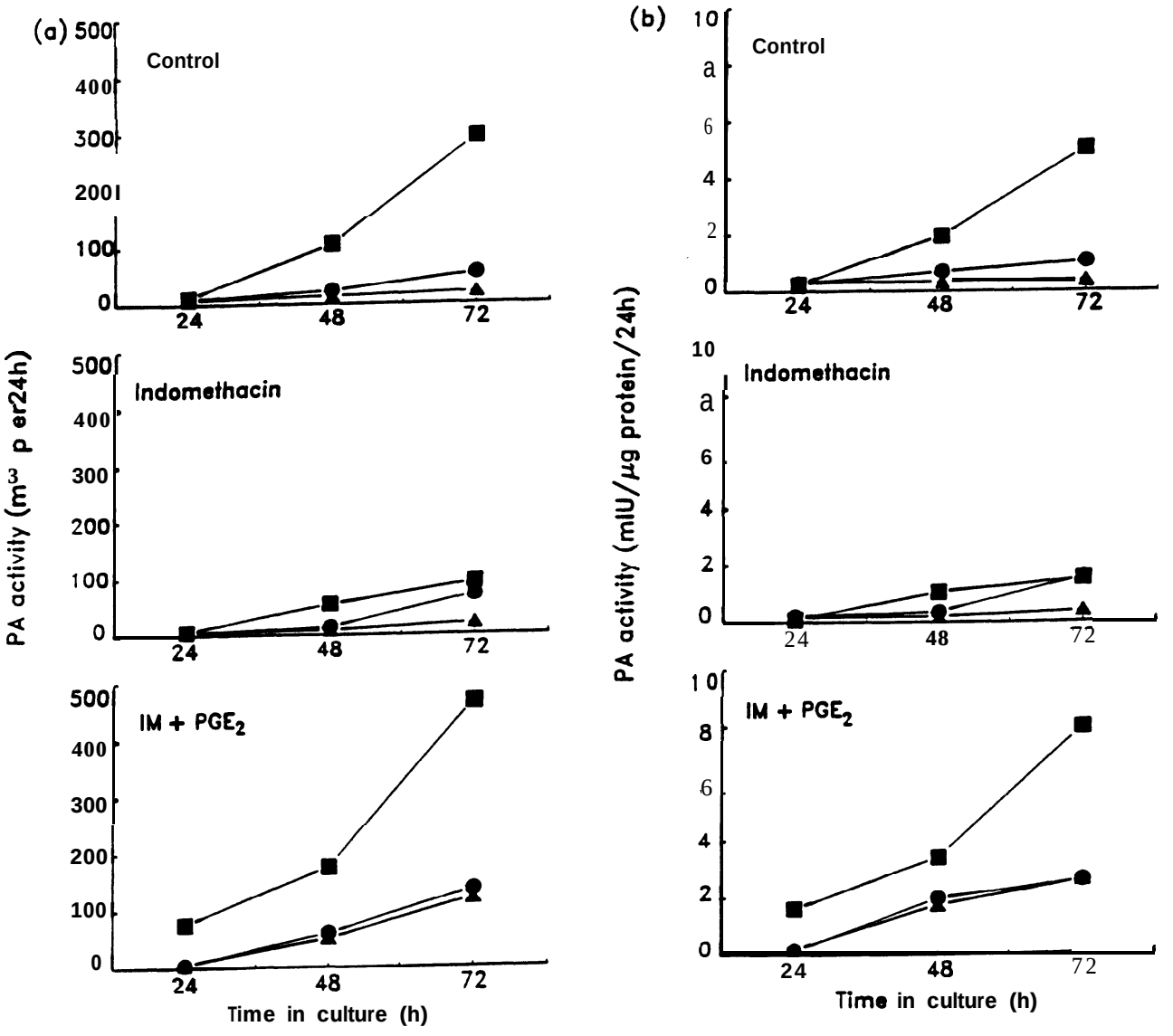

Fig. 2. Plasminogen activator (PA) activity **(a)** and PA activity per unit of cellular protein **(b)** in the medium from endometrial stromal cells obtained from uteri at the equivalent of day 4 (●), day 5 (■), or day 6 (A) of pseudopregnancy and cultured for 24, 48 or 72 hr under control conditions, with indomethacin (10^{-5} M) or with prostaglandin E_2 (PGE_2, 1 μg/ml) plus indomethacin. Each point represents the mean ± SEM of four observations; where the SEM is not apparent, it is smaller than the symbol.

of day 4, 5, or 6 of pseudopregnancy. For total PA activity (Fig. 2A), analysis of variance indicated a highly significant ($P < 0.001$) three-way interaction between the effects of day of pseudopregnancy, time in culture, and treatment during culture. For control cultures, irrespective of the day of pseudopregnancy on which the cells were obtained, total PA activity increased significantly from 24 to 72 hr in culture. However, for cells obtained from day 5 of pseudopregnancy, total PA activities at 48 and 72 hr in culture were greater than corresponding controls for cells from day 4 or day 6.

When indomethacin was added to the culture medium, total PA activity still increased significantly ($P < 0.01$) from 24 to 72 hr for all cultures. For cells obtained from uteri at day 6 of pseudopregnancy, treatment with indomethacin had no significant effect when compared with the corresponding control. By contrast, for day 5 cells at 24, 48, and 72 hr in culture and for day 4 cells at 24 and 48 hr in culture, indomethacin treatment resulted in significantly ($P < 0.01$) lower total PA activity. For day 4 cells at 72 hr in culture, indomethacin treatment was associated with a small but significant ($P < 0.05$) increase in PA activity.

The addition of PGE_2 with indomethacin resulted in total PA activities that were significantly higher ($P < 0.01$) than in the same cells under control conditions or when treated with indomethacin, except at 24 hr in culture for day 4 and day 6 cells, where activities were not different from those of cells treated with

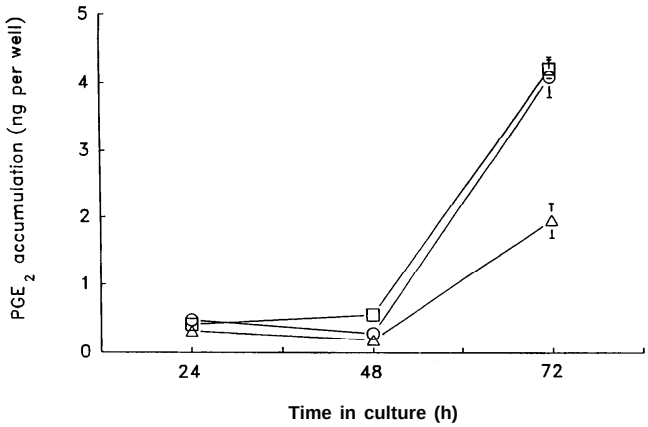

Fig. 3. Accumulation of prostaglandin E_2 in the medium of endometrial stromal cells obtained from uteri at the equivalent of day 4 (O), day 5 (□), or day 6 (△) of pseudopregnancy and cultured for 24, 48, or 72 hr under control conditions, with the medium being replaced every 24 hr. Each point represents the mean ± SEM of four observations; where the SEM is not apparent, it is smaller than the symbol.

indomethacin. Total PA activity was greatest ($P < 0.01$) for cells obtained from day 5 uteri and did not differ between day 4 and day 6 cells.

Because there were changes in cellular protein during culture, and because these changes depended both on the source of the cells and on time in culture, PA activity per unit of cellular protein also was analyzed (Fig. 2B). Analysis of variance revealed a significant ($P < 0.001$) three-way interaction between the effects of day of pseudopregnancy, time in culture, and treatment. For control cultures, PA activity per unit of protein increased significantly ($P < 0.01$) with time for cells obtained from day 4 and day 5 uteri but not for day 6 uteri. PA activity per unit of protein and its increase during culture were greatest for cells obtained from uteri at day 5.

When the cells were cultured in the presence of indomethacin, PA activity per unit of cellular protein increased ($P < 0.01$) from 24 to 72 hr in culture. For day 4 and day 6 cells, treatment with indomethacin had no significant effect when compared with the corresponding controls, except for day 4 cells at 72 hr, when activity was slightly but significantly ($P < 0.05$) increased. By contrast, indomethacin treatment of day 5 cells resulted in decreased ($P < 0.01$) activities at all times. At 48 hr, PA activity per unit cellular protein was greatest for day 5 cells; at 72 hr it was least for day 6 cells.

Treatment of the cells with PGE_2 plus indomethacin resulted in PA activities per unit of cellular protein that, within each time and day, were greater than those for controls or indomethacin-treated cultures, except for day 4 and day 6 cells at 24 hr. At 24, 48, and 72 hr in culture, activities were greatest for day 5 cells, whereas there were no differences between day 4 and day 6 cells.

The accumulation of PGE_2 in the medium of cells cultured under control conditions is presented (Fig. 3); PGE_2 concentrations were not detectable (<200 pg per well) for cells cultured in the presence of indomethacin. Analysis of variance of the log-transformed data from control cultures revealed a significant ($P < 0.01$) interaction between the effects of day and time in culture. For each day of pseudopregnancy, PGE_2 accumulation at 72 hr was greater than that at 48 or 24 hr. At 24 hr in culture, accumulation by day 6 cells was less ($P < 0.01$) than by day 4 cells; accumulation by day 5 cells was intermediate and did not differ from that of either day 4 or day 6, cells. Accumulation of PGE_2 at 48 hr differed significantly ($P < 0.05$) among all three days, being greatest for day 5 cells and least for day 6 cells. At 72 hr, PGE_2 accumulation did not differ between day 4 and day 5 cells and was significantly ($P < 0.01$) greater than for day 6 cells.

Estradiol-Induced Changes in Uterine Sensitization and Secretion of Plasminogen Activator In Vitro

Endometrial stromal cells from uteri exposed in vivo to differing amounts of estradiol were cultured in a series of repeated experiments, and the effects of manipulating PGE_2 concentrations on total PA activity in the medium and on PA activity per unit of cellular protein were determined. For total PA activity (Fig. 4A), analysis of variance indicated a highly significant ($P < 0.001$) three-way interaction among the effects of estradiol, time in culture, and treatment during culture. For control cultures, total PA activity increased significantly ($P < 0.001$) from 24 to 72 hr, irrespective of estradiol treatment in vivo. When measured after 24 hr of culture, total PA activity was greatest ($P < 0.01$) for cells exposed in vivo to the intermediate dose of estrogen. By contrast, after 48 and 72 hr of culture, PA activity was directly related to the dose of estrogen, being greatest for cells exposed to the high dose of estradiol and lowest for the low dose of estradiol.

When indomethacin was present in the culture medium, PA activity increased ($P < 0.001$) with time in culture. Compared with the corresponding controls, treatment with indomethacin resulted in significantly ($P < 0.01$) decreased PA activities, except for low-estradiol cells at 24 hr of culture, where indomethacin had no significant ($P > 0.05$) effect. As with the control cultures, PA activities were directly related to the dose of estradiol, except at 24 hr, when intermediate-estradiol cells had the highest ($P < 0.01$) activities.

When PGE_2 plus indomethacin was added to the medium, total PA activity was increased ($P < 0.01$) above that of respective control or indomethacin-treated cells at each time, except for low-estradiol cells at 72 hr, where the activities of control and PGE_2 plus indomethacin-treated cells did not differ ($P > 0.05$). The PA activity was greatest ($P < 0.01$) at each time for cells obtained from animals treated with the high dose of estradiol and least for those with the low dose of estradiol.

To allow for possible treatment effects on protein content of cultured cells, PA activity per unit of cellular protein (see Fig. 4B) also was analyzed. Analysis of

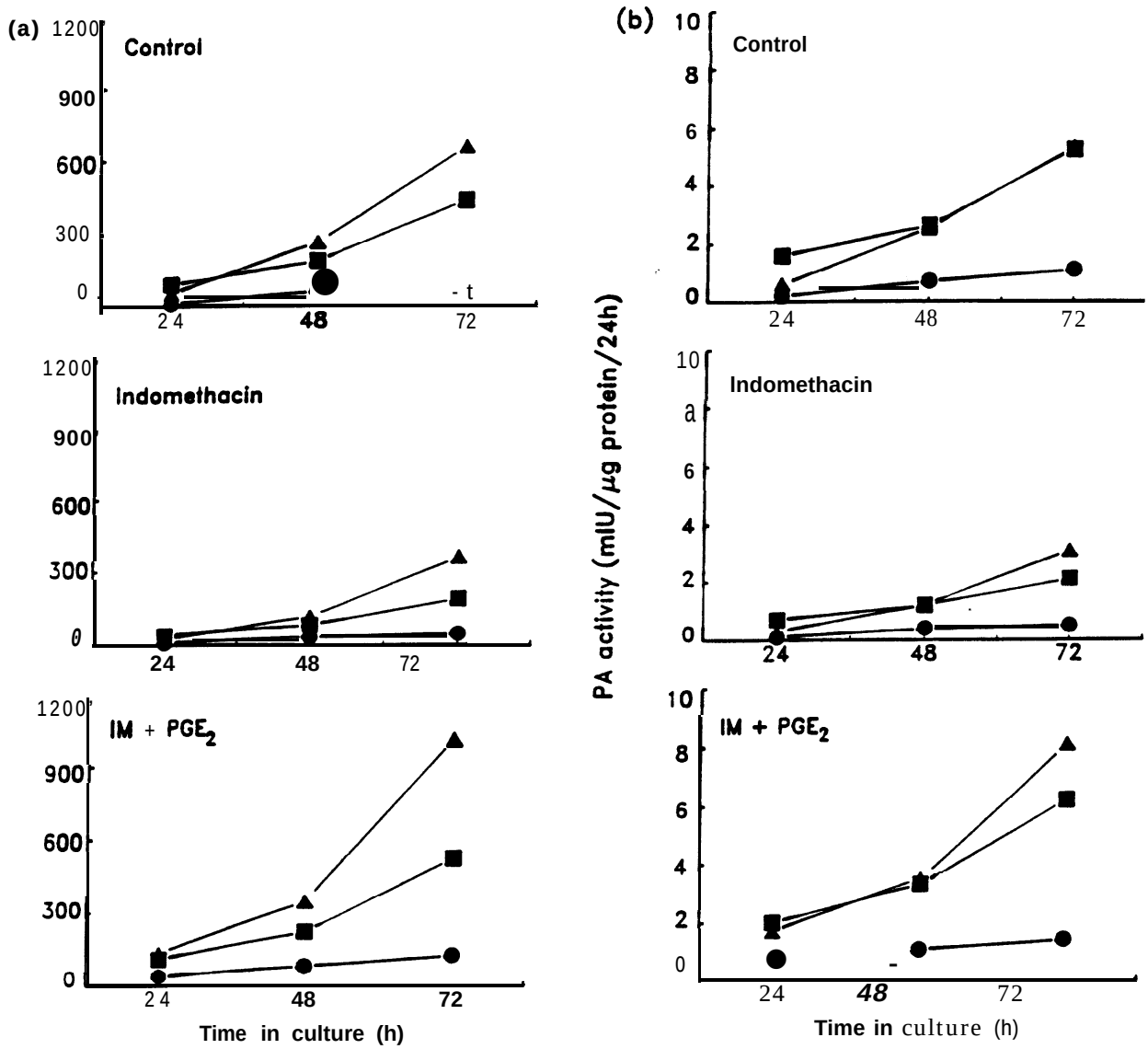

Fig. 4. Plasminogen activator (PA) activity **(a)** and PA activity per unit of cellular protein **(b)** in the medium from endometrial stromal cells obtained from uteri treated on day 4 with low (●), intermediate (■), or high (A) doses of estradiol. The cells were cultured for 24, 48, or 72 hr under control conditions, with indomethacin (10^{-5} M), or with prostaglandin E_2 (PGE_2, 1 μg/ml) plus indomethacin. Each point represents the mean ± SEM of four observations; where the SEM is not apparent, it is smaller than the symbol.

variance revealed a highly significant ($P < 0.001$) three-way interaction among the effects of dose of estradiol, time in culture, and treatment during culture. For control cultures, the time courses of PA activity per unit of protein differed depending on prior treatment with estradiol. In all cases, PA activities increased ($P < 0.001$) with time in culture. At all times, low-estradiol cells had activities that were lower ($P < 0.01$) than those for intermediate- and high-estradiol cells. At 24 hr in culture, intermediate-estradiol cells had significantly ($P < 0.01$) higher PA activity per unit of protein than high-estradiol cells; at 48 and 72 hr in culture, there were no differences.

Compared with the corresponding controls, treatment with indomethacin resulted in significantly ($P < 0.01$) reduced PA activity per unit of protein, except for low-estradiol cells at 24 hr in culture, where indomethacin had no effect ($P > 0.05$). Activities increased ($P < 0.01$) with time in culture. Low-estradiol cells had activities per unit of protein that were lower ($P < 0.01$) than for intermediate- and high-estradiol cells at all times. Compared with high-estradiol cells, activity in intermediate-estradiol cells was higher ($P < 0.01$) at 24 hr in culture, not different ($P > 0.05$) at 48 hr, and lower ($P < 0.01$) at 72 hr.

Treatment of the cells during culture with indomethacin plus PGE_2 resulted in PA activities per unit protein that, within each time and estradiol treatment, were greater ($P < 0.01$) than those of controls or indomethatin-treated cultures. Activities increased ($P < 0.01$) with

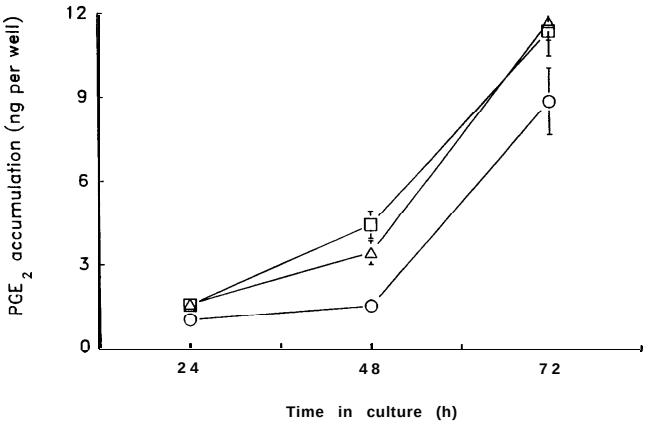

Fig. 5. Accumulation of prostaglandin E_2 in the medium of endometrial stromal cells obtained on day 5 from the uteri of rats treated on day 4 with low (○), intermediate (Cl), or high (△) doses of estradiol. The cells were cultured for 24, 48, or 72 hr under control conditions, with the medium being replaced every 24 hr. Each point represents the mean ± SEM of four observations; where the SEM is not apparent, it is smaller than the symbol.

time in culture. Low-estradiol cells had activities that were lower ($P < 0.01$) than those of intermediate- and high-estradiol cells at all times. At 24 hr in culture, PA activity of intermediate-estradiol cells was higher ($P < 0.01$) than that of high-estradiol cells; at 48 hr there was no difference ($P > 0.05$), while at 72 hr it was higher ($P < 0.01$) for the high-estradiol cells.

The accumulation of PGE_2 in the medium of cells cultured under control conditions is shown (Fig. 5); for cells cultured with indomethacin, the concentrations of PGE_2 were below the sensitivity of the assay (< 100 pg per well). Analysis of variance of the log-transformed data from control cultures indicated a significant ($P < 0.005$) interaction between the effects of prior treatment with estradiol and time in culture. Accumulation of PGE_2 increased ($P < 0.01$) for each of the estradiol treatments from 24 to 48 hr and then again from 48 to 72 hr. Within each time, PGE_2 accumulation by low-estradiol cells was lower ($P < 0.01$) than that by intermediate- and high-estradiol cells, except at 72 hr, when the accumulation for low-estradiol cells was less ($P < 0.05$) than that for high-estradiol cells but not different ($P > 0.05$) from that for intermediate-estradiol cells. Accumulation of PGE_2 by intermediate- and high-estradiol cells did not differ ($P > 0.05$) at any time.

To determine if estradiol treatment in vivo affected the type of PA activity secreted in vitro, amiloride was added to the PA assay of samples of conditioned media collected at 72 hr from intermediate- and high-estradiol cells that had been cultured under control conditions or with indomethacin plus PGE_2. For intermediate-estradiol cells, PA activities when amiloride was present, as a percentage of activities in its absence, were 6.3 ± 0.5% and 12.1 ± 2.5% for control and indomethacin plus PGE_2 cultures, respectively; the corresponding percentages for high-estradiol cells were 16.3 ± 1.7% and 15.0 ± 1.3% (n = 4 in all cases). Analysis of

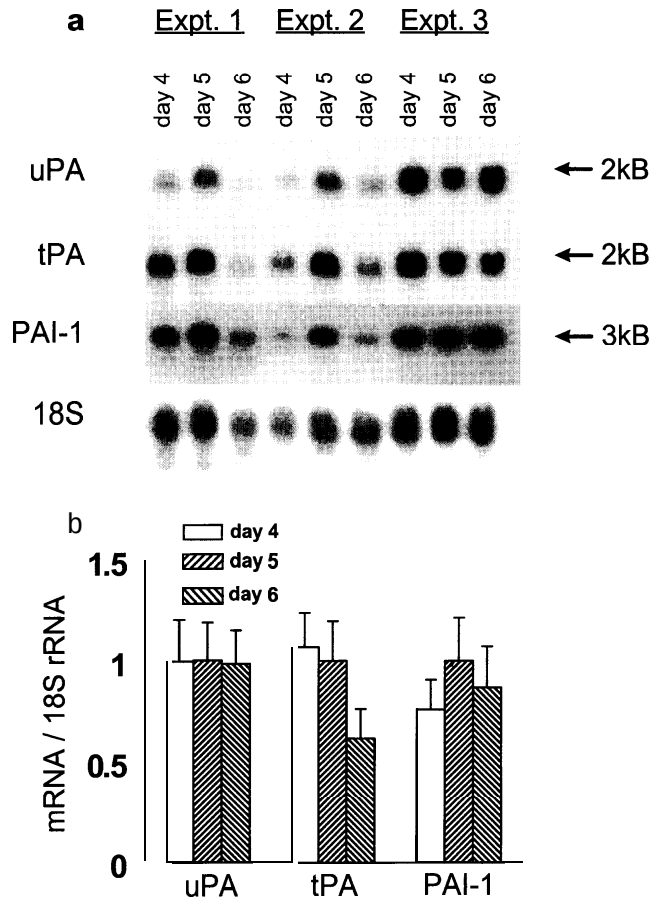

Fig. 6. Northern blot analysis of total RNA (10 μg) extracted from rat endometrial stromal cells obtained from uteri at the equivalent of day 4, day 5, or day 6 of pseudopregnancy and cultured for 72 hr under control conditions. **(a)** Autoradiographs of the membrane after sequential hybridization with ^{32}P-labelled cDNA probes for urokinase-type plasminogen activator (uPA), tissue-type plasminogen activator (tPA), plasminogen activator inhibitor 1 (PAI-l), and 18S ribosomal RNA (18S). **(b)** Mean ± SEM ratios of mRNA/18S ribosomal RNA signals, as determined by image analysis, with the ratios for day 5 arbitrarily set at 1.

variance indicated that the percentages were higher ($P < 0.005$) for the high-estradiol cells, with no effect attributable to the culture conditions, although the interaction between the effects of estradiol treatment and culture conditions approached significance ($0.05 < P < 0.1$).

Northern Blot Analyses

To determine if there were changes in steady-state concentrations of mRNAs for and PAIs, Northern blot analyses were performed on total RNA obtained from differentially sensitized endometrial stromal cells cultured for 72 hr under control conditions. For both temporal and estradiol-induced changes in sensitization, RNA was obtained from three separate cultures using different cell preparations. In both analyses, a single species of mRNA was identified when Northern blots were probed sequentially for uPA, tPA, and

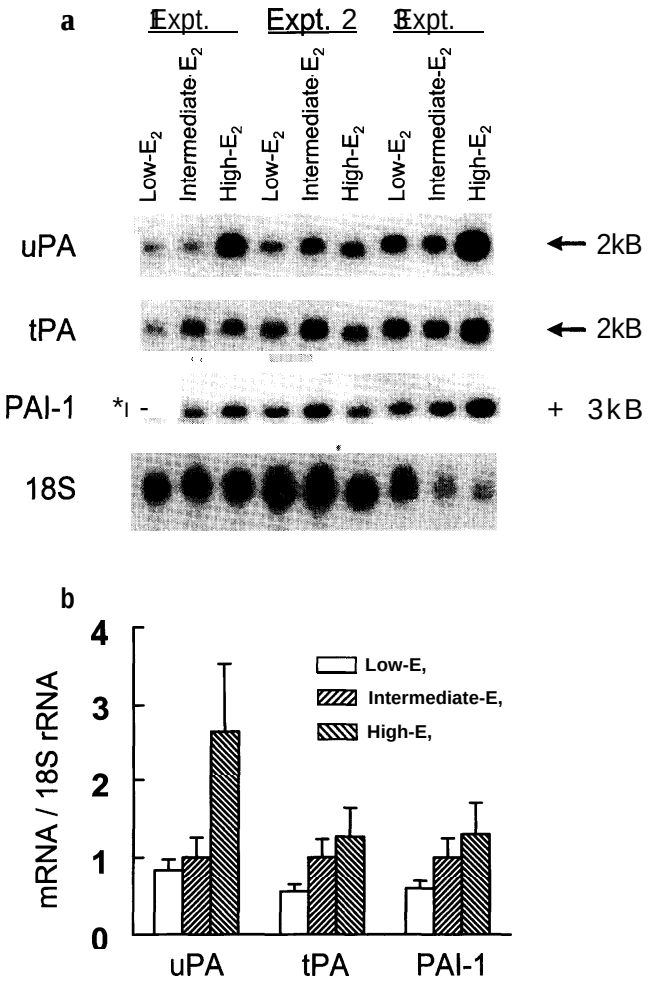

Fig. 7. Northern blot analysis of total RNA (10 μg) extracted from endometrial stromal cells obtained from the uteri of rats treated on day 4 with low, intermediate, or high doses of estradiol and killed on day 5. The cells were cultured for 72 hr under control conditions. **(a)** Autoradiographs of the membrane after sequential hybridization with ^{32}P-labelled cDNA probes for urokinase-type plasminogen activator (uPA), tissue-type plasminogen activator (tPA), plasminogen activator inhibitor 1 (PAI-l), and 18S ribosomal RNA (18S). **(b)** Mean SEM ratios of mRNA/18S ribosomal RNA signals, as determined by image analysis, with the ratios for the intermediate estradiol cells arbitrarily set at 1.

(Figs. 6A and 7A); no signal was obtained for PAI-2 (data not shown). As assessed by the ratios of the mRNA signals to 18S rRNA signals (see Figs. 6B and 7B), the only significant ($P < 0.05$) difference was in the steady-state concentrations of mRNA for tPA, which were lower in cultured cells from day 6 uteri compared with those from day 4 and day 5 uteri.

DISCUSSION

In the present study we sought to determine if the amount of PA activity secreted into the medium by rat endometrial stromal cells reflected the extent to which the cells had undergone decidualization. We have shown previously, using ALP activity as a marker, that the ability of rat endometrial stromal cells to undergo decidualization in vitro depends on the degree of uterine sensitization at the time the cells were obtained (Kennedy and Ross, 1997); this uterine sensitization is both time and hormone dependent. We anticipated that if secretion of PA activity is also a marker for decidualization, cells from animals treated with the intermediate dose of estradiol and killed on day 5 would secrete the greatest amounts of PA. This was the case when cells obtained on the equivalent of day 4, 5, or 6 of pseudopregnancy were compared; under control conditions, the secretion of PA activity was substantially greater for day 5 cells, whether the activities were expressed on a per-well basis or per unit of cellular protein. However, it was not the case when cells were obtained from animals treated with differing amounts of estradiol on day 4. Whereas decidualization in vivo, as determined by uterine mass, and in vitro, as determined by ALP activity (Kennedy and Ross, 1997), is greatest for animals receiving the intermediate dose of estradiol, secretion of PA activity was greatest for cells from animals receiving the high dose of estradiol. Thus secretion of PA activity into the medium apparently does not reflect the process of decidualization in vitro, and consequently, PA activity is not a marker for decidualization.

Decidualization in vivo and in vitro is mediated, at least in part, by PGs, particularly of the E series (Kennedy, 1994). The secretion of PA activity by cultured rat endometrial cells from sensitized uteri is also regulated, in part, by PGE_2 (Zhang et al., 1996). In the present study, indomethacin at concentrations that reduced PGE_2 accumulation in the medium to levels that were nondetectable, significantly reduced PA secretion for most cells, thereby suggesting that PA secretion under control conditions reflects, in part, endogenous PG production. The cells from the differentially sensitized uteri had varying abilities to produce PGE_2 during culture, as indicated by differences in PGE_2 accumulation in the medium. However, the ability of the cells to secrete PA activity is not simply explained by differences in PG production because when exogenous PGE_2 was added to the cultured cells along with indomethacin, PA activities still differed and depended on the treatment given in vivo. If differences in PGE_2 production were the sole explanation for the variation in the secretion of PA activity in vitro, then exposure to the exogenous PGE_2 should have resulted in similar amounts of PA activity being secreted, irrespective of the state of sensitization of the uteri from which the endometrial stromal cells were obtained.

The chromogenic assay for PA activity used in the present study measures net activity; changes in activity measured in a sample may be a consequence of a change in total PA activity or a change in PAI concentrations. Our previous study (Zhang et al., 1996) indicated that the PA activity secreted by maximally sensitized endometrial stromal cells was primarily uPA and that indomethacin and PGE_2 affect PA activity not by altering PA1 secretion but rather in part by modulating the rate of transcription of the uPA gene and/or by altering

the stability of its transcripts. In the present study we used amiloride, a specific inhibitor of uPA activity (Vassalli and Belin, 1987), to determine the nature of the increase in PA activity observed in the conditioned media from high-estradiol cells. Compared with intermediate-estradiol cells, amiloride inhibited a significantly smaller proportion of PA activity in the conditioned medium from high-estradiol cells, suggesting that a relatively greater proportion of the PA activity secreted by these cells was tPA. However, Northern blot analyses of total RNA extracted from cells after 72 hr in culture under control conditions did not reveal any significant differences between intermediate- and high-estradiol cells in steady-state mRNA concentrations of tPA or uPA Either the Northern blot analyses are not sufficiently precise to detect the changes in transcript concentrations that produce these changes in secretion of PA activity or the regulation is at a posttranscriptional level.

Despite the differences in PA activity observed under control conditions in the conditioned media from the various cell preparations, the only significant difference in the concentrations of transcripts for uPA, tPA, and PAI-1, as determined by Northern blot analyses, was for those of tPA, which were lower for day 6 cells. No transcripts for PAI-2 were detected, in agreement with previous observations (Zhang et al., 1996); this may be a consequence of very low expression of the PAI-2 gene or of failure of the mouse-derived probe to hybridize to rat mRNA The failure to detect more differences in transcript concentrations may arise because Northern blot analyses are not sufficiently precise to detect the relatively small changes in concentrations of the transcripts that produce the changes in PA activity, or alternatively, the regulation is at the posttranscriptional level.

In summary, endometrial stromal cells from differentially sensitized rat uteri secrete differing amounts of PA activity when cultured, indicating that the secretion of PA in vitro is controlled in part by the endocrine milieu to which they are exposed prior to culture. Furthermore, PA secretion by the cultured cells is regulated in part by PGE_2. However, the amounts of PA activity secreted by the cultured cells do not reflect decidualization in vitro, and consequently, PA activity is not an appropriate marker for decidualization in vitro.

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