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Effect of Estrogen on the Gestational Profiles in Gene Expression of Placental Lactogen I, II and Pit-1 in the Rat Placenta

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To investigate gestational profiles in gene expression of placental lactogen I (PL-I), PL-II and Pit-1, RNA samples were extracted from the placentas of pregnant day 12 to 20 at 2 day intervals. Northern blots showed changes in gene expression of PL-I, -II and Pit-1. Sizes of PL-I and -II mRNA were changed and amounts of PL-I, -II and Pit-1 mRNA increased during progress of gestation. To examine the effect of estrogen on the gene expression of PL-I, -II and Pit-1, pregnant female rats were ovariectomized (OVX) and daily injected with 17β-estradiol (OVX + E). OVX markedly lowered the amount of PL-I and -II mRNA, and shifted mRNA size from 1 kb to 1.3 kb in PL-I mRNA and 0.6 kb to 1 kb in PL-II mRNA, respectively. OVX had no effect on the mRNA size of Pit-1, but markedly attenuated Pit-1 mRNA level. Estrogen injection reversed the effect of OVX on the size-shift but not on the amount of PL-I and -II mRNA. Replacement of E partially recovered OVX-induced inhibition of Pit-1 mRNA level. Present results suggest that estrogen may play a pivotal role on the gene expression of PL-I and -II such as alternative RNA splicing and/or polyadenylation, and Pit-1 may be involved in the gene expression of PL-I and -II by estrogen.

KEY WORDS: Ovariectomy, Estrogen, Placental Lactogens, Pit-1, Rat Placenta

The placenta of a number of species produces several members of prolactin (PRL)-growth hormone (GH) gene family (Ogren and Talamantes, 1988; Soares *et al.*, 1991). The well-known members of this family in the mouse and rat are placental lactogen (PL)-I and PL-II (Robertson *et al.*, 1982; Robertson *et al.*, 1990). Rat PL-I (rPL-I), the early form, is dominant in maternal serum from pregnant day 11 to 13, while rPL-II, the late form, is present in maternal blood in increasing amounts from pregnant day 12 until parturition (Robertson and Friesen, 1981; Robertson *et al.*, 1982).

The gene expression of GH and PRL in the pituitary is regulated by a pituitary specific transcription factor, Pit-1 (Ingraham *et al.*, 1990; Karin *et al.*, 1990; Li *et al.*, 1990). Pit-1 is a member of POU-homeo family of transcription factors (Ingraham *et al.*, 1988; 1990). Pit-1 can bind and activate the promoters of human placental lactogen (hPL) and placental growth hormone variants (GH-V) as well (Lemaigre *et al.*, 1989; Nachtigal *et al.*, 1989; Nickel *et al.*, 1991). Recently, Pit-1 was reported to be locally synthesized in the rat and human placentas (Bamberger *et al.*, 1995; Lee *et al.*, 1996). Therefore, Pit-1 may be involved in the regulation of gene expression of the rat PLs.

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Secretion of PL-I and PL-II is dually regulated by the maternal factors and fetal factors (Robertson *et al.*, 1984a, b). Estrogen from maternal circulation has an inhibitory effect on the PL-I and PL-II secretion, while undefined fetal factors are activatory for the release of PL-I and PL-II. However, precise mechanism for PL-I and -II gene expression by maternal estrogen is still unclear. Therefore, present study aims to determine the gestational profiles of PL-I, -II and Pit-1, and the regulatory effect of estrogen on the gene expression of PL-I, -II and Pit-1 in the rat placenta using Northern blot analysis.

Materials and Methods

Animals and tissue preparations

Timed pregnancy was generated by housing the Sprague-Dawley female rats with males. The presence of a copulatory plug or sperms in the vaginal smear was defined as day 0 of pregnancy. On the day 14 of gestation, maternal rats were ovariectomized and daily injected with 4 μg of 17β-estradiol (E, Sigma). Removal of maternal ovaries was performed as previous reports (Robertson *et al.*, 1984a,b). Rats were sacrificed on the day 18 of gestation and the placental tissues were removed. Present experimental groups were intact, ovariectomized (OVX), and ovariectomized and subsequent administration of E (OVX + E). To examine the gestational profiles of PL-I, PL-II and Pit-1 gene expression, placentas were removed from uterus on the pregnant day 12 to 20 at 2 day intervals.

cDNA and cRNA probes

To exclude the possible detection of other POU-homeo family gene products, the rat Pit-1 cDNA (generously provided by Dr. R.A. Maurer, Oregon Health Science University, Portland, Oregon) was reconstructed. The EcoRI fragment (about 440 bp) of Pit-1 cDNA structure which corresponds to the trans-activation domain of Pit-1 and is Pit-1 specific, was inserted into pBluscript II KS (Stratagene). After linearization with PstI, ^{32}P-labeled cRNA probes were synthesized using T3 RNA polymerase. PL-I and PL-II cDNA (generously gifted by Dr. M.C. Robertson, University of Manitoba, Canada) were labeled with ^{32}P-dCTP using random priming method (Feinberg and Vogelstein, 1984). The specific activities of probes were about 1×10^9 cpm/ml.

RNA extraction and Northern blot hybridization

Total RNA from placenta was extracted using the acid guanidium thiocyanate-phenol chloroform method (Chomczynski and Sacchi, 1987). For Northern blot analysis, RNA fractions (10 μg for detection of PL-I and PL-II mRNA, and 20 μg for detection of Pit-1 mRNA) from placentas were electrophoresed on a 1% agarose/ 2.2 M formaldehyde gel at 100 V for 1.5 h. RNA was then transferred to Nytran membrane (0.45 μm pore size; Schleicher and Schuell). The membranes were prehybridized with 10 ml of hybridization buffer at 42°C for 2 h. Hybridization buffer consisted of 50% deionized formamide, 5× SSC (1× SSC: 0.15 M NaCl and 0.015 M sodium citrate), 5× Denhardt's solution (1× Denhardt's solution: 0.01% polyvinylpyrrolidone, 0.01% Ficoll and 0.01% BSA), 0.1% SDS and 2 mg of heat-denatured salmon sperm DNA. Hybridization was carried out in a hybridization incubator (Stuart scientific) with hybridization buffer plus PL-I and -II cDNA probes or Pit-1 cRNA probe. Hybridization temperatures were adjusted at 42°C and 55°C for PL-I and -II cDNA probes, and Pit-1 cRNA probe, respectively. After overnight hybridization, the membranes were washed at high stringency to exclude the non-specific signals and exposed to X-ray film (Hyperfilm β-max, Amersham) for 1-4 days.

Results and Discussion

Gestational profiles of PL-I, PL-II and Pit-1 gene expression

In the present study, we elucidated the gestational profiles of PL-I, PL-II and Pit-1 gene expression during pregnant day 12 to 20 using Northern blot hybridization (Figs. 1, 2 and 3). Fig. 1 shows the gestational profile of PL-I-like mRNA. In the day 12 of pregnancy, 1 kb band of PL-I

mRNA was detected. In the placenta of pregnant day 14, any detectable transcript of PL-I was not found. In the placentas of pregnant day 16 to 20, PL-I-like mRNA bands were present. PL-I was known to be expressed mid-pregnancy until pregnant day 12 (Robertson *et al.*, 1990), while late gestational PL-I-like variant (PL-Iv), of which cDNA has sequence homology of 99% with PL-I cDNA, was reported to be expressed from pregnant day 16 to term (Robertson *et al.*, 1991). Therefore, during the pregnant day 16 to 20 in the present study, they looked like PL-Iv mRNA bands that were detected by hybridization with PL-I cDNA probe. Interestingly, Northern blot analysis of RNA from placentas of day 16 generated two discrete bands of PL-Iv mRNA, which are about 1 kb and 1.3 kb, respectively. However, placentas from pregnant days 18 and 20 showed only the 1.0 kb band. After day 16 of gestation, the density of PL-Iv mRNA gradually increased along with the days of gestation.

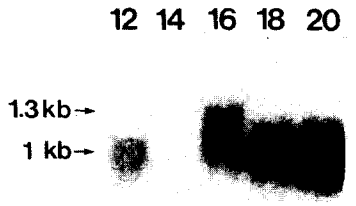

Fig. 1. Gestational profiles of PL-I and PL-Iv mRNA determined by Northern blot hybridization. Placenta of pregnant day 12 showed 1 kb band of PL-I mRNA. Placentas from pregnant day 16 to 20 showed PL-Iv mRNA, while placenta from pregnant day 14 did not generate positive signals of PL-I or PL-Iv mRNA. Placenta from pregnant day 16 showed 2 discrete bands of PL-Iv mRNA, which are 1.3 kb and 1 kb, respectively. Arabic numerals on the lanes indicate pregnant days.

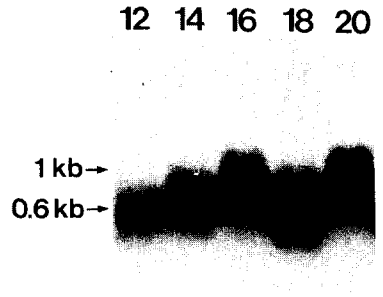

Fig. 2. Gestational profiles of PL-II mRNA during pregnant day 12 to 20. Northern blots of placental RNA with PL-II cDNA probe generated 2 discrete hybridization signals of PL-II mRNA, which are 0.6 kb band of pregnant days 12, 14 and 18, and 1 kb band of pregnant days 16 and 20. Arabic numerals on the lanes indicate the designated days of pregnancy.

Northern blot analysis showed changes in profile of PL-II mRNA during pregnant day 12 to 20 (Fig. 2). PL-II mRNA bands were gradually intensified with progression of gestation from day 12 to 18. Two different mRNA bands of PL-II were present in the placenta. Those were PL-II mRNA band of 0.6 kb in the placentas of pregnant days 12, 14 and 18, and 1.0 kb in pregnant days 16 and 20, respectively. This result is quite different from previous report that showed only a 1 kb band of PL-II mRNA (Duckworth *et al.*, 1986; Campbell *et al.*, 1989). It is difficult to explain the reason of this discrepancy. It may be originated from different RNA splicing or change of polyadenylation. The mechanisms are not known yet.

Northern blots of Pit-1 mRNA showed the discrete two bands of transcripts (Fig. 3), which are about 2.5 kb and 1.2 kb, as described previously in the rat pituitary and rat pituitary adenomoa GH_3 cells (Ingraham *et al.*, 1988; Day and Day, 1994). Pit-1 mRNA level increased until pregnant day 16 and then remained relatively constant until day 20. Therefore, Pit-1 may play an important role in the placenta especially in the late pregnancy.

Regulation of PL-Iv, PL-II and Pit-1 gene expression by ovariectomy and subsequent administration of estrogen

Removal of maternal ovary and subsequent replacement of E resulted in a great changes of PL-Iv, PL-II and Pit-1 gene expression (Figs. 4, 5 and 6). Two discrete bands of PL-Iv mRNA, which

are very similar to the mRNA bands found in the placenta of pregnant day 16, were generated by OVX (Fig. 4). The 1 kb band among 2 bands of PL-Iv mRNA seemed to be the same with those of intact and E treated groups. E treatment to the OVX maternal rats reversed the effect of OVX, that is, only 1 kb mRNA signal was present in the E treatment group. These results suggest that disappearance of estrogenic influence is responsible for the appearance of alternative PL-Iv mRNA form in the placenta of pregnant day 16. The amount of mRNA also changed greatly by OVX and subsequent administration of E. Messenger RNA signal of intact group was much denser than those of OVX and OVX + E groups. Therefore, it may be plausible to explain that ovarian factors including E activate the PL-Iv gene expression directly or indirectly.

OVX shifted the size of PL-II mRNA band from 0.6 kb to 1.0 kb, while administration of E reversed the size-shift effect of OVX (Fig. 5). It may also be due to the influence of estrogen that band of PL-II mRNA was changed over pregnant day 12 to 20, because OVX shifted the mRNA band from 0.6 kb to 1.0 kb and subsequent replacement of E returned the band from 1 kb to

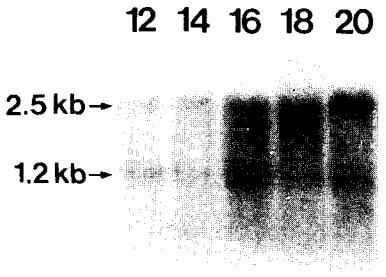

Fig. 3. Northern blot analysis of Pit-1 mRNA during pregnant day 12 to 20. RNA (20 μg) from placental tissues was electrophoresed and hybridized with ^{32}P-labeled Pit-1 cRNA probe. Arabic numerals on the lanes indicate the designated days of pregnancy.

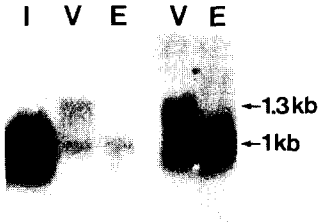

Fig. 4. Effects of ovariectomy and subsequent replacement of E on the regulation of PL-Iv mRNA determined by Northern blot hybridization. Right panel is the result of longer exposure than left panel. Experimental groups: I, intact; V, OVX; E, OVX + E (See Materials and Methods for details).

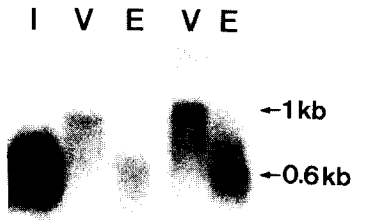

Fig. 5. Effects of ovariectomy and subsequent injection of E on the regulation of PL-II mRNA. Right panel is the longer exposed result of left panel. Experimental groups are the same as those of Fig. 4.

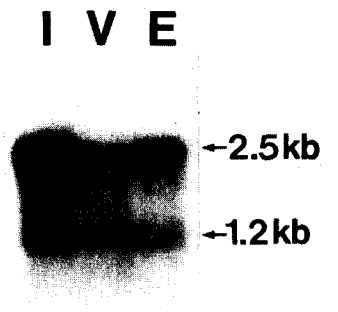

Fig. 6. Effect of ovariectomy and daily injection of E on the regulation of Pit-1 mRNA level. RNA samples (20 μg) from placentas of intact (I), OVX (V), and OVX + E (E) groups were electrophoresed and hybridized with rat Pit-1 cRNA probe.

0.6 kb in size. OVX and subsequent injection of E also affected the amount of PL-II mRNA, that is, mRNA levels in OVX and OVX + E groups were much lower than that in intact group.

Northern blot analysis of Pit-1 mRNA showed that OVX and subsequent administration of E also affected the expression of Pit-1 (Fig. 6). OVX had no effect on the pattern of Pit-1 mRNA bands, but markedly attenuated densities of Pit-1 mRNA bands. However, administration of E to the OVX maternal rats partially reversed the OVX-dependent suppression of Pit-1 mRNA level. Present effect of E on the placental Pit-1 mRNA appears to be well coincident with previous report that E enhanced Pit-1 mRNA level of the rat pituitary (Lee *et al.*, 1995). In the pituitary lactotrophs, Pit-1 was known to be essential for the estrogenic regulation of PRL gene expression (Day *et al.*, 1990; Nowakowski and Maurer, 1994). Moreover, our unpublished data showed that the site of Pit-1 expression migrates from junctional zone to labyrinth zone of the rat placenta during gestational progress are well consistent with the previous report that the site of PL-II expression shifted from junctional zone to labyrinth zone during the second half of pregnancy (Campbell *et al.*, 1989). Taken together, these results also lead to suggest that Pit-1 is involved in the estrogenic regulation of PLs.

OVX clearly attenuated mRNA levels of PL-Iv and PL-II, which could not be compensated by E replacement. Therefore, the amounts of the gene expression seem to be not dependent upon maternal estrogen but dependent upon other factor(s) of maternal ovary. Expression of Pit-1 gene was affected by OVX and E replacement, which suggests that OVX itself is able to change the regulatory environment for Pit-1 gene expression. In addition, circulatory E may change the environmental cues around placental Pit-1 gene, then, affect Pit-1 gene expression. In the pituitary lactotroph, it has been well known that many extracellular regulators such as thyrotropin releasing hormone (TRH) and dopamine regulate gene expression of PRL through the Pit-1 mediation (Ingraham *et al.*, 1990; Elsoltz *et al.*, 1991). Moreover, normal gene expression of PRL needs not only Pit-1 but also estrogen-receptor complex (Day *et al.*, 1990; Lee *et al.*, 1995). Still now in the placenta, there is no evidence about the regulation of Pit-1 gene expression by environmental cues. However, it is quite possible that the regulation of Pit-1 gene expression in placenta may be similar with that in lactotroph. Thus, E may play an important role for the regulation of Pit-1 as it does in lactotroph. It is also possible that ovarian factors as well as E may influence on the environmental cues such as placental dopamine. There is no evidence about E responsive element in the rat PL-Iv and PL-II genes. Therefore, E may indirectly affect the gene expression of PL-Iv and PL-II via other environmental cues and Pit-1 mediation.

It is quite plausible that E may influence mechanisms of alternative splicing and/or change of polyadenylation of PL-Iv and PL-II transcripts. Still now, direct evidence for the alternative splicing and change of polyadenylation of PLs by E is not available. Therefore, further studies are needed to elucidate the precise mechanisms involved in the effect of E on the size-shift of PL mRNA.

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흰쥐 태반에서 Placental Lactogen I과 II 그리고 Pit-1의 유전자 발현에 미치는 에스트로겐의 영향

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임신중기에서 말기에 이르는 흰쥐의 태반에서 Placental Lactogen I(PL-I), II 그리고 Pit-1 의 유전자 발현 변화를 Northern blot hybridization으로 조사하였다. 그 결과, 임신시기에 따라 PL-I과 PL-II의 mRNA 양과 크기에 변화가 나타났다. 이들 유전자 발현에 미치는 에스트로겐의 영향을 조사하기 위하여, 임신 14일째 쥐의 난소를 제거하고(OVX), 이후 매일 에스트로겐을 투여한 후(OVX + E), 임신 18일째 태반을 회수하여 PL-I, II 그리고 Pit-1의 유전자 발현을 Northern blot hybridization으로 조사하였다. OVX 군의 경우, PL-I의 mRNA 크기는 1 kb에서 1.3 kb로 PL-II의 mRNA는 0.6 kb에서 1 kb로 변화하였다. OVX + E 군에서는 PL-I과 PL-II의 mRNA가 정상대조군과 같은 상태로 환원하였다. 정상대조군에 비하여 OVX와 OVX + E 군에서 PL-I과 PL-II의 mRNA 양 또한 현저히 감소하였다. 한편, 난소제거와 에스트로겐 투여는 Pit-1의 mRNA 크기에는 영향을 미치지 못한 반면, mRNA 양은 난소제거시 감소하였다가, 에스트로겐을 투여하면, 부분적으로 회복되는 경향을 보였다. 이러한 본 실험의 결과는 에스트로겐이 PL-I과 PL-II의 RNA splicing이나 polyadenylation 등을 포함한 유전자 발현에 영향을 미치며, Pit-1이 이 과정에 개입하는 것을 시사하는 것이다.