

Co-Localization of Placental Lactogen-I, Placental Lactogen-II, and Proliferin in the Mouse Placenta at Midpregnancy¹

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ABSTRACT

This study was undertaken to determine whether mouse placental lactogen (mPL)-I, mPL-II, and proliferin (PLF) are expressed by the same population of placental giant cells at midpregnancy. Tissue sections from Day 9 of pregnancy were analyzed by double immunofluorescence staining. Sections were stained for PLF by use of a rhodamine-conjugated second antibody, and for mPL-I or mPL-II by use of a fluorescein-conjugated second antibody. All three proteins were present in most of the same giant cells. The distribution of mPL-I and PLF among giant cells *in vitro* was also examined. When placental cells from Day 7 of pregnancy were cultured for 5 days, > 90% of the cells that immunostained for mPL-I also immunostained for PLF on the first 3 days of culture. Thereafter, the percentage of cells that contained both proteins declined rapidly while the percentage that contained only PLF increased, suggesting continued differentiation of the cells *in vitro*. These data demonstrate that the same trophoblast giant cells express mPL-I, mPL-II, and PLF simultaneously at midpregnancy, suggesting that their gestational profiles in maternal blood during this period result at least partly from changes in gene expression in one population of cells and not from differentiation of several subsets of giant cells, each expressing only one member of the gene family.

INTRODUCTION

The placentas of a number of species produce polypeptides that are members of the prolactin (PRL)-growth hormone (GH) gene family [1, 2]. The best-studied members of this family in the mouse are mouse placental lactogen (mPL)-I [3], mPL-II [4], and proliferin (PLF) [5]. The known biological activities of mPL-I and mPL-II are similar to those of PRL [3, 4, 6, 7]. The functions of PLF have not been determined, although they are known to differ from those of the mPLs [8, 9]. The gestational profiles of these proteins in maternal serum differ considerably from one another. Mouse PL-I appears in maternal serum on Day 6 of pregnancy [10]. Its concentration increases rapidly beginning on Day 8 and reaches maximal values on Day 10. After Day 11, its concentration decreases abruptly and remains low for the rest of pregnancy. PLF has been detected in maternal blood by Day 8 of pregnancy [11]. It is present at high concentration between Days 10 and 12, and then its concentration declines to low values by Day 16. Mouse PL-II appears in the maternal blood on Day 9 of pregnancy [12, 13]. Its concentration increases until about Day 14 and then plateaus in some mouse strains or continues to increase for the remainder of pregnancy in others.

There is evidence that each of these proteins is produced by giant cells at midpregnancy [11, 14–19], but it is

not clear whether the same giant cell in fact produces all three proteins at the same time *in vivo* or whether the proteins are produced by different subpopulations of giant cells. To address this question, in the present study we have examined the co-localization of these proteins in the conceptus at midpregnancy.

MATERIALS AND METHODS

Hormones and Antibodies

Recombinant mPL-I, mPL-II, and PLF were purified as previously described [4, 6, 20]. Rabbit antisera to mPL-I, mPL-II, and PLF have been described [6, 11, 12]. A monoclonal antibody to PLF of the IgG₃ subclass was generated in mice [21].

Cell Dissociation and Culture

Conceptuses were collected on Day 7 of pregnancy from Swiss Webster mice (Simonsen Laboratories, Gilroy, CA); the presence of a vaginal plug was used as an indicator of Day 0 of pregnancy. The fetus and decidua basalis were removed. The remaining tissue was dispersed in collagenase, and the cells were fractionated on a Percoll gradient as described previously [22]. Cells banding at a density of 1.044 g/ml, which produce mPL-I and mPL-II [22, 23], were collected and plated. The cells were suspended in culture medium (NCTC-135 containing 20 mM HEPES, 25 mM NaHCO₃, 1.65 mM cysteine, 50 mg streptomycin/ml, and 50 U/ml penicillin G, pH 7.3) supplemented with 5% (vol/vol) fetal bovine serum, and were plated at a density of 2.0×10^5 to 3.0×10^5 cells/cm² in plastic multiwell plates. The cells were allowed to attach for 2 h, and the medium was replaced with serum-free culture medium. The cells were

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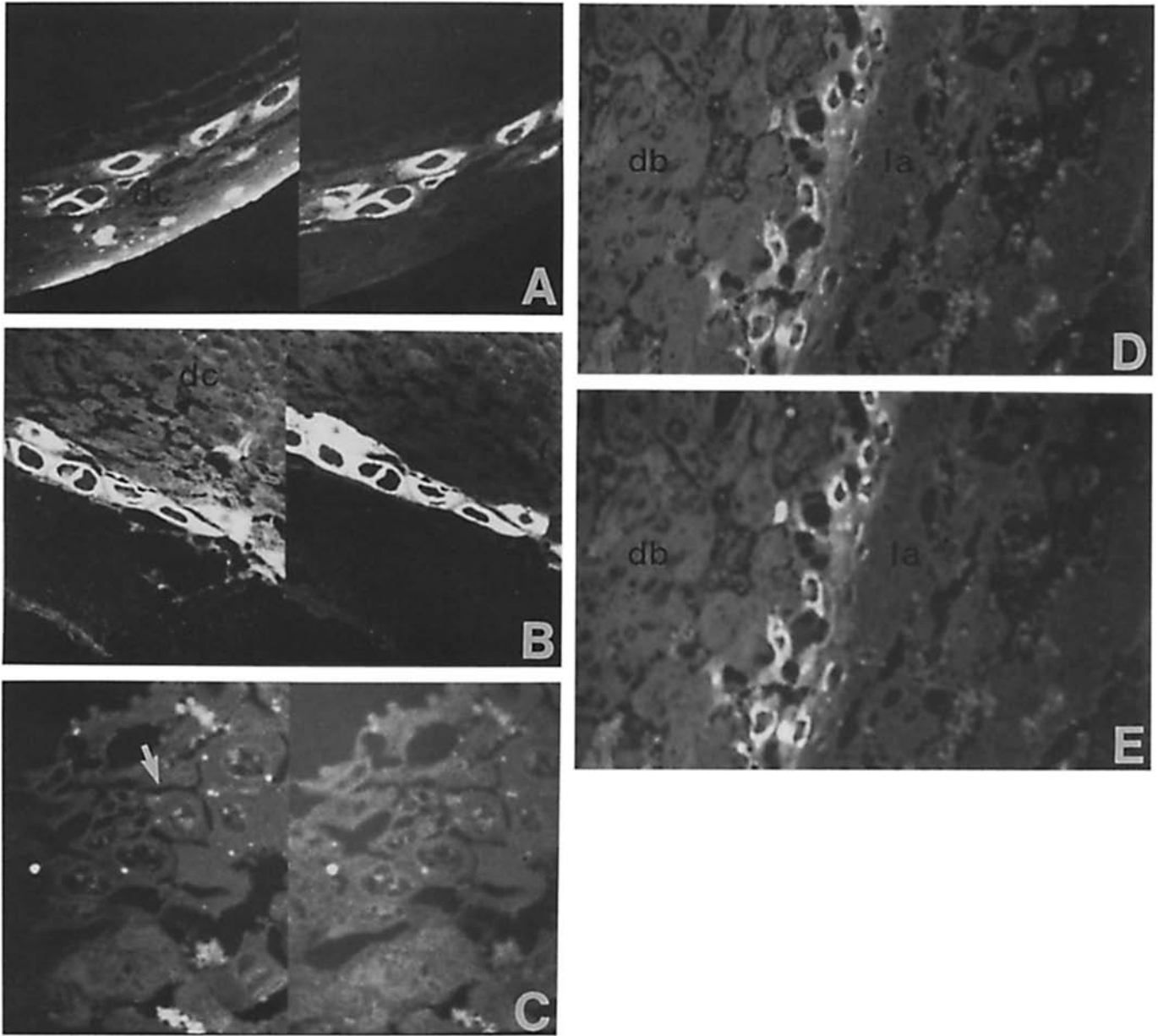


FIG. 1. Double immunofluorescence staining for mPL-I, mPL-II, and PLF in mural region (A and B) and basal zone (C-E) on Day 9 of pregnancy. A and B) Sections were stained first for PLF (left side of each panel) and then for mPL-I (A, right side) or mPL-II (B, right side). D and E) Sections were stained first for PLF (D) and then for mPL-I (E). C) Control section stained with mouse IgG₃ (left side) and nonimmune rabbit serum (right side). Arrow = giant cell; dc = decidua capsularis; db = decidua basalis; la = labyrinth.

incubated at 37°C under an atmosphere of 95% air:5% CO₂ for up to 5 days. The day the cells were plated was considered Day 0 of culture. The medium was changed daily.

Immunostaining

Conceptuses were collected on Days 7, 9, and 10 of pregnancy. They were fixed in Bouin's solution, dehydrated, embedded in paraffin, and sectioned at 7 μm. The sections were stained for mPL-I, mPL-II, or PLF with avidin-biotin immunoperoxidase or immunoglucose oxidase kits

(Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions [14, 24]. Tissue sections were also subjected to double immunofluorescence staining. First, they were stained for PLF by incubating them with a monoclonal antibody to PLF (20 μg/ml) for 1 h, followed by incubation with rhodamine-conjugated anti-mouse IgG (Cappel, Durham, NC) at a dilution of 1:25 (vol/vol) for 30 min. Then they were stained for mPL-I or mPL-II by incubating them for 1 h with polyclonal rabbit antiserum specific for one of the proteins, followed by incubation for 30 min with flu-

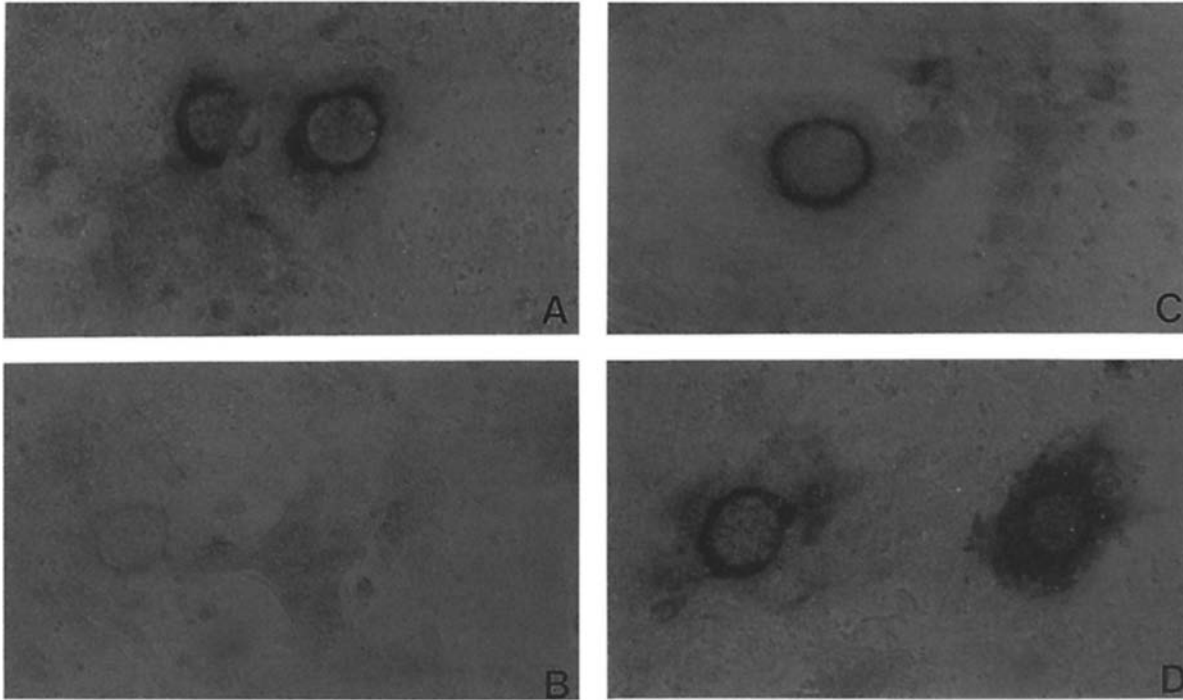


FIG. 2. Specificity study with monoclonal antibody to PLF. Cells from Day 7 of pregnancy were cultured for 3 days and stained with monoclonal antibody to PLF (A), or with anti-PLF monoclonal antibody that had been preincubated overnight at 4°C with 5 µg/ml PLF (B), 5 µg/ml mPL-I (C), or 5 µg/ml mPL-II (D). Immunoglucose oxidase method was used. Note that staining with anti-PLF antibody was strongly attenuated by preincubation with PLF but not with the other proteins. When the same study was repeated using each of the other antisera, staining was attenuated when antiserum was preincubated with protein against which it was raised; preincubation with one of the other proteins did not attenuate staining. ×260.

orescein-conjugated anti-rabbit IgG (Vector Laboratories) at a dilution of 1:100 (vol/vol). The anti-mPL-I and anti-mPL-II antisera were used at dilutions of 1:1500 (vol/vol) and 1:500, respectively. All steps were carried out at room temperature.

Cultured cells from Day 7 of pregnancy were stained sequentially for mPL-I and PLF. The cells were first stained for one of the proteins with an avidin-biotin immunoperoxidase kit as above, and the number of stained cells was determined. The cells were then stained for the other protein with an avidin-biotin immunoglucose oxidase kit as above, and the number of stained cells was counted. The percentage of cells staining for only one of the proteins and for both proteins was determined as described in the legend to Figure 3.

RESULTS

Localization of mPL-I, mPL-II, and PLF in the Conceptus

Tissue sections from Days 7, 9, and 10 of pregnancy were initially stained for mPL-I, mPL-II, and PLF by immunoperoxidase and immunoglucose oxidase methods in order to compare their localization within the conceptus. Specific staining for mPL-I, mPL-II, and PLF was present in some giant cells in both the mural and polar regions of the conceptus on Day 7 of pregnancy (data not shown). Staining

for each of these proteins was also present in giant cells in both the mural region and basal zone on Days 9 and 10 of pregnancy (data not shown), confirming previous reports [11, 14–19].

In order to determine whether the same giant cells produce all three of these proteins *in vivo*, sections of Day 9 conceptuses were initially stained for PLF by use of a rhodamine-conjugated second antibody, and then they were stained for one of the other proteins with use of a fluorescein-conjugated second antibody. For each examination, one section from each of three conceptuses was examined; each section contained more than 100 stained cells. Representative areas of the sections are shown in the figures. In both the mural region (Fig. 1, A and B) and basal zone (Fig. 1, D and E), most of the giant cells that contained PLF also stained for mPL-I (Fig. 1, A and E) or mPL-II (Fig. 1B and data not shown). A few cells stained for only PLF, mPL-I, or mPL-II.

The specificity of the immunostaining methods was verified by demonstrating that preincubation of each of the antisera or the monoclonal antibody with 5 µg/ml of the protein against which it was directed strongly attenuated staining, but preincubation with any of the other proteins did not (Fig. 2). Replacing the primary antiserum or monoclonal antibody with nonimmune rabbit serum or mouse IgG₃, respectively, also markedly reduced staining (Fig. 1C).

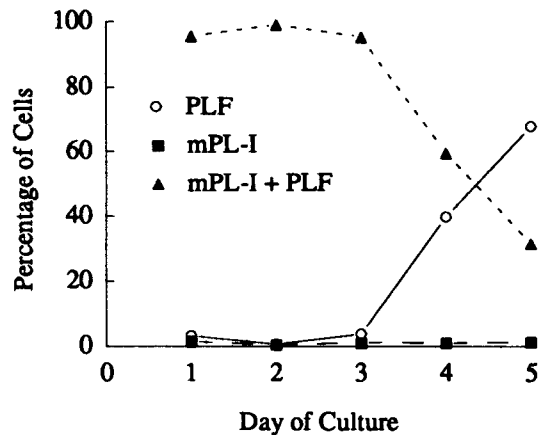


FIG. 3. Theoretical percentages of immunostained cells from Day 7 of pregnancy that contained mPL-I, PLF, or both mPL-I and PLF as a function of time in culture. Cells (10^5) were plated in 96-well plates. On each day of culture, six wells were stained with anti-mPL-I antiserum, and number of stained cells was counted. Same wells were then stained with anti-PLF antiserum, and total number of stained cells was determined. Percentage of cells that contained only PLF was calculated as $(\text{number stained with both antisera}) - (\text{number stained with anti-mPL-I antiserum}) / (\text{number stained with both antisera})$. Six additional wells were then stained with anti-PLF antiserum, and number of stained cells was determined. These wells were then stained with anti-mPL-I antiserum, and total number of stained cells was counted. Percentage of cells that contained only mPL-I was calculated as $(\text{number stained with both antisera}) - (\text{number stained with anti-PLF antiserum}) / (\text{number stained with both antisera})$. Percentage of cells that contained both mPL-I and PLF was calculated as $(100 - [\text{percentage containing only mPL-I}] - [\text{percentage containing only PLF}])$. Numbers of cells that stained for both hormones did not differ significantly between wells that were first stained for mPL-I and then for PLF, and wells that were stained for both proteins in reverse order ($p > 0.05$). Number of cells that stained for both hormones for each day was as follows (mean \pm SEM; $n = 12$): Day 1, 69.8 ± 2.1 ; Day 2, 110.0 ± 4.6 ; Day 3, 122.9 ± 4.0 ; Day 4, 101.4 ± 5.9 ; Day 5, 57.4 ± 1.8 .

In the double immunofluorescence staining experiments, signal was not detectable in the rhodamine channel when cells were stained for mPL-I or mPL-II using fluorescein-conjugated second antibody. Similarly, signal could not be detected in the fluorescein channel when cells were stained for PLF using rhodamine-conjugated second antibody (data not shown).

Expression of mPL-I and PLF In Vitro

The results of immunostaining conceptuses for mPL-I, mPL-II, and PLF indicated that a single giant cell produces all three of these proteins on Day 9 of pregnancy. We then determined whether an individual cell retains the ability to produce the PLs and PLF in vitro, using a culture system that contains cells from throughout the fetal placenta. We have previously demonstrated that the same giant cell can produce both mPL-I and mPL-II in vitro in cultures from Days 7 and 9 of pregnancy [24], and therefore only mPL-I was monitored here.

Cells from Day 7 of pregnancy were immunostained for mPL-I and/or PLF on several days of culture to examine temporal changes in the expression of these proteins in

vitro. On the first three days of culture, between 90 and 99% of the cells that immunostained for mPL-I also immunostained for PLF (Fig. 3). The percentage of stained cells that contained both proteins declined on Days 4 and 5 of culture, and by the fifth day, only 20–30% of the stained cells contained both mPL-I and PLF. The percentage of immunostained cells that contained only PLF was low ($< 5\%$) during the first 3 days of culture, but it increased dramatically on Days 4 and 5. The percentage of immunostained cells that contained only mPL-I was very low ($< 2\%$) throughout the 5-day culture period.

DISCUSSION

The results of double immunofluorescence staining of conceptuses indicate that mPL-I, mPL-II, and PLF are all produced by the same giant cell in vivo on Day 9 of pregnancy. The presence of mPL-I, mPL-II, and PLF in the same giant cells at midpregnancy suggests that changes in gene expression in one population of giant cells underlie the gestational changes in the concentrations of these proteins in maternal serum during this period and that each of the proteins is not produced by a different subset of giant cells. These data confirm and extend previous reports suggesting that several placental members of the PRL-GH-PL gene family can be expressed by the same cell. Lee et al. [11] reported localization of mPL-II and PLF to the same cells in experiments in which sequential tissue sections from Day 12 of pregnancy were examined for the presence of each protein individually. We recently demonstrated that the same giant cells produce both mPL-I and mPL-II in placental cell cultures initiated on Days 7 and 9 of pregnancy [24].

The localization pattern of mPL-I and PLF in the placental cells in vitro was consistent with the in vivo data. When cells from Day 7 of pregnancy were cultured, almost all of the cells that immunostained for mPL-I also contained PLF for the first three days of incubation, which suggests that these proteins are co-localized in vivo as early as Day 7. In a previous study examining mPL-I and mPL-II production in vitro [24], we observed that about 90% of the PL-containing cells immunostained for both mPL-I and mPL-II on the third day of incubation in cultures initiated on Day 7 of pregnancy. Therefore, these cells produce mPL-I, mPL-II, and PLF simultaneously in vitro as they do in vivo.

The pattern of mPL-I and PLF localization in cells from Day 7 of pregnancy changed during the 5-day culture period, suggesting that cell differentiation was occurring. As noted above, almost all of the cells that contained mPL-I on the first three days of culture also contained PLF, but then the percentage of immunostained cells that contained both proteins declined markedly while the percentage that contained only PLF increased. It is not known whether the cells that contained only PLF on the fourth and fifth days of culture were the same cells that initially produced both mPL-I and PLF or whether some or all of these cells represent a

subset that never produced mPL-I. In our earlier study examining expression of mPL-I and mPL-II in vitro [24], we observed a shift from mPL-I to mPL-II production in some of the cells. Thus, a shift from mPL-I to PLF production in one population of cells is certainly also possible. The continuing presence of PLF-containing cells in the cultures in the presence of a declining population of mPL-I-containing cells bears some similarity to the gestational patterns of the proteins in maternal serum at midpregnancy, where the mPL-I concentration declines much more rapidly than the PLF concentration after their peak values are attained on Day 10 of pregnancy [10, 11], and suggests that this culture system may be useful for examining factors regulating the gestational change in mPL-I and PLF gene expression during midpregnancy.

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