Characterization of Rat Mid-Pregnancy-Specific Placental Lactogen Produced by Baculovirus / Insect Cell Expression System

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Abstract. The placenta produces prolactin (PRL)- or growth hormone-like hormones known as placental lactogens (PLs) which play roles in the maternal endocrine system and fetal growth development. We previously cloned a cDNA encoding a novel rat PL, PL-I mosaic (PL-Im), which is specifically expressed at mid-pregnancy. In this study, the biological activity of PL-Im was evaluated by producing the recombinant protein (recPL-Im) by means of a baculovirus/insect cell expression system. RecPL-Im demonstrated binding activity for PRL receptor in a radioreceptor assay (RRA) system with rat liver membrane fraction and ¹²⁵I-ovine PRL (oPRL). In addition, the activity of recPL-Im is comparable to oPRL in the Nb2 cell proliferation assay. Interestingly, recPL-Im stimulated progesterone secretion in a concentration-dependent manner in a primary culture of luteal cells from pregnant rat, whereas oPRL had no effect on luteal cells. The luteotropic function of recPL-Im was accompanied by suppression of the secretion of 20 α -dihydroprogesterone (20 α -OHP) in the culture. PL-Im therefore has ability to bind PRL-receptor and express PRL-like activity. In addition, recPL-Im has luteotropic hormone (LTH) activity and its activity is qualitatively different from that of PRL.

Key words: Placental lactogen, Luteotropic, Corpus lutea.

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The rat placental PRL family consists of molecules structurally similar to PRL and growth hormone [1]. We successfully cloned cDNA encoding two members of it, PL-Im and PRL-like protein-D (PLP-D) [2, 3]. PL-Im mRNA is expressed at mid-pregnancy in the rat placenta and its structure is highly homologous (over 90%) with PL-I (mid-pregnancy-specific PL) [4] and PL-I variant (PL-Iv) (late-pregnancy-specific PL) [5]. Recently it was suggested that PL-Im is identical to PL-I based on the observation that the original-

ly identified PL-I, as distinct from PL-Im, could not be isolated from the cDNA libraries of Rcho-1 cells and midgestation placental tissue, or from a rat genomic library. The structure of PL-Im is therefore closely related to that of PL-I and they are the only PLs expressed at mid-pregnancy. It is still unclear, however, whether PL-Im and PL-I are identical proteins.

PL-Im cDNA has two possible N-glycosylation sites [2], but PLP-D has only one [3]. The recombinant PLP-D do not stimulate the proliferation of the rat lymphoma cell line, Nb2 cells, revealing that this placental PRL-like molecule has activity distinct from that of PRL but the molecular char-

acteristics of PL-Im remained to be evaluated. In rodents, PRL is one of the luteotropic factors that are essential for continuing progesterone secretion by the corpora lutea during early pregnancy [6, 7]. PRL secretion, however, ceases during mid-pregnancy following the initiation of PL secretion [8–10]. After placentation, mid-pregnancy-specific-PL is considered to be the primary candidate for the luteotropic factor that regulates progesterone secretion [11–13] so it is conceivable that PL-Im has luteotropic activity.

We previously purified 20 α -hydroxysteroid dehydrogenase (20 α -HSD) from rat ovary [14] and cloned its cDNA [15]. PRL expresses its luteotropic action by inhibiting the enzyme activity of 20 α -HSD, which converts progesterone to the inactive steroid, 20 α -OHP [14, 15]. As a result, it has been well documented that there is a reciprocal relationship between the secretion of progesterone and 20 α -dihydroprogesterone (20 α -OHP). Evaluation of the increase in the progesterone level and the decrease in 20 α -OHP is one of the indexes of specific luteotropic mechanisms.

In the present study, we produced recombinant PL-Im (recPL-Im) by a baculovirus/insect cell expression system to investigate whether the recPL-Im has PRL-like activity in the radioreceptor assay and in the proliferation assay of Nb2 cell growth. In addition, its luteotropic action was evaluated by using primary cultured luteal cells prepared from pregnant rats.

Materials and Methods

Reagent

Antiserum (N19) for detecting recPL-Im was a gift from Dr. M. Soares [16].

oPRL was provided by the National Hormone and Pituitary Program NIDDK-oPRL-20 (AFP-10677C, 31 international unit per mg), TC 100 insect medium, lipofectin, Fisher's medium (FM) and Dulbecco's Modified Eagle's medium (DMEM) were purchased from GIBCO BRL (Grand Island, NY, USA), Ex-cell 400 medium and fetal calf serum (FCS) were from JRH Biosciences (Lenexa, KS, USA), BaculogoldTM was from Pharmingen (San Diego, CA, USA), bactotriptose broth was from DIFCO (Detroit, MI, USA), tunicamycin, streptomycin, deoxyribonuclease (DNase), progesterone and 4-pregnen-20 α -ol-3-one (20 α -OHP) were from

Sigma Chemical (St Louis, MO, USA), Block ace was from Snow Brand (Sapporo, Japan), Horseradish peroxidase-conjugated anti rabbit IgG was from Jackson Immunoresearch Lab. (Baltimore, MD, USA), the ECL Western blotting kit was from Amersham (Tokyo, Japan), ¹²⁵I-rPRL and ³H-Progesterone was from Du Pont/NEN Research Products (Boston, MA, USA), donor horse serum (HS) was from Summit Biotechnology (Ft. Collins, CO, USA), WST-1 and Hepes were from Dojin Chemical (Kumamoto, Japan), Collagenase (type 2) was from Worthington Biochemical (Freehold, NJ, USA), dextran T-70 was from Pharmacia LKB (Uppsala, Sweden) and Norit A activated charcoal was from NACALAI TESQUE (Kyoto, Japan). All the other reagents used were obtained from Wako Pure Chemicals (Osaka, Japan).

Animals

Adult Wistar strain rats (Imamichi Institute for Animal Reproduction, Ibaraki, Japan) were kept under a lighting schedule of 14 h light and 10 h darkness (lights on from 0500 h to 1900 h) and allowed food and water *ad libitum* during the evening of the proestrus day, each female was housed with a male and the day when sperm were observed in a vaginal smear was designated day 0 of pregnancy.

Production of recPL-Im by using recombinant baculovirus

Recombinant virus production was carried out as described by Matsuura *et al.* [17, 18]. In brief, a 690 bp fragment of the PL-Im cDNA, which encodes entire PL-Im protein, was subcloned into a pAcYM1 vector (pAcIm). Then a recombinant baculovirus, AcIm, was obtained by transfecting Sf9 cells with linearized baculovirus DNA (BaculogoldTM) and the pAcIm.

Sf9 cells in serum-medium (TC 100 medium supplemented with 10% FCS, 0.26% bactotriptose broth and 200 nM kanamycin) and Tn5 cells in serum-free-medium (Ex-cell 400, 1 $\times$ 10⁷ cells/100-mm dish) were cultured for 1 h at 27 C. Then AcIm was added to the cells at a multiplicity of infection of 5–10, samples were taken after incubation for 4 days at 27 C, and centrifuged to separate the cells from the culture medium, both of which were stored at -20 C until required for various assays.

Tunicamycin treatment

When Tn5 cells were infected with AcIm, tunicamycin (final concentration 10 mg/ml) was added to the cell cultures [19, 20]. As controls, Tn5 cells infected with AcIm were incubated identically in the absence of tunicamycin. After being incubated for 4 days at 27 °C, the test and control cells were harvested and subjected to SDS-PAGE and Western blot analysis.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

SDS-PAGE was carried out as described by Noda *et al.* [14] with 12.5% (w/v) polyacrylamide gels under reducing conditions. Western blot was performed by the modified method described by Miura *et al.* [21] with anti-rabbit IgG and ECL developed with the ECL reagent.

Radioreceptor assay (RRA) and Nb2 cell proliferation assay

The binding activity of recPL-Im to PRL-R was measured by radioreceptor assay (RRA) with ¹²⁵I-rPRL and hepatic microsomal membranes prepared from the rats on day 14 of pregnancy, as described by Furuyama *et al.* [22]. Briefly, the microsomal fraction (10 µg protein equivalent) and ¹²⁵I-rPRL with or without recPL-Im or standard oPRL were incubated for 2 h at 25 °C in 20 mM Tris-HCl buffer (pH 7.8) containing 10 mM CaCl₂ and 0.1% BSA followed by two successive washings with the buffer. After centrifuging at 3,000 rpm for 15 min at 4 °C, the precipitate was counted in a gamma counter.

The Nb2 cell proliferation assay was performed as described by Tanaka *et al.* [23]. The cells were re-suspended ($1-2 \times 10^5$ cells/ml) in Hepes / 10 HS-medium and plated in 96-well culture plates (Costar, Cambridge, USA) at a concentration of 2×10^4 cells/200 µl/well in the presence or absence of various concentrations of standard oPRL or recPL-Im and they were incubated for 48 h. The number of cells in each well was determined by a calorimetric assay with WST-1.

Luteal cell culture

Luteal cell culture was carried out by the modified method described by Matsuyama *et al.* [24]. Briefly, newly formed corpora lutea were removed from the rats on day 10 of pregnancy, washed with

buffer A (25 mM Hepes, 137 mM NaCl, 5 mM KCl and 0.7 mM Na₂HPO₄), and dissociated at 37 °C with enzyme solution (buffer A containing 0.4% (w/v) collagenase, 0.4% (w/v) BSA and 10 mg/ml DNase). The cells were resuspended at a concentration of 12.8×10^4 cells/ml in DMEM and plated in 96-well culture plates (Costar, Cambridge, USA) at a concentration of 3.2×10^4 cells/250 µl/well in the presence or absence of various concentrations of oPRL or recPL-Im. The cultures were incubated as described above for 24 h, and the culture media were collected and hormone assays were carried out.

Hormone assay

Progesterone and 20α-OHP were measured by radioimmuno assay (RIA), as described by Matsuyama *et al.* [24].

Statistics

The data shown are the means ± SEM. Differences between means were analyzed by Welch's two-sided test and those at P values of less than 0.025 or 0.05 were considered significant.

Results

Expression of recPL-Im in insect cells

recPL-Im was detected in AcIm-infected culture media and cell lysate, but not in AcNPV infected-culture (control) media by Western blot analysis with N19 antiserum (Fig. 1A). The molecular weight (MW) of recPL-Im secreted into the medium ranged from 28 to 30 kDa and that of recPL-Im in the cell lysate was 26 to 30 kDa. The heterogeneity was assumed to be glycosylation, because PL-Im has two possible N-glycosylation sites. To confirm this, the cells were cultured in the presence of tunicamycin (Fig. 1B). The MW of recPL-Im was 28–29 kDa in the control medium and 26 kDa in the presence of tunicamycin, and thus the MW was clearly reduced by inhibiting N-glycosylation, indicating that the recPL-Im has N-linked oligosaccharides.

In the present study, recPL-Im secreted into the medium was used in the experiment to evaluate the biological activity of PL-Im otherwise indicated.

Binding activity of recPL-Im to PRL-R

The binding activities of recPL-Im for PRL-R

Fig. 1. Production of recPL-Im by baculovirus / insect cell system. A) Western blot analysis showing the expression of recPL-Im and the effect of tunicamycin on recPL-Im. Sf9 cells were cultured with AcIm (recombinant virus) or AcNPV (wild-type virus). B) Tn5 cells infected with AcIm were incubated with medium containing tunicamycin at 10 μg/ml (+) or with normal medium (-) as the control. At 96 h after infection, cells and culture medium (total volume 10 ml) were collected. Twenty milliliters of each culture medium and an aliquot corresponding to 1/200 th of the cell lysate infected with AcIm were analyzed by SDS-PAGE, followed by Western blot. The molecular weight marker is indicated at left. C) Binding Activity of recPL-Im to PRL-R in RRA. Binding activities of recPL-Im (○) and control (+) were compared with that of oPRL (□) by RRA. Microsomal membrane fractions of the rat livers were incubated for 2 h with ¹²⁵I-oPRL in the presence of increasing doses of each culture medium or oPRL. Medium or oPRL doses were expressed as the volume (μl) or weight (ng) added to each assay tube. Binding was determined by subtracting values for tube blanks from total radioactivity.

were examined by RRA (Fig. 1C). Serial dilution of the medium containing recPL-Im inhibited ¹²⁵I-rPRL binding competitively in parallel to binding of the oPRL standards. Control media showed no binding activity. It therefore became evident that PL-Im has the ability to bind PRL receptor.

Based on the binding activity in the RRA system, the amount of recPL-Im contained in one μl of the medium was calculated to be 4.71 ng of oPRL equivalent (oPRL eq.).

Mitogenic action of recPL-Im on Nb2 cells

RecPL-Im was subjected to the Nb2 proliferation assay (Fig. 2A). The concentration dependent increase in the number of cells was evoked by recPL-Im and oPRL. Stimulation of cell proliferation was detectable with as little recPL-Im as about 0.1 ng/ml of oPRL eq./ml of recPL-Im and maximum at about 1.5 ng/ml of oPRL eq. so that recPL-Im has a potent stimulating action on Nb2 cell growth, and the action was comparable to that of oPRL.

Fig. 2. Biological activity of recPL-Im. A) Mitogenic action of recPL-Im on Nb2 cells. Mitogenic activities of recPL-Im (□) or control (+) were compared with that of authentic pituitary oPRL (●) by noting the proliferation stimulative effect on Nb2 cells. Cultures of 1×10^5 viable Nb2 cells were incubated for 48 h at each dose. The lower horizontal line shows the zero oPRL control cell population value. The concentration

Luteotropic action of recPL-Im on rat luteal cell cultures

Luteal cells prepared from pregnant rat (day 10) were incubated for 24 h with various concentrations of recPL-Im or oPRL (0 to 2 μ g of oPRL eq./ml) and the progesterone and its metabolite 20 α -OHP were evaluated. We previously reported that PRL showed luteotropic action in the presence of macrophages but PRL alone cannot stimulate progesterone secretion in a primary culture of luteal cells [24]. Again, oPRL did not stimulate progesterone (Fig. 2B) secretion and did not suppress secretion of 20 α -OHP in the primary culture system (Fig. 2C), confirming that PRL alone is not enough to stimulate progesterone [24].

In contrast, progesterone secretion was increased in a concentration-dependent manner by recPL-Im. (Fig. 2B) so that recPL-Im had the ability to stimulate progesterone secretion from luteal cells in culture. RecPL-Im significantly inhibited 20 α -OHP secretion and there was a reciprocal relationship between the progesterone and 20 α -OHP concentrations (Fig. 2C).

PL-Im therefore has luteotropic activity and this activity seems to be qualitatively distinct from that of PRL.

Discussion

By using the recPL-Im obtained with the baculovirus/insect cell system, it became clear that PL-Im has a PRL-like action in the RRA and Nb2 cell proliferation assay, and it has luteotropic activity in the primary culture of corpus luteum from pregnant rat. The luteotropic activity of PL-Im is, however, not identical to that of PRL. In addition,

of recPL-Im in the medium was estimated as oPRL equivalent (ng/ml) based on the result of RRA shown in Fig. 1C (One μ l of recPL-Im medium was equivalent to 4.71 ng of oPRL). B), C) Luteotropic activity of recPL-Im in the culture of rat luteal cells. Luteotropic actions of recPL-Im (□) were compared with that of oPRL (●) by measuring progesterone (B) and 20 α -OHP (C) secretion from cultured luteal cells. Luteal cells prepared from the rats on day 10 of pregnancy were cultured for 24 h in the presence of increasing concentrations of recPL-Im or oPRL. The amount of progesterone and 20 α -OHP secreted into the medium were measured by RIA (*P<0.05).

because PL-Im was glycoprotein, but PRL is not glycosylated, the placental PRL-like molecules vary widely in structure and function.

Proteins of the placental PRL family are grouped according to their ability to bind the PRL receptor (PRL-R). Whereas PL-I, PL-II and PL-IV can bind to the PRL-R, the other PLPs apparently cannot [1]. With the exception of PL-II, all members of the placental PRL family have one or two putative N-glycosylation sites [1], asparagine-Xaa-serine/threonine (Asn-Xaa-Ser/Thr), where Xaa can be any amino acid except proline. PL-Im has two putative N-glycosylation sites at Asn⁷⁹ and Asn¹²⁸ [2]. The apparent MW of recPL-Im showed relatively wide range in the Western blot, and it (more than 26 kDa) was bigger than predicted from its nucleotide sequence of the cDNA [2]. In the presence of tunicamycin, the MW was reduced and heterogeneity disappeared, indicating that recPL-Im has N-linked glycoside(s).

The corpus luteum is the main source of progesterone secretion throughout the whole period of pregnancy in the rat. It is well established that the pituitary derived PRL is an essential luteotropic factor during early pregnancy [6, 7]. But maternal PRL secretion, which is characterized by two daily surges, is not continued and ceases at mid-pregnancy [25, 26] when placental lactogens start to be secreted. The member of the placental PRL family has been assumed to be one of the luteotropic factors during pregnancy after placentation [13]. In accord with this concept, the mid-pregnancy-specific PLs are secreted again in late pregnancy and are associated with the prolonged secretion of progesterone from the corpora lutea beyond the normal pregnancy period in the prolonged pregnancy model, which is developed by induction of a new set of corpora lutea following successive treatment with PMSG and hCG at mid-pregnancy [1, 27]. The present study clearly demonstrated that recPL-Im has luteotropic activity in a primary culture of luteal cells of pregnant rat.

The potency of the recPL-Im is comparable to that of oPRL in the Nb2 cell assay when the amount of the recombinant protein is normalized by PRL-

RRA, indicating that PL-Im has potent mitogenic activity. In contrast to PL-Im, PL-IV displayed very little proliferative activity on Nb2 cells although it interacts with PRL-R resulting in the activation of Jak2 [28, 29]. Judging from the above, the function of PL-Im appears to differ essentially from that of PL-IV, though they can be classified as part of a extremely high homologous PRL subfamily that also includes PL-I. This finding would help to make sense of the molecular diversity of the rat placental PRL family.

Many attempts have been done to demonstrate the luteotropic action of PRL [13]. Indeed, PRL was proved to have luteotropic activity during pseudopregnancy and in early pregnancy [6, 7]. The luteotropic activity was, however, observed only within the limited life-span of the corpus luteum, which is about 2 weeks, because a continuous supply of PRL to a pseudopregnant rat cannot extend the luteal phase, and the period of PRL action was limited until mid-half of the pregnancy in the rat [30]. In this context, it is noteworthy that recPL-Im has a biological function qualitatively different from that of PRL. Treatment of luteal cells obtained on day 10 of pregnancy with recPL-Im resulted in a concentration-dependent increase in progesterone secretion, but PRL did not show any signs of activity in the experiment. This is in accord with previous studies indicating that PRL did not have a stimulatory action on the *in vitro* culture of dispersed luteal cells of pseudopregnant rat [31, 32]. In the present study, direct evidence was provided that mid-pregnancy-specific PL, PL-Im, has a luteotropic effect on luteal cells in mid-pregnancy, and the function of PL-Im appears to be qualitatively different from that of PRL.

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