

Expression of Decidual Prolactin-Related Protein in the Rat Decidua*

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ABSTRACT

It is well established that rat decidual tissue produces a PRL-like hormone(s) that binds to the PRL receptor on both the corpus luteum and the decidual cells and initiates profound changes in the endocrine milieu required for the establishment of pregnancy. The recent cloning of a decidual PRL-related protein (dPRP) prompted us 1) to determine whether the expression of this gene is triggered by decidualization of the endometrial stromal cells, 2) to examine the temporal and cell-specific pattern of its expression, and 3) to examine the role of both decidual signals and PRL on levels of its messenger RNA (mRNA). Total RNA was isolated from uteri of either nonpregnant rats or pseudopregnant rats with or without decidual tissue. A 1-kilobase mRNA species hybridizing strongly with the dPRP probe was present in decidualized uteri. No dPRP mRNA could be detected in uteri not subjected to decidualization. Developmental studies indicated a constant high level of dPRP mRNA in the decidual tissue until day 12 of pseudopregnancy, followed by a marked decline at a time when extensive cell death occurs in the decidua, suggesting that dPRP is constitutively expressed in this tissue. To examine the cell-specific expression of dPRP, antimesometrial decidua was separated from mesometrial

decidua, and the large antimesometrial cell population was separated from the small mesometrial cells by elutriation. The results of Northern analysis revealed clearly that dPRP is abundantly and solely expressed in the large antimesometrial cells. No dPRP mRNA could be detected in the mesometrial cells and in numerous other endocrine and nonendocrine tissues. A faint signal was observed, however, in the trophoblast. Despite the very strong paracrine regulation between the antimesometrial and mesometrial cells and the high levels of PRL receptor expression in these cells, both *in vivo* and coculture experiments revealed no regulation of dPRP gene expression by either PRL or mesometrial cell signal, adding further support to the possibility that once induced, dPRP remains constitutively expressed.

In summary, the results of this investigation revealed that the expression of dPRP in endometrial stromal cells is triggered by the induction of decidualization and that this gene is selectively and abundantly expressed in a defined cell population located in the antimesometrial region of the uterus. Thus, dPRP is not only a useful indicator of decidualization, but is also an excellent marker for the differentiated antimesometrial cells. (*Endocrinology* 135: 1422-1427, 1994)

DECIDUALIZATION, involving major transformation of the uterine endometrial stromal cells, is a remarkable feature of the maternal response to pregnancy in many mammalian species. It is particularly striking in these species, including humans and rats, in which placentation is characterized by extensive invasion of trophoblastic cells. In the rat, the uterine endometrial stroma undergoes dramatic growth and differentiation in response to either blastocyst implantation or artificial stimuli to form decidual tissue. A complex relationship exists between decidual and ovarian signals. Ovarian progesterone is essential for the survival of the decidual tissue, which, in turn, is responsible for controlling a cascade of events in the ovary necessary for pregnancy to continue (1-10). Decidual cell actions were found to be directed at least in part through the production of a PRL-related hormone that binds to PRL receptor (11-14) and has luteotropic functions similar to those of PRL (3-6, 8). The decidual luteotropic activities have been attributed to a 28- to 29-kilodalton protein(s) produced by a defined cell population located in the antimesometrial decidua (9, 11).

Rat decidual tissue, does not express the pituitary PRL gene (9), and the decidual hormone(s) differs from PRL even though it binds to the same receptors (1, 11). Recently, Roby *et al.* (15) have succeeded in isolating, from a rat decidual tissue complementary DNA (cDNA) library, a clone that encodes for a decidual PRL-related protein (dPRP). dPRP is a new member of the PRL-GH family. Its gene is localized to rat chromosome 17, which also carries other members of the PRL gene family. Nucleotide sequence analyses of the dPRP cDNAs predicted a mature secretory protein of 211 amino acids with two putative *N*-linked glycosylation sites. The predicted dPRP amino acid sequence contains six cysteine residues located in positions homologous to the cysteines of PRL. The similarity of positioning of cysteines within the PRL and dPRP sequences suggests a correspondence in folding and, thus, biological activities.

The recent cloning of dPRP has prompted us to examine 1) the tissue specificity of dPRP expression, 2) its temporal expression in decidual tissue during development, 3) the definite cell population within the decidua that expresses the dPRP gene and, finally, 4) the role of both decidual signals and PRL in dPRP expression.

Materials and Methods

Materials

RPMI-1640 (without glutamine) was purchased from Mediatech (Washington DC), collagenase (type I) was obtained from Worthington Biochemical Corp. (Freehold, NJ), and Dispase (type II) and DNase I

Received April 29, 1994.

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* Presented in part at the 26th Annual Meeting of the Society for the Study of Reproduction, Fort Collins, CO, 1993. This work was supported by NIH Grants HD-12356 (to G.G.), HD-29036 (to M.J.S.), and T32-HL-07692 (to Y.G.).

† NIH Merit Awardee (HD-11119).

were from Boehringer Mannheim Biochemicals (Indianapolis, IN). Antibiotic-antimycotic, sodium pyruvate, and glutamine were obtained from Gibco-BRL (Gaithersburgs, MD), Hanks' Balanced Salt Solution (HBSS; without Ca^{2+} and Mg^{2+}), nonessential amino acids, D-glucose, and fluorescein diacetate were purchased from Sigma Chemical Co. (St. Louis, MO); fetal bovine serum was obtained from Hyclone (Logan, UT). Nitrocellulose and GeneScreen Plus nylon membranes were purchased from Schleicher and Schuell (Keene, NH) and New England Nuclear Research Systems (Boston, MA), respectively. [^{32}P]deoxy-CTP was purchased from Amersham (Arlington Heights, IL).

Animals

Pseudopregnancy was induced in Sprague-Dawley rats by mating females with vasectomized males at the Holtzman facilities (Madison, WI). The day a vaginal plug was found was designated day 1 of pseudopregnancy. Rats were housed in a controlled environment (22 C) and kept under a photoperiod of 14 h of light and 10 h of darkness. Purina rat chow and water were provided *ad libitum*. Animal care and handling conformed with the NIH guidelines for animal research. The experimental protocols were approved by the Institutional Animal Care and Use Committee.

Surgical procedures

Decidualization of uterine endometrium was induced by scratching the antimesometrial surface of both uterine horns on day 5 of pseudopregnancy with a hooked needle under ether anesthesia. In one set of experiments, day 8 pseudopregnant rats were treated with either vehicle or ergocryptine (0.4 mg, sc) to selectively inhibit PRL secretion and killed 24 h later; control rats were treated with vehicle (0.25 ml 70% ethanol) only. Rats were killed by an overdose of ether. The uterine horns were isolated and washed thoroughly in ice-cold PBS to remove excess blood. The decidual tissues were exposed, and the antimesometrial and mesometrial tissues were dissected out, as described previously (13, 16), and rinsed several times with ice-cold PBS. Dissected antimesometrial and mesometrial tissues from 5–10 rats were pooled and processed separately. Trophoblast tissues were carefully dissected from placentas of day 19 pregnant rats. Uterine horns were obtained from either pseudopregnant or nonpseudopregnant rats, and all other tissues, except the testes, were from pregnant rats.

Dispersion and separation of decidual cells

Minced decidual tissue was incubated in a water-jacketed Cellstrir (Wheaton Scientific, Millville, NJ) at 37 C for 40 min with 50 U/ml collagenase, 2.4 U/ml Dispase, and 200 U/ml DNase I with mild stirring. Decidual cells were filtered through nylon mesh and centrifuged at $200 \times g$ for 5 min at 4 C. Cell pellets were gently resuspended in 10 ml HBSS elutriation buffer (HBSS with 25 mM HEPES and 0.1% BSA, pH 7.4).

To separate the large antimesometrial cells from the small mesometrial cells, as previously described (17, 18), dispersed cells were eluted using a JE-6B elutriator rotor fitted with a Sanderson chamber (Beckman, Palo Alto, CA). The cells were washed three times and resuspended in 2 ml culture medium (RPMI-1640 supplemented with 10% fetal bovine serum, $2 \times$ antibiotic-antimycotic, $1 \times$ glutamine, $1 \times$ nonessential amino acids, $1 \times$ sodium pyruvate, and 0.5% D-glucose). Viable cell numbers were determined by fluorescein diacetate stain and the trypan blue exclusion method.

Northern blot analysis

Total RNA from dissected tissues or elutriated and cultured cells was isolated by the guanidinium-thiocyanate-phenol-chloroform methods (19). Total RNA were fractionated by electrophoresis through a 1% agarose gel containing 0.74 M formaldehyde under denaturing conditions. The amount of RNA loaded onto the gels was checked by ethidium bromide staining. Fractionated RNA was transferred to GeneScreen nylon membranes by the capillary technique according to the manufacturer's instruction. For dPRP analysis, blots were prehybridized and hybridized in buffer [50 mM piperazine- N,N' -bistethanesulfonic acid, 100 mM NaCl, 50 mM sodium phosphate, 1 mM EDTA, and 5% sodium

dodecyl sulfate (SDS), pH 6.8] containing denatured salmon sperm DNA (100 $\mu\text{g}/\text{ml}$) at 65 C for 3 and 16 h, respectively. A randomly labeled 0.9-kilobase (kb) rat dPRP cDNA (15) was used for hybridization. After hybridization, blots were washed four times (5 min each) in $2 \times \text{SSC}$ ($1 \times \text{SSC} = 0.15 \text{ M NaCl}$ and 15 mM sodium citrate, pH 7.0) with 0.1% SDS at room temperature, followed by two additional washes (15 min each) in $0.1 \times \text{SSC}$ with 0.1% SDS at 50 C. For PLP-B analysis, a randomly labeled full-length PLP-B cDNA (1.1 kb; pRP9-1), kindly provided by Dr. Mary Lynn Duckworth (University of Manitoba, Manitoba, Canada), was used as probe for hybridization. The blot was hybridized and washed as described previously (20). Ethidium bromide staining confirmed that the ribosomal RNAs were intact and that equal amounts of RNA were loaded in each lane.

Culture and coculture of mesometrial and antimesometrial decidual cells

Antimesometrial decidual cells (3×10^6) were plated into six-well culture plates. Mesometrial cells (3×10^6) were seeded into Falcon Cell Culture Inserts with Cyclopor membranes ($\sim 5\text{-cm}^2$ area; Becton Dickinson, Lincoln Park, NJ). Mesometrial and antimesometrial cells were cultured either independently or together. For the coculture experiments, inserts containing mesometrial cells were inserted into the wells above the antimesometrial cells, as depicted in Fig. 8. Cells were cultured for 48 h in RPMI-1640 culture medium at 37 C under an atmosphere of 5% CO_2 -95% air, with a medium change every 24 h.

Results

Effect of decidualization on dPRP expression

To determine whether decidualization of stromal cells induces the expression of dPRP, Northern analysis was performed on total RNA obtained from uteri of either nonpseudopregnant rats or pseudopregnant rats with or without decidual tissue. As shown in Fig. 1, a 1-kb messenger RNA (mRNA) species hybridizing strongly with the dPRP probe was present in RNA preparations from decidualized uteri. Only a faint signal was present in the uteri of pseudopregnant rats without decidual tissue, and no dPRP mRNA was detected in nonpseudopregnant uteri. These results indicate that decidualization of the endometrial stroma markedly stimulates the expression of the dPRP gene and further establishes dPRP as a specific marker for endometrial stromal cell decidualization.

Developmental and cell-specific expression of dPRP in the decidua

Once we established that decidualization induces the expression of the dPRP gene, we examined the changes in dPRP mRNA throughout pseudopregnancy. The results shown in Fig. 2 indicate that levels of dPRP mRNA remain elevated throughout pseudopregnancy and decline after day 12, at a time when extensive cell death occurs in the decidua, suggesting that dPRP is constitutively expressed in decidual tissue.

Because PRL-like activity is found only in the large cells located in antimesometrial decidua (7, 9, 10), we examined whether dPRP expression is also confined to this cell population. We first examined dPRP expression in antimesometrial and mesometrial decidual tissues separated by dissection and found (Fig. 3) that dPRP is principally expressed in the antimesometrial tissue at each stage examined. Because tissue contamination could not be prevented during dissection, we

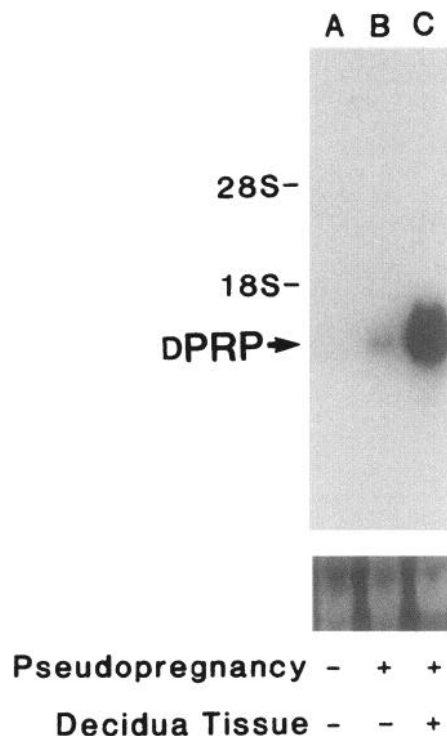


FIG. 1. Effect of decidualization on dPRP gene expression. Total RNA was isolated from rat uteri. Twenty micrograms were loaded in each lane and hybridized with the dPRP cDNA probe, as described in *Materials and Methods*. The exposure time was 24 h. Lane A, RNA from uteri of nonpregnant rat; lane B, RNA from pseudopregnant uterus without decidua; lane C, RNA from uteri bearing decidua.

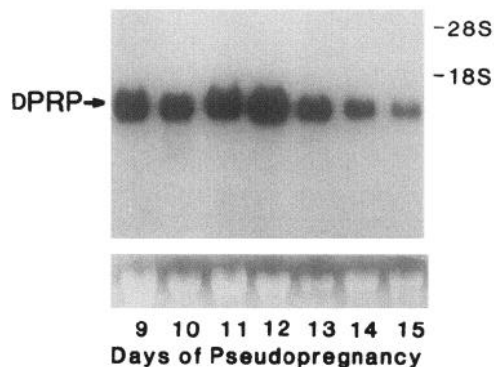


FIG. 2. Developmental expression of dPRP in the decidua. Total RNA was isolated from freshly dissected tissues obtained on different days of pseudopregnancy. Twenty micrograms were loaded in each lane and hybridized with dPRP cDNA probe. The exposure time was 1.5 h.

separated the large antimesometrial cells from the small mesometrial cells by elutriation. The results of Northern analysis performed on both separated tissues and cells (Fig. 4) indicate clearly that dPRP is expressed solely in the large antimesometrial cells. No signal could be detected in the mesometrial cells, even with long exposure.

Tissue-specific expression of dPRP

To determine whether dPRP gene is expressed in other tissues, total RNA was isolated from several endocrine and nonendocrine tissues and subjected to Northern analysis.

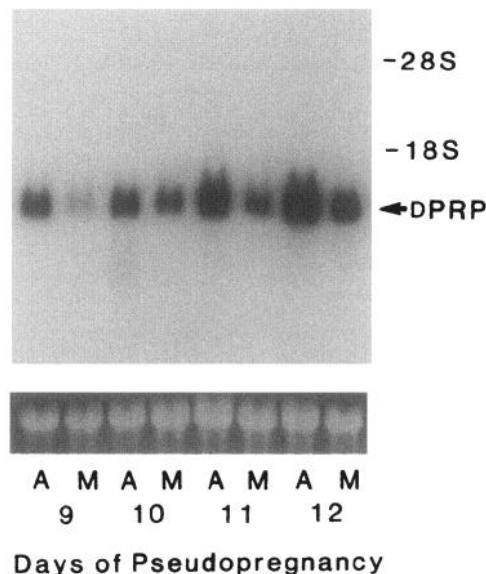


FIG. 3. dPRP mRNA content in antimesometrial and mesometrial decidua throughout decidual development. Total RNA was isolated from freshly dissected antimesometrial (A) and mesometrial (M) decidua. Twenty micrograms of total RNA were loaded in each lane and hybridized with the dPRP cDNA probe. The exposure time was 1 h.

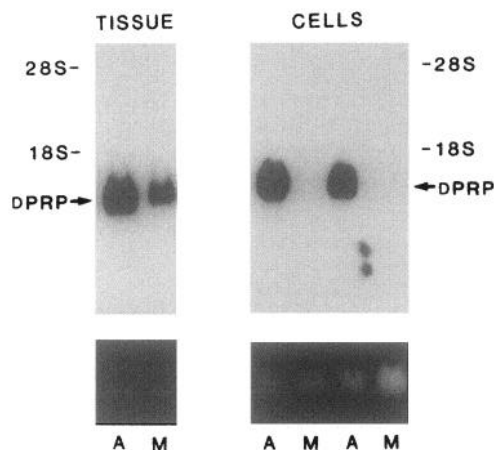


FIG. 4. Cell-specific expression of dPRP mRNA in the decidua. Total RNA was isolated from either freshly dissected antimesometrial (A) and mesometrial (M) decidua (*left panel*) or antimesometrial (A) and mesometrial (M) decidua cells (*right panel*) separated by elutriation. Twenty micrograms of total RNA were loaded in each lane and hybridized with the dPRP cDNA probe. The exposure time was 1 h for tissue and 16 h for cells.

The results (Fig. 5) indicate that whereas dPRP mRNA was abundant in the decidua, no message was detected in the corpus luteum, testes, adrenal gland, liver, heart, or small intestine, suggesting that dPRP may be highly specific to the decidua. However, a faint signal was observed in the placenta. Because the placenta is comprised of both decidual and trophoblastic tissue, it became of importance to determine whether dPRP is expressed in isolated trophoblast. We also compared the expression of dPRP with that of another PRL-related hormone, termed PRL-like protein-B (PLP-B), which was reported to be present in the trophoblast and also in the decidua, albeit in lower abundance (20). Northern

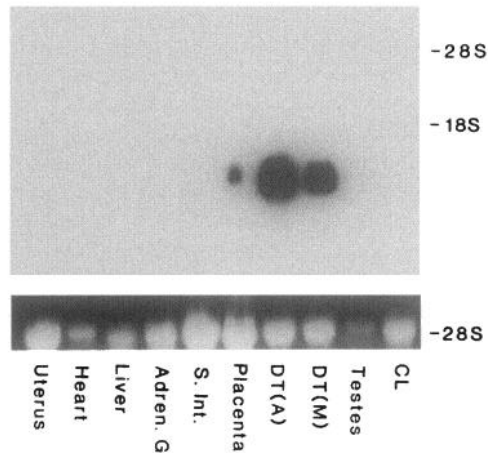


FIG. 5. Tissue-specific expression of dPRP. Total RNA was isolated from uterus, heart, liver, adrenal gland (Adren. G), small intestine (S.Int.), placenta, antimesometrial decidua [DT(A)], mesometrial decidua [DT(M)], testes, and corpora lutea (CL). Twenty micrograms of total RNA were loaded in each lane, fractionated by 1% agarose-0.74 M formaldehyde, and hybridized with the dPRP cDNA probe, as described in *Materials and Methods*.

analysis was carried out with RNA preparations from antimesometrial and mesometrial decidua and from trophoblast tissue isolated by dissection from the decidua. The results (Fig. 6) revealed that dPRP mRNA is expressed in the decidual tissue with much greater abundance than PLP-B. A trace amount of dPRP mRNA was detected in the trophoblast. It is not yet clear whether this is due to contamination with some decidual tissue, cross-hybridization with other PRL-related mRNAs known to be expressed in the trophoblast (21, 22), or very low expression of dPRP itself in this tissue. Interestingly, although two PLP-B transcripts (1.2 and 0.9 kb) were detected in both trophoblast and decidua, they were differentially expressed in the two tissues. The 1.2-kb species is found more abundantly in the decidua.

Regulation of dPRP in the decidua

Because of a very strong paracrine regulation between antimesometrial and mesometrial cells (23, 24), we investigated whether the absence of dPRP message in the mesometrial cells was due to an inhibitory signal from the antimesometrial cells. We also examined whether the products of the mesometrial cells affect the expression of dPRP by antimesometrial cells. We isolated antimesometrial and mesometrial cells by elutriation and cultured them either independently or in coculture, as shown in Fig. 7. No dPRP mRNA could be detected in the mesometrial cells even when these cells were cultured in the absence of antimesometrial cells. We also could not detect any difference in the expression of dPRP mRNA in antimesometrial cells whether they were cultured in the presence or absence of mesometrial cells.

Because decidual cells possess binding sites for PRL (14) and express the two forms of the PRL receptor gene (25), we investigated the possibility that PRL may regulate the expression of dPRP in the decidua. PRL secretion was blocked with ergocryptine treatment, as previously described (1, 3). Decidual tissues were obtained from rats treated with or without

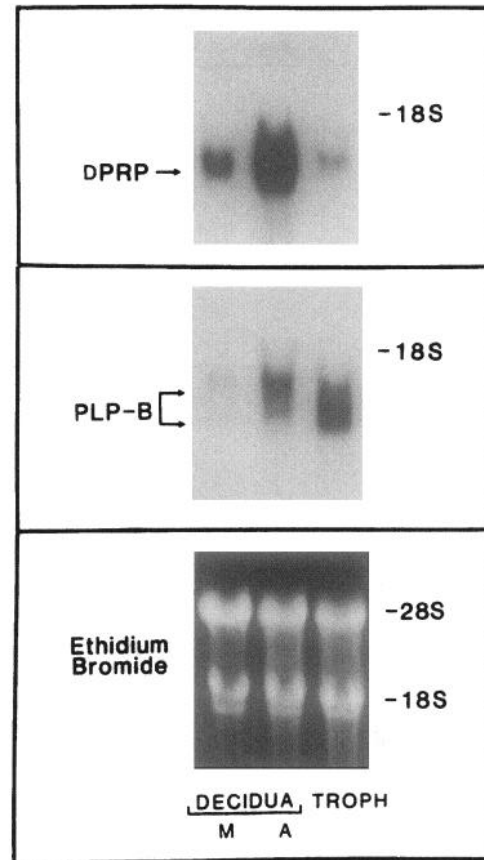


FIG. 6. Expression of dPRP and PLP-B mRNAs in decidual and placental tissues. Decidual tissue was isolated from day 13 pseudopregnant rats. Antimesometrial (A) and mesometrial (M) decidual tissues were separated by dissection. Trophoblast (TROPH) was isolated from day 19 pregnancy. Total RNA was isolated from both tissues. Twenty micrograms of total RNA were loaded in each lane and hybridized with the PLP-B cDNA. After stripping ($0.01 \times$ SSC and 0.01% SDS; 100 C; 2 min, five times), the blot was hybridized with the dPRP cDNA. Ethidium bromide staining of the blot is present in the lower panel.

the dopamine agonist, and the level of dPRP mRNA was examined in pooled antimesometrial and mesometrial decidual tissues by Northern analysis (Fig. 8). No apparent effect of PRL on dPRP expression could be detected. Here, also, dPRP mRNA was predominantly located in the antimesometrial tissue.

Discussion

The present study demonstrates that the decidual PRL-related protein gene is highly specific to decidual tissue. It is abundantly expressed in the decidua and more specifically in a defined cell population confined to the antimesometrial region of the uterus. The expression of the dPRP gene is triggered by the induction of decidualization, indicating that dPRP is not only a useful indicator of decidualization, but is also an excellent marker for antimesometrial cell transformation. These cells, which originate from endometrial stromal cells located in the antimesometrial region of the uterus, decidualize first and become very large. Decidualization occurs at a later stage in the mesometrial region where cells remain less differentiated and much smaller. Our find-

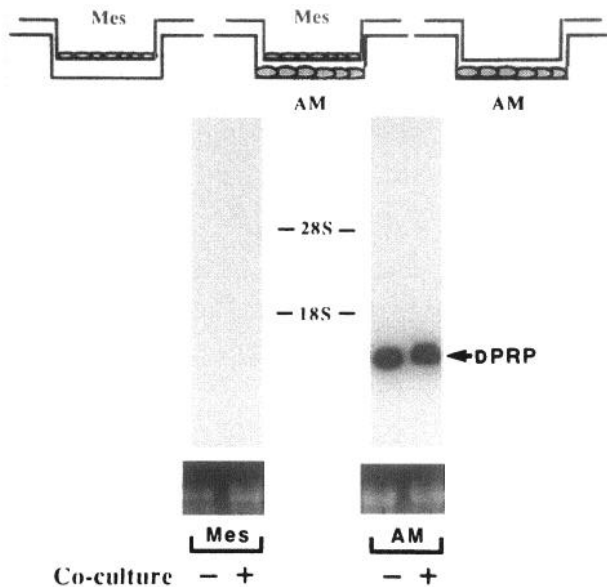


FIG. 7. Culture and coculture antimesometrial and mesometrial cells. Antimesometrial (A) and mesometrial (M) decidua cells were isolated from day 9 pseudopregnant rats and separated by elutriation. Cells were either cultured independently or cocultured, as depicted, for 48 h. Ten micrograms of total RNA were loaded in each lane and hybridized with the dPRP cDNA probe. The exposure time was 4 days.

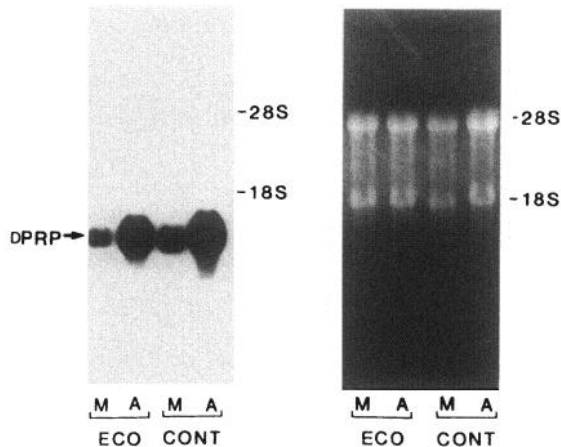


FIG. 8. Effect of ergocryptine treatment on dPRP mRNA. Total RNA was isolated from dissected antimesometrial and mesometrial decidua tissues of rats treated with either ergocryptine (0.4 mg/rat) or ethanol (CONT; 250 μ l/rat). Twenty micrograms of total RNA were loaded in each lane and hybridized with the dPRP cDNA probe. Ethidium bromide staining of the blot is present in the right panel, as some difference in the amount of RNA between lanes was observed. The exposure time was 24 h.

ing that dPRP gene is expressed only in the large antimesometrial cells together with our previous report (9, 13, 24) that these cells are the sole source of the PRL-like hormone secreted by the decidua suggest that during decidualization, only this cell population acquires the specific factors responsible for this cell-restricted expression. Mesometrial decidua cells may be either inherently unable to express dPRP gene or may be prevented actively from such expression by inhibitory signals originating from antimesometrial cells. Such a paracrine regulation does indeed exist between the two decidua cell populations (10, 23). We have recently reported

(23) that in contrast to dPRP, α_2 -macroglobulin is produced by mesometrial cells. *In vivo*, the antimesometrial cells neither expressed the gene for nor synthesized this protease inhibitor. Yet, once these cells were separated from mesometrial cells and cultured independently, they became able to express α_2 -macroglobulin mRNA. Our results, however, do not support such a possibility for the expression of dPRP in mesometrial cells. No dPRP mRNA could be detected in mesometrial cells even when they were cultured in the total absence of antimesometrial cells. This suggests that the two decidua cell types may either originate from different stem cells with unique tissue-specific factors or, alternatively, they may be subjected to differentiation signals that cause the activation of different genes. Whatever the reason for this cell-specific expression, once dPRP is induced, levels of its mRNA remain constantly elevated throughout decidual development. It decreases later in pseudopregnancy, probably because of the extensive cell death that takes place in the antimesometrial cells at this stage (26, 27). Developmental expression of dPRP contrasts with decidual expression of follistatin and activin by antimesometrial cells (26, 28). The follistatin gene is expressed for only a few days, and its mRNA disappears abruptly after day 10, whereas activin mRNA only becomes detectable after day 11. This suggests that activin and follistatin are differentially regulated in antimesometrial cells during development, whereas dPRP may be constitutively expressed. However, in contrast to activin and follistatin, which are produced by many different tissues (29), dPRP appears to be highly specific to antimesometrial decidua cells. dPRP could not be detected in the numerous endocrine and nonendocrine tissues examined. Very low levels of dPRP mRNA were detected, however, in day 18 placenta. This mRNA was not necessarily due to the presence of decidua tissue in the placenta, as similar signal was detected in isolated trophoblast. Another PRL-related gene, termed PLP-B, was previously reported to be expressed in both trophoblast and decidua (20, 30). PLP-B clone was initially isolated from a day 18 rat placental library and was found to be abundantly expressed in the trophoblast of late pregnancy (30). Further studies (20) indicated that PLP-B mRNA was also detectable in the antimesometrial decidua. This led Croze *et al.* (20) to suggest that PLP-B may be the decidua luteotrophic hormone.

Our investigation has revealed that dPRP is expressed in the decidua tissue with much greater abundance than PLP-B. We originally thought that PLP-B mRNA, detected in the decidua with the PLP-B cDNA, may be due to cross-hybridization with dPRP. However, careful analysis of the results indicate that the predominant decidua PLP-B transcript is 1.2 kb, whereas dPRP is 1 kb, as previously reported (15, 20). These findings indicate, therefore, that at least two PRL-related genes are expressed in the decidua. Both PLP-B and dPRP mRNAs encode for proteins in the 28- to 29-kilodalton range, and the luteotropic activity reported earlier may encompass both proteins.

Because antimesometrial cells not only secrete PRL-like hormone(s) but also express PRL receptors (14, 25), we investigated the role of PRL in dPRP expression. We also examined whether mesometrial cell signals affect dPRP expression in the antimesometrial cells. These experiments

stemmed from our previous findings, which revealed that large amounts of PRL-like hormone accumulate in the culture medium only when antimesometrial cells are cultured in the absence of mesometrial cells (9) or when mixed decidual cells are continuously perfused with medium (12). We interpreted these findings to mean that either mesometrial cells are a site of degradation of the hormone, or mesometrial signals may inhibit its expression. The results of this investigation indicate that neither PRL nor products of the mesometrial cells affect dPRP expression in antimesometrial cells, adding further support to the possibility that once induced, dPRP remains constitutively expressed. However, what triggers dPRP gene expression in the defined antimesometrial cell population at the inception of decidualization remains a mystery and is the subject of further investigation in our laboratory.

Acknowledgments

Special thanks are extended to Dr. Mary Lynn Duckworth for the generous donation of the PLP-B probe. We also thank Linda Alaniz-Avila for the photography, Rosemary Clepper for animal care, and Vivian Rogala for secretarial assistance.

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