Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 217–221, January 1985
Medical Sciences

Identification and partial characterization of a prolactin-like hormone produced by rat decidual tissue

(luteotropic hormone/pseudopregnancy/decidual hormone/progesterone production)

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Communicated by C. Ladd Prosser, September 10, 1984

ABSTRACT **Previous studies have strongly, but indirectly, suggested that rat decidual tissue produces a prolactin-like hormone, decidual luteotropin, which markedly affects luteal cell function. However, it was also found that extracts of decidual tissue do not cross-react with antisera to either rat or ovine prolactin (PRL). The purpose of this study was to determine whether the decidual tissue contains a substance that binds to PRL receptors in rat luteal membranes and, if so, to identify, quantitate, and characterize this molecule with the use of an ovarian radioreceptor assay. Decidual tissue was induced in day 5 pseudopregnant rats by scratching the antimesometrial wall of the uterus; it was collected on day 9 and homogenized and extracted. Decidual tissue extracts bound specifically to ovarian PRL receptors. Graded dilutions of the extracts yielded curves that were parallel to the ovine PRL standard, indicating that decidual luteotropin competes for the same receptor sites on rat luteal membranes. To determine the levels of decidual luteotropin throughout pseudopregnancy, decidual tissue was obtained on each day between days 6–12. The PRL-like activity was detectable in decidual tissue as early as day 6, reached a maximum on day 9, and declined thereafter. The elution profile obtained from gel filtration of a day 9 decidual tissue extract displayed a major component of decidual luteotropin eluting at a V_e/V_o ratio of ≈2.0. Column chromatography indicated that decidual luteotropin corresponds to a protein with a molecular weight of 23,500. The hormone was heat labile, digestible by trypsin, and appears to contain disulfide linkages. In summary, this study reports the identification, quantitation, and partial characterization of a PRL-like hormone produced by the decidual tissue of the rat.**

In the rat, the decidual tissue is the result of proliferation, hypertrophy, and differentiation of endometrial stromal cells, induced during pregnancy after contact of the blastocyst with the endometrium or by artificial stimulation of the uterus on the 5th day of pseudopregnancy. Stromal cells proliferate and differentiate into decidual cells between the 5th and 11th day of pseudopregnancy (1, 2). After this period, no evidence for further growth is seen. Mitoses are few, cells do not continue to enlarge (2), and the decidual tissue begins to regress. Apparently, when growth stops, degeneration begins (1).

Several functions have been attributed to decidual cells. It has been postulated that the decidual tissue maintains pregnancy by protecting the maternal tissue from destructive invasion by the trophoblastic cells of the placenta (3) and by protecting the fetoplacental unit from immunological rejection by the mother (4). Because of its high glycogen content, a nutritive role for the decidual tissue has also been postulated (5). In the rat, early studies demonstrated that decidual tissue also prolongs the life span of the corpus luteum (6, 7). Recently, several secretory products have been associated with the decidual tissue, and there is increasing evidence to support an endocrine role for this tissue. Decidual cells secrete prostaglandins in rats (8, 9), and both relaxin and prolactin (PRL) in humans (10–12). Gibori *et al.* (13) first hypothesized that the decidual tissue of the rat secretes a PRL-like hormone that may be responsible for the prolongation of corpus luteum function. They found that inhibition of PRL secretion in pseudopregnant rats causes a precipitous decline in luteal progesterone synthesis only in rats without decidual tissue. In the presence of decidual tissue, corpora lutea continue to secrete progesterone as if PRL had not been removed from the circulation. Subsequently, it was found that the effect of the decidual tissue is exerted through a substance, a decidual luteotropin, which reaches its sites of action by passage through the systemic circulation (14). Studies in pregnant rats have also revealed that the maternal part of the placenta produces a substance that sustains luteal cell function after PRL withdrawal (15).

In humans, decidual PRL has been found to be identical to its pituitary homologue (11, 16). Antiserum developed against human PRL recognizes decidual PRL (11, 12, 16), making quantitation of the hormone relatively easy. In the rat, decidual luteotropin does not cross-react with antisera to either rat or ovine PRL (13). This has hampered the characterization of this hormone. Since the corpus luteum appears to be a target tissue for the decidual hormone (13), we developed a radioreceptor assay (RRA) that uses highly luteinized ovaries as the source of PRL receptors. With this assay we were able to identify, quantitate, and partially characterize rat decidual PRL-like hormone.

MATERIALS AND METHODS

Hormones and Drugs. Ovine PRL [NIADDK-oPRL-16; 31 international units (IU)/mg], bovine PRL (NIADDK-bPRL-6; 30 IU/mg), rat PRL (NIADDK-rPRL-B-3; 20 IU/mg), ovine luteinizing hormone (NIADDK-oLH-21; 2.5 IU/mg), ovine follicle stimulating hormone (NIADDK-oFSH-13; 15 IU/mg), and ovine PRL for radioiodination (NIH-oPRL-I-1; 35 IU/mg) were provided by the National Hormone and Pituitary Program of the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases. Pregnant mare serum gonadotropin (Gestyl) and human chorionic gonadotropin (Pregnyl) were obtained from Organon. The molecular weight marker kit was purchased from Pharmacia, Ultrogel AcA 54 was from LKB, silica gel was from Fisher, and all other chemicals were from Sigma.

Animals. Pseudopregnant rats (250–275 g) were obtained from Holtzman (Madison, WI). The day a vaginal plug was found was considered day 1 of pseudopregnancy. The rats were kept at 26°C under a controlled photoperiod of 14 hr of

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Abbreviations: PRL, prolactin; RRA, radioreceptor assay; IU, international units.

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light and 10 hr of dark, with free access to Purina rat chow and water.

Surgery. A clean, but not aseptic, technique was used during the surgical procedure, with ether as anesthetic. Decidual tissue was induced on day 5 of pseudopregnancy by scratching the antimesometrial surface of both uterine horns with a hooked needle. Rats were sacrificed daily from days 6–12 of pseudopregnancy with an overdose of ether, and the uterine horns were dissected out and slit lengthwise. Decidual tissue was obtained from each animal, weighed, and then snap-frozen separately for subsequent determination of PRL-like activity.

Muscle (biceps femoris) was also obtained for use as a control tissue and similarly treated. For the gel filtration studies, a pool of decidual tissue was obtained from rats on day 9 of pseudopregnancy.

Tissue Extraction. Either decidual tissue or muscle (30%, wt/vol) was homogenized in a 16 × 100 mm glass test tube with a Polytron homogenizer for 10 sec at 4°C in buffer [50 mM Tris·HCl/1 mM EDTA/5% (vol/vol) glycerol, pH 7.4] containing 25 μg of bacitracin, 10 μg of pepstatin A, and 174 μg of phenylmethylsulfonyl fluoride per ml. The homogenate was centrifuged at 3000 × *g* for 10 min at 0°C. The supernatant was recentrifuged at 25,000 × *g* for 30 min at 0°C to remove lysosomes. The 25,000 × *g* supernatant was used for the determination of PRL-like activity. For the gel filtration study, the 25,000 × *g* supernatant was centrifuged at 100,000 × *g* for 90 min at 0°C to obtain the cytosolic fraction.

RRA. ^{125}I-labeled ovine PRL was prepared by the lactoperoxidase method (17) and purified on Ultrogel AcA 54 gel packed in a disposable 10-ml syringe. Specific activity as calculated by displacement assay (18), ranged between 60 and 80 μCi/μg (1 Ci = 37 GBq). The binding capacity of each tracer preparation determined after incubation with excess receptors was 45%–60%.

Ovaries were obtained from pregnant mare serum/human chorionic gonadotropin-primed 25-day-old Sprague–Dawley-derived rats (19). Membranes from these highly luteinized ovaries were prepared as described (20) with a few modifications. Just prior to the assay, the membranes were washed twice with assay buffer (50 mM Tris/20 mM $MgCl_2$/1 mM EDTA/0.1% bovine serum albumin) and centrifuged at 10,500 × *g* for 10 min.

The ovarian RRA for PRL-like hormones is a modification of a previously described procedure (21). Incubations were carried out in duplicate for 16 hr at 22°C in 12 × 75 mm glass tubes. For the standard curve, a total volume of 500 μl consisted of 100 μl of ovarian membranes, 100 μl of ^{125}I-labeled ovine PRL (100,000 cpm/ng), 100 μl of ovine PRL standards (1–10,000 ng/ml), and 200 μl of assay buffer. Tissue extracts were assayed in duplicate at concentrations ranging from 25 to 200 μl. The separation of bound from free tracer was achieved by the addition of 1 ml of ice-cold assay buffer containing 0.5% silica gel, followed by centrifugation at 1500 × *g* for 20 min. The supernatant was then aspirated, and the silica gel pellet was washed with 3 ml of assay buffer and counted. The lower limit of sensitivity for the assay was 0.5 ng per tube. The saturation study and Scatchard analysis were performed as described (20).

Time Course Determination. To determine the levels of decidual luteotropin throughout pseudopregnancy, decidual tissue was isolated from rats each day from days 6 to 12 of pseudopregnancy. Decidual tissue obtained from individual rats was weighed separately. Muscle (biceps femoris) was used as a tissue control. Tissue extracts were assayed in duplicate and in at least two aliquot sizes.

Gel Filtration. The elution pattern of the cytosolic fraction obtained from the decidual tissue was determined by applying a 9- to 10-ml sample to a column of Ultrogel AcA 54 (2.5 × 90 cm), eluted with 50 mM Tris·HCl/1 mM EDTA/5% (vol/vol) glycerol, pH 7.4. Elution fractions (10 ml) were analyzed by RRA for PRL-like activity. The column was calibrated with a low molecular weight marker kit. For the determination of the molecular weight of decidual luteotropin, the experiment was repeated twice.

Concanavalin A Sepharose Chromatography. To determine whether decidual luteotropin is a glycoprotein, 1.5 ml of a cytosolic extact of decidual tissue obtained from day-9 pseudopregnant rats was applied to a column (10-ml syringe) packed with Concanavalin A Sepharose 4B. The unadsorbed sample (non-glycosylated fraction) was eluted with a starting buffer (0.1 M sodium acetate buffer, pH 6.1, containing 1 mM $MgCl_2$/1 mM $MnCl_2$/1 mM $CaCl_2$/1 M NaCl), collected, and pooled. The adsorbed sample (glycosylated fraction) was eluted with 0.3 M α-D-methyl mannoside in the startng buffer. The eluates were pooled separately. The pooled fractions were dialyzed extensively and then lyophilized, reconstituted, and checked for the presence of PRL-like activity by RRA.

Treatment with Dithiothreitol, Trypsin, and Heat. Dithiothreitol (100 μl of a 5 mM solution in assay buffer) was incubated with 100 μl of day-9 decidual tissue extract (40%, wt/vol) for 60 min at 37°C. The control tubes were incubated with 100 μl of buffer only. After incubation, the contents were dialyzed overnight at 4°C with three changes of Tris buffer. These samples were subsequently assayed for PRL-like activity. In another set of experiments, 100 μl of 5 mM dithiothreitol was added to samples and then assayed for PRL-like activity without the dialysis step. To determine whether decidual luteotropin is a protein, tissue was extracted in the absence of proteolytic inhibitors. Tissue extract (100 μg) was incubated with 100 μg of trypsin (15,000 units per mg of protein) in a 100-μl vol for 60 min at 37°C. The control tubes were incubated with 100 μl of Tris buffer. At the end of incubation, 200 μg of trypsin inhibitor (10,000 units per mg of protein) was added to all the tubes. The tubes were then assayed for the presence of PRL-like activity. Decidual tissue extracts were incubated for 16 hr at 22°C, 1 hr at 37°C, or boiled for 25 min. The tissue extract was then assayed for PRL-like activity. The activity in the control tubes (kept at 4°C) was taken as 100%.

Statistics. The data were analyzed for differences by Student's *t* test and by analysis of variance. A difference was considered significant if the *P* value was <0.05.

RESULTS

Membranes of highly luteinized ovaries were used as a source of PRL receptors, because the corpus luteum is clearly a target tissue for decidual luteotropin. The results shown in Fig. 1 indicate that PRL binds to rat luteal membranes with high affinity (K_d 4.6×10^{-10} M) and specificity. Ovine PRL, bovine PRL, and rat PRL were all capable of displacing the ^{125}I-labeled ovine PRL tracer from luteal PRL receptors in a dose-related manner. Ovine luteinizing hormone and ovine follicle-stimulating hormone had no effect. The inter- and intra-assay coefficients of variation were 9% and 5%, respectively. The sensitivity of this radioreceptor assay was 0.5 ng per tube. Graded dilutions of decidual tissue extracts yielded curves that were parallel to the ovine PRL standard, indicating that the decidual PRL-like hormone and ovine PRL compete for the same receptor sites on rat luteal membranes (Fig. 1). Comparable doses of muscle extract were unable to displace the ^{125}I-labeled ovine PRL from the ovarian binding sites (Fig. 1).

The results of the time course experiments are presented in Fig. 2. PRL-like activity was detectable in decidual tissue as early as day 6, reached a maximum concentration on day 9, and declined thereafter. Muscle processed in a similar manner was found to be devoid of any PRL-like activity.

To characterize the decidual PRL-like hormone, decidual

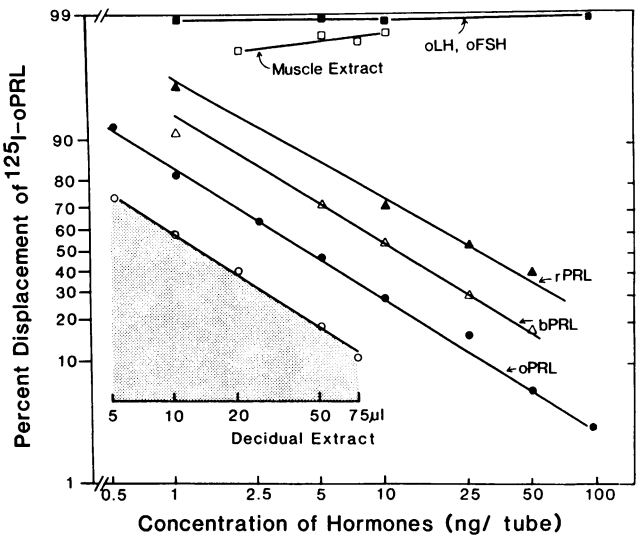

FIG. 1. Competition of ^{125}I-labeled ovine PRL (^{125}I-oPRL) in the luteal RRA with bovine (b) PRL, rat (r) PRL, and a serial dilution of decidual tissue extract. For the hormonal specificity of PRL binding, luteal membranes were incubated with ^{125}I-oPRL (100,000 cpm per 100 µl) alone or with increasing concentrations (1–100 ng) of oPRL, bPRL, rPRL, ovine luteinizing hormone (oLH), and ovine follicle-stimulating hormone (oFSH). For the competition of ovine PRL with decidual tissue extracts, 40% (wt/vol) homogenates of day 9 decidual tissue were prepared and 5–75 µl were assayed in quadruplicate.

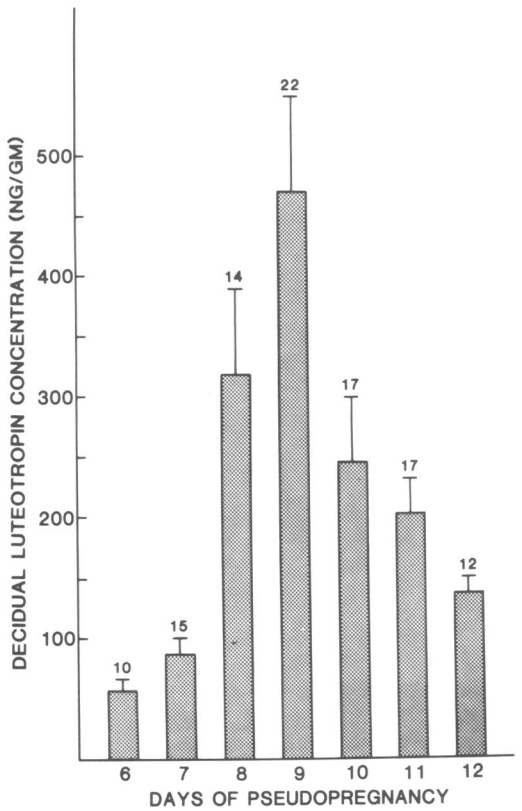

FIG. 2. Tissue concentration of the decidual PRL-like molecule throughout pseudopregnancy (ng per g wet weight). Bars represent mean ± SEM with the number of animals indicated at the top of each bar.

tissue obtained from day-9 pseudopregnant rats was routinely used. It was found that storage of decidual tissue samples at −20°C resulted in a loss of PRL-like activity. However, hormone levels remained stable in samples that were stored at −70°C. Homogenization of decidual tissue in the absence of protease inhibitors also resulted in a significant decrease in activity.

Decidual luteotropin is not heat stable. Incubation of decidual extracts in moderate heat for long periods or at increased temperatures for a short period resulted in a reduction in PRL-like activity (Table 1). Trypsin digestion of decidual tissue extract completely destroyed the binding activity. Extracts incubated in the absence of trypsin contained 37.2 and 93.3 ng of PRL-like hormone per g of tissue. No PRL-like activity was found in extracts incubated in the presence of trypsin.

Chromatography on Sepharose concanavalin A suggests that decidual luteotropin does not contain any carbohydrate moieties available to interact with the concanavalin-A. All PRL-like activity was found in the unadsorbed fraction. No activity whatsoever was obtained in the glycosylated fraction.

Incubation of decidual tissue extracts with dithiothreitol or addition of dithiothreitol to the assay tubes induced a 64% and a 95% loss in activity, respectively (Fig. 3). Pretreatment of receptors with the same concentration of dithiothreitol had no effect on their subsequent capacity to bind PRL or the decidual PRL-like hormone. These findings suggest the presence of disulfide linkages in the decidual PRL-like molecule.

The elution profile of a day-9 decidual tissue extract obtained from gel chromatography displayed two peaks of activity in the ovarian RRA (Fig. 4). The major component of

Table 1. Effect of temperature on decidual PRL-like activity

Temperature, °C	Time, hr	Decidual luteotropin, % activity lost
20	16	33
37	1	16
100	0.25	100

A decidual tissue extract (25,000 × *g* supernatant of homogenate) obtained from day 9 pseudopregnant rats was incubated in triplicate at the indicated temperature and time. Extract incubated at 4°C (73 ng of PRL-like activity per g) was taken as 100%.

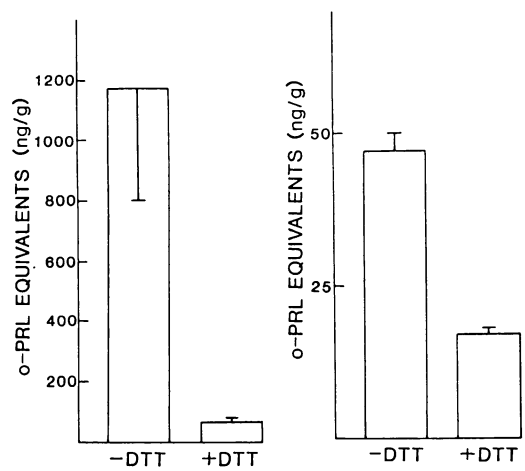

FIG. 3. Effect of dithiothreitol on the decidual PRL-like hormone. (*Left*) Day-9 decidual tissue extract was assayed for PRL-like activity in the presence of 100 µl of 5 mM dithiothreitol. Results represent mean ± SEM of 3 determinations. (*Right*) Decidual tissue extracts were preincubated with 100 µl of 5 mM dithiothreitol for 60 min at 37°C. Controls were incubated with 50 mM Tris·HCl buffer. Control and dithiothreitol-treated samples were then dialyzed overnight against three changes of 50 mM Tris·HCl buffer. Samples were then assayed for PRL-like activity. Results represent mean ± SEM of 3 determinations. o-PRL, ovine PRL; −DTT, control; +DTT, extracts incubated with dithiothreitol.

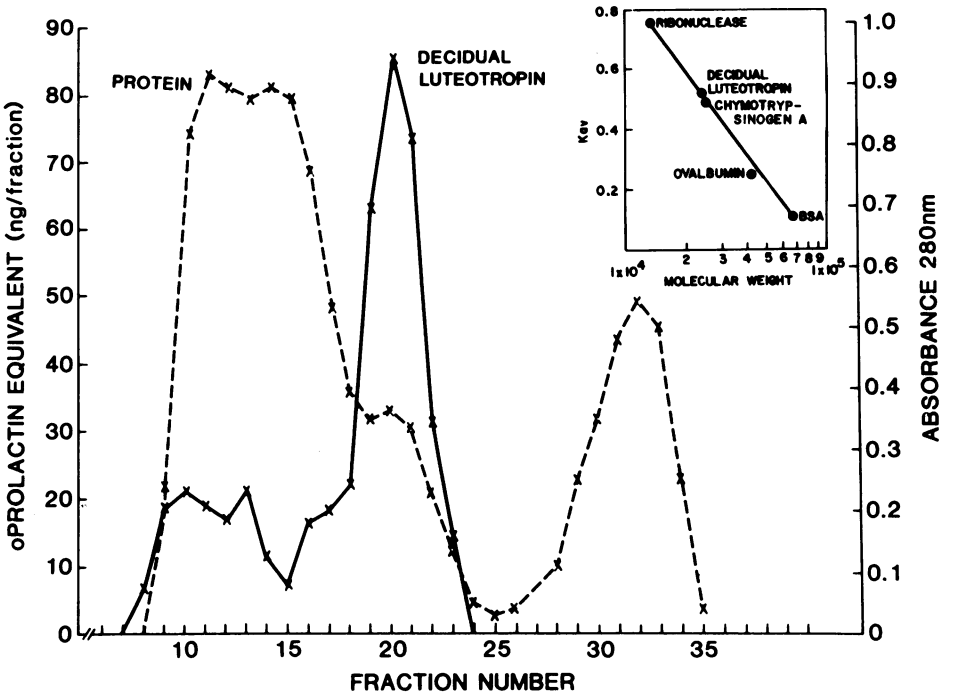

FIG. 4. Chromatography of decidual tissue extract on a column of Ultrogel AcA 54. For estimation of approximate molecular weight of decidual luteotropin (*Inset*), the K_{av} corresponding to peak protein concentration of each marker was plotted against its molecular weight, and the resulting graph was used to estimate the molecular weight of the decidual PRL-like molecule. Marker proteins used and their molecular weights are as follows: ribonuclease A, 13,700; chymotrypsinogen A, 25,000; ovalbumin, 43,700; bovine serum albumin (BSA), 67,000. oProlactin, ovine prolactin.

the decidual PRL-like molecule has a V_e/V_o ratio of ≈2.0. A small shoulder eluted with a V_e/V_o ratio of 1.7, which may represent a precursor or aggregate of the PRL-like hormone. There was 60%–80% recovery of the PRL-like activity from the column fractions. Ultrogel AcA 54 column chromatography of molecular weight standards (blue dextran, bovine serum albumin, ovalbumin, chrymotrypsinogen, and ribonuclease) indicated that decidual hormone corresponds to a protein with a molecular weight of ≈23,500 (Fig. 4).

DISCUSSION

This study reports the identification, quantitation, and partial characterization of the PRL-like hormone produced by the decidual tissue of the rat. This hormone competes with PRL in a dose-related manner for the same receptor site on the luteal membrane. However, it differs from PRL in that it is not recognized by antisera developed against rat or ovine PRL (13). Despite the apparent differences, there are several chemical and physiological similarities between these two hormones. Comparable to PRL, the decidual hormone appears to be a protein with disulfide bridge(s) and with a molecular weight of ≈23,500. Both hormones bind to luteal cells and markedly affect ovarian steroidogenesis *in vivo* (13, 15, 22–24). *In vitro* studies indicate that a decidual extract stimulates luteal cell production of progesterone and enhances luteinizing hormone-stimulated steroidogenesis (unpublished observations).

Initial attempts to demonstrate the presence of the decidual PRL-like hormone were difficult because of the unstable nature of this peptide. Storage of tissue samples at −20°C and homogenization in the absence of protease inhibitors both resulted in a significant decrease in activity. The proteolytic degradation of the PRL-like hormone was inhibited to a great extent by the addition of phenylmethylsulfonyl fluoride, pepstatin A, and bacitracin during the extraction procedure. Inclusion of glycerol also helped stabilize the activity of this protein. In addition, it was observed that proteolytic enzymes from the ovarian receptor homogenates degraded the hormone activity. Extensive washing of the ovarian receptor preparation helped resolve this problem.

The pattern of the decidual PRL-like hormone concentration in the decidual tissue shows a gradual increase during the first 4 days of decidualization. A peak concentration is reached on day 9, followed by a gradual decline. These levels correlate closely to the pattern of decidual tissue growth and regression. In the rat, decidual cells proliferate vigorously for ≈5 days after induction. Thereafter, the cells stop dividing and begin to regress (2, 5). The decline in decidual luteotropin levels corresponds to the disappearance of the luteotropic effect of the decidua. From day 11, the decidual tissue of either pregnant or pseudopregnant rats loses its luteotropic activity and becomes unable to sustain luteal cell production of progesterone (13, 15, 23). In humans, the appearance of PRL in the endometrium also coincides with the onset of decidualization at the time when implantation would occur and increases in proportion to the degree of decidualization (25, 26).

No substantial evidence exists to confirm access of human decidual PRL to the maternal circulation. Decidual PRL is released principally into the amniotic fluid (11, 27), where it may control membrane transport of electrolytes and substrates (28, 29). However, substantial proof is lacking. In contrast, an ovarian role for rat decidual luteotropin has already been established. Decidual luteotropin reaches the ovaries via the peripheral circulation (14) and appears to affect both luteal and follicular function. It causes an increase in the rate of progesterone secretion both *in vivo* (30) and *in vitro* (31, 32) and maintains luteal production of progesterone after procedures that depress PRL (13–15). Twenty-four hours after implantation in pregnant rats or induction of decidual tissue in pseudopregnant rats, pituitary PRL is no longer required, most probably because of the emergence of decidual luteotropin (13–15). However, decidual luteotropin cannot sustain luteal production of progesterone in the absence of the pituitary (23). Pituitary luteinizing hormone is

required for luteal steroidogenesis and a synergism between luteinizing hormone and decidual luteotropin from day 8 until day 11 of pseudopregnancy has been reported (33). Recently, it was found that decidual luteotropin markedly inhibits the follicular production of both testosterone and estradiol (unpublished observations).

It remains to be determined whether decidual luteotropin acts locally to affect placental function and fetal growth. Both decidual tissue (34, 35) and trophoblast (36) contain PRL receptors, and decidual luteotropin competes in a dose-dependent manner with ovine PRL for the same receptor sites in the decidual tissue (37). In the rat, the trophoblast begins secreting a placental lactogen after day 10 of pregnancy (38). The appearance of this lactogen corresponds to the decline in the level of decidual luteotropin. This temporal relationship suggests that decidual luteotropin may act locally on trophoblastic tissue to inhibit placental lactogen secretion. The possibility that the decidual tissue may delay the ontogeny of placental lactogen is also supported by the findings of Glasser and McCormack (39). They reported that trophoblastic tissue obtained from blastocyst outgrowths in culture begins secreting rat placental lactogen at a very early stage of pregnancy (as early as day 6), whereas trophoblasts grown adherent to the decidual tissue *in vivo* produce placental lactogen only from day 10.

Therefore, the rat placenta appears to produce two PRL-like hormones with luteotropic activity. The hormone produced early in pregnancy originates from the decidual tissue, whereas the luteotropin produced at mid-pregnancy originates from the trophoblast (38). The two hormones compete with PRL for the same receptor sites in luteal cells (Fig. 1; ref. 20) and affect corpus luteum function in a similar manner (24, 40, 41). However, rat placental luteotropin possesses a larger molecular weight (38) and the duration of its effect is longer than decidual luteotropin. Rat placental luteotropin affects corpus luteum function for at least 4 days (41), whereas the effect of decidual luteotropin disappears within 24 hr after the removal of its source (13). In addition, findings from several laboratories suggest strongly that placental luteotropin, but not decidual luteotropin, inhibits the secretion of PRL (42–44).

The ethical and practical limitations for *in vivo* experimentation in humans reduce the possibility of investigating and understanding the role of decidual prolactin in the physiology of human reproduction. Speculations about the function of decidual prolactin were based on occasional findings in pregnant women and *in vitro* experiments with human membranes. Results of the present investigation provide evidence that the rat can be used as an experimental animal model to further investigate the role of the PRL-like hormone produced by the decidua.

This work was supported by grants from the National Institutes of Health (HD12356) and the National Science Foundation (PCM 8112225).

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